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ISOLATION AND OPTIMIZATION OF ORGANIC SOLVENT TOLERANT AMYLASE PRODUCING BACILLUS ISOLATE RG-11

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Abstract: *It is the first instance when thermo-solvent tolerant, antibiotic and heavy metal resistance amylase producing strain of Bacillus isolated from soil sample was capable of growing in the presence of 30% (v/v) cyclohexane. The strain exhibited resistance against heavy metals (Cr^{6+}, As^{3+}, Cs^{+1}, Pb^{2+}) and various antibiotics (penicillin, lincomycin, cloxacillin, pefloxacin, tetracycline, cefuroxime and cefazoline). In un-optimized modified nutrient starch broth (pH 7.0 and 55°C), the strain produced 3500 U/ml amylase. The presence of organic solvents n-dodecane, n-decane, iso-octane, n-octane and toluene, enhanced the amylase production. The amylase was not only stable but also its activity enhanced in the presence of 30 % (v/v) solvents n-dodecane, iso-octane, n-decane, xylene, Toluene, n-haxane, n-butanol, Acetone, Methanol, and cyclohexane, after prolonged incubation (7 days). The unique property of solvent tolerance, antibiotic and heavy metal resistance proves the potential candidature of this isolate not only for peptide bond synthesis, but also for industrial use and bioremediation strategies involved in environmental clean up.*

Keywords: *Bacillus, Heavy metal resistance, Solvent stability, Thermotolerant.*

Introduction: Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units. These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet out the industrial demands. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. The history of amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff. This was followed by several reports of digestive amylases and malt amylases. It was much later in 1930, that Ohlsson suggested the classification of starch digestive enzymes in malt as - and - amylases according to the anomeric type of sugars produced by the enzyme reaction. -Amylase (1,4- -D-glucan-glucanhydrolase, EC. 3.2.1.1) is a widely distributed secretary enzyme of different origin.

Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyze from the non-reducing end, successively resulting in short end products. Today a large number of enzymes are known which hydrolyze starch molecule into different products and a combined action of various enzymes is required to hydrolyze starch completely.

Materials and Methods

1. Isolation, Screening and Identification of Organic Solvent Tolerant Amylase Producing Bacteria: The soil samples were collected aseptically from different site of university campus to isolate amylase producing bacteria. 1.0 gm soil was suspended in 9.0 ml sterile distilled water, agitated for a min and 0.1 ml suspension was spread over nutrient starch agar plates (pH 7.0) containing, 2.0%, starch; 0.5%,

peptone; 0.3%, beef extract; 0.3, NaCl; 2%, agar. The inoculated plates were overlaid with 7.0 ml of acetone, toluene, benzene, hexanol, heptane, tetrachloroethane, carbon tetrachloride, dodecane isooctane, and n-octane and incubated at 37^0C, till sufficient growth appeared. After sufficient growth incubated plates were overlaid with Gram's iodine reagent (0.01 M I_2-KI solutions). If a strain was amylolytic then it started hydrolyzing the starch present in the surrounding and in the zone degradation there was no blue color formation. Selection was done as per colonies with and without clear and transparent zone as amylase producing (Amy+) and amylase non-producing (Amy-) strain, respectively. Bacterial colonies showing clear zones were selected, streaked twice on starch agar plates for purification and maintained as pure culture over nutrient agar slants (pH 7, 4°C). The isolate having maximum clearance zone was selected for further studies. The selected bacterial isolate RG-01 was identified by morphological and biochemical characterization as per the Bergey's Manual of Systematic Bacteriology (1989).

2. Solvent Tolerance, Antibiotic and Heavy Metal Resistance Studies on Efficient Bacterial Strain RG-01: In order to determined organic solvent tolerance, the strain RG-11 was grown in starch agar broth (pH 7.0, adjusted using 1M Na_2CO_3 solution) containing (%): starch, 2.0; peptone, 0.5; beef extract, 0.3 and NaCl, 0.5. The broth (50 ml) was inoculated with a loopful bacterial culture and incubated at 37°C on static condition. From this cultured broth, 5.0 ml mother culture of 0.8 OD (A620: 1 cm cuvette) containing 3.2 x 10^8 cfu/ml was transferred to 25 ml sterile starch nutrient broth containing 30% (v/v) dodecane, benzene, toluene, hexanol, heptane, isooctane, noctane, carbon tetrachloride, tetrachloroethane, acetone. To prevent the solvent evaporation, the mouth of Erlenmeyer flask was plugged with butyl rubber stoppers. Growth in the absence of solvent under similar conditions served as control. The culture broth (5.0 ml) was drawn aseptically from each flask periodically after every 12 h and growth was assessed spectrophotometrically by turbidity measurement (A620: 1 cm cuvette).

The antibiotic sensitivity pattern was studied by disc diffusion method. The antibiotics (µg/disc) used were: ciprafloxacin (10), kanamycin (30), tetracycline (30), ampicillin (25), norfloxacin (10), penicillin (5) and methicillin (5) nalidixic acid (30) gentamicin (30). Antibiotic impregnated discs were placed over freshly prepared bacterial lawn on nutrient starch agar plates and incubated at 37°±1°C for 24 h. The isolate was classified as resistant or sensitive by the presence/absence of inhibition zone of bacterial growth around antibiotic discs.

To determine the heavy metal resistance pattern, 0.1 ml of bacterial culture of 0.8 OD (A_{620}; 1cm cuvette) was spread aseptically on nutrient starch agar plates, supplemented with different concentrations (µg ml^{-1}) of the following heavy metals: Ni (0.0-800), Zn (0.0-00), Cr (0.0-1000), and Hg (0.0-400). The metal salts used were nickel chloride ($NiCl_2$), zinc chloride ($ZnCl_2$), potassium dichromate [$K_2Cr_2O_7$], and mercurous chloride ($HgCl_2$). Bacterial growth was observed during 24-48 h at 37°±1°C.

3. Crude Enzyme Preparation and Enzyme Assay: To obtain crude enzyme 24 h old cultures were transferred to microcentrifuge tubes and centrifuged at 10000 rpm for 10 min. Cells were discarded and resultant supernatant was used as the crude enzyme for various enzyme assay. -Amylase was assayed by measuring the reducing sugar released by reaction on starch. Amylase assay was done (Nelson somogy method) by using a reaction mixture consisting 500 µl of substrate solution (1.0% soluble starch in 1.0 M phosphate buffer pH 7.0), 100 µl of the enzyme solution and 1ml volume make up by adding 400 µl distilled water. The reaction mixture was incubated for 10 min at 37°C. Reaction was stopped by adding 1 ml of alkaline copper tartrate solution. The reaction mixture was heated to 100°C for 10 min and cooled, then added arsenomolybdate solution for color stabilization. Optical density of each sample with reaction mixture was taken at 620 nm in a spectrophotometer (Shimadzu, Japan). One unit of enzyme activity was defined as the amount of enzyme that liberates 1.0 µg of glucose min^{-1} ml^{-1}.

4. Bacterial Growth, Amylase Production and Effect of Organic Solvents: As this strain was isolated in the presence of hexanol it was imperative to study the effect of other organic solvents on bacterial growth. Hence, to study the effect of various organic solvents on growth and amylase production, bacterial growth was carried out for 24 h at 37°C in 100 ml starch nutrient broth (as above) in the presence of 50 ml of various individual organic solvent. Each flask was inoculated and incubated as described earlier. Mouth of each flask was tightly plugged using butyl rubber stopper to avoid solvent

evaporation. A flask with no solvent served as control. At 12 h interval, 5 ml culture broth was withdrawn, centrifuged at 10,000 rpm (4°C) for 10 min and cell free supernatant was used as enzyme for amylase estimation. The cell pellet obtained was dried and bacterial growth was assessed in the terms of cell dry weight (mg/ml).

4. Effect of Temperature on Enzyme Activity and Stability: The influence of temperature on activity of amylase was studied by incubating the reaction mixture at different temperatures (35-65°C). The enzyme was incubated at different temperatures 35-65°C for 1 h to study the stability of the enzyme. The residual amylse activity was measured by conducting the reaction at temperature 37°C and pH 7.0. The activity of the enzyme was considered as 100% under standard assay conditions.

5. Effect of pH on Amylase Activity and Stability: The effect of pH on amylase activity was measured in the pH range of 6 to 10, using the appropriate buffers at concentration of 100 mM (6.0–7.5, sodium phosphate; 8.0–9.0, Tris–HCl; 9.5–12.0, glycine–NaOH) under standard assay conditions. To study stability as a function of pH, 100 µL of the purified enzyme was mixed with 100 µL of the buffer solutions and incubated at 37°C for 1 h then aliquots of the mixture were taken to measure the residual amylase activity (%) under standard assay conditions.

6. Effect of Incubation Periods: The effect of incubation periods on amylase activity at optimized pH and temperature was determined using starch (0.1%, w/v, pH, 7.0) as substrate at different incubation (12, 24, 36, 48, 60 and 72). Prior to treatment, the temperature of substrate solution was stabilized at required value and then amylase activity was measured.

7. Effect of Carbon Sources: Various starchy carbon sources viz. soluble starch, potato starch, wheat bran, glucose, fructose, lactose, xylan, and sawdust at 2.0% were individually added in the medium and inoculated with bacterial culture separately with their respective optimized pH, temperature then incubated for 24 h. The best source of carbon was further optimized in different concentration viz. 1.0, 2.0, 3.0, 4.0, 5.0, 6.0%, 7.0% and 8.0% (w/v) for amylase production.

8. Effect of Nitrogen Sources: Different organic and inorganic nitrogen sources viz. beef extract, yeast extract, peptone, ammonium sulphate, ammonium nitrate, potassium nitrate, urea and ammonium dihydrogen orthophosphate were individually added into the basal medium at 0.5%. Active culture of individual bacterium was inoculated with 1.0 ml inoculum having 3.2×10^8cfu ml^{-1}. The best source of nitrogen was further optimized in different concentration viz. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% (w/v) for amylase production.

Results and Discussions

1. Isolation, Screening and Identification of Thermotolerant Amylase Producing Bacterial Cultures: Ten (10) bacterial isolates producing variable amylolitic zones on starch agar plates which stained with iodine solution were isolated from the soil samples. The zones of clearance by isolates reflect their extent of amylolitic activity. Those having clearance zone greater than >1.5 cm were considered as significant. Among 10 bacterial isolates, 5 exhibited good amylase activity which was reassessed by loading their culture broth in the wells on starch agar plates which stained with iodine solution (pH 7.0). The culture broth of good amylase producers cleared more than >1.5 cm zone within 3-4 h of incubation at $37^o \pm 1^oC$, thereby indicating an extra-cellular nature of the amylase. The isolate RG-11, showing maximum clearance zone diameter was selected for further studies.

The efficient strain RG-11 was rod-shaped, Gram-positive, aerobe and facultative, motile, with positive Voges Proskaver, catalase and oxidase test. It grew over a wide range of pH (5-12), temperature (15-85°C), NaCl concentration (0.0-16%), and was able to hydrolyze Gelatin, Esculin, Starch, Tween 20 and 40 and produce acid from glucose. The strain was halotolerant as it grew in the presence of 0.0-12% NaCl, but did not require salt for its physiological activities. On account of morphological and biochemical characteristics, it was identified as *Bacillus sp.*

Table 1: Morphological, physiological and biochemical characteristics of the selected RG-11 isolate.

Characteristics	Results	Characteristics	Results
Morphological tests		Gelatin hydrolysis	+
Gram's reaction	+	Esculin hydrolysis	+
Shape	Cylindrical	Starch hydrolysis	+
Motility	+	Urea hydrolysis	_
Physiological tests		Nitrate reduction	_
Growth on NaCl (%) 2.0-8.0	+	Ornithine decarboxylase	_

Growth at pH 7.0-12.0	+	Lysin decarboxylase	_
Growth at temp. 25-95°C	+	Catalase test	+
Growth under anaerobic condition	+	Oxidase test	+
Biochemical tests		Arginin decarboxylas	_
Growth on MacConkey agar	_	Tween 20 hydrolysis	+
Indole test	_	Tween 40 hydrolysis	+
Methyl red test	_	Gas production from glucose	_
Voges Proskauer test	+	**Acid production from**	
Citrate utilization	_	Dextrose	+
H_2S production	_	Lactose	_

(+): Positive; (–): negative.

2. Solvent Tolerance, Antibiotic Sensitivity Assay and Heavy Metal Resistance Pattern: The bacterial growth was slightly reduced (10-20%) in the presence of benzene (30%, v/v) as compared to the control. Although, the initial growth was slow up to 24 h in the presence of hexanol, it was fairly high compared to the extent growth reported by other researchers. Similar growth behavior by P. aeruoginosa PST-01 was reported by Ogino et al., (2001), where 23% (v/v) hexanol was supplemented for growth and tolerance studies. Gupta et al., (2006) also reported a decreased growth of *P. aeruoginosa* PseA strain in the presence of 33% (v/v) hexanol who observed 50% reduction in dry cell mass. Therefore, the tolerance and growth efficiency of our strain RG-11 was certainly better than that reported by other researchers. The reduced availability of air and hence oxygen may not be the reason of decreased growth response in the presence of hexanol, as both the control as well as organic solvent containing media flasks were plugged with butyl rubber stoppers. It was, therefore, hexanol responsible for the reduced growth in the experimental flask compared to the control.

Table 2: Bacterial growth in different organic solvent at different incubation

S.N.	Organic Solvent	12h	24h	36h	48h	60h	72h	84h
1.	n-dodecane	2.044	2.330	2.488	2.938	3.476	3.062	3.098
2.	Tolune	1.864	1.500	1.186	1.152	1.116	0.970	0.982
3.	Heptane	1.656	1.174	1.254	1.178	1.082	0.904	0.918
4.	Carbon tetrachloride	1.698	1.448	1.332	1.294	1.246	1.208	1.192
5.	Iso-octane	1.632	1.462	1.202	0.998	0.954	0.770	0.676
6.	n-octane	1.730	1.696	1.738	1.752	2.245	2.388	2.612
7.	Hexanol	2.902	2.868	2.652	2.560	2.314	2.280	2.222
8.	Tetrachloro ethane	3.868	3.976	4.192	3.528	3.406	3.244	3.108
9.	Benzene	1.872	1.562	1.254	1.174	1.165	1.140	0.976
10.	Acetone	4.070	4.170	4.280	4.780	4.530	4.496	4.344

The strain RG-11 was resistant against penicillin and tetracycline, and while other antibiotics created variable inhibition zones of bacterial growth around the discs. The antibiotics may be arranged in the following order of growth inhibition zone (mm dia.): kanamycine (24)> ciprofloxacin (20)> gentamycin (20)> norfloxacin (17)> methicillin (14)> nalidixic acid (12)> amicillin (10)> penicillin (00)> tetracycline (00). Antibiotics, for which the isolate is resistant, may be supplemented to fermentation medium during enzyme production so as to check the contamination by other sensitive isolates.

Table 3: Effect of Antibiotics on Amylase producing bacterial resistant and sensitivity

S.N.	Antibiotics (µg)	Resistant/ Sensitive	Zone (mm)
1.	Penicillin (5)	R	00
2.	Amicillin (25)	S	10
3.	Norfloxacin (10)	S	17
4.	Ciprafloxacin (10)	S	20
5.	Kanamycin (30)	S	24
6.	Tetracycline (30)	R	00
7.	Methicillin (5)	S	14
8.	Nalidixic acid (30)	S	12
9.	Gentamicin (30)	S	20

A variable level of tolerance was noted among various heavy metals under study. The strain RG-11 tolerated very high concentrations (µg/ml) of certain heavy metals which may be arranged in the following order of tolerance: nickel (800)> zinc (750)> cromium(600)> mercuric(400). Amylase producing bacteria having antibiotic resistance and heavy metal

tolerance was not reported. The very high level of tolerance against chromium reveals the possible application of this isolate in bioremediation of such metals from natural contamination sites. In leather industries, this isolate may be useful for dehairing process as the use of chromium is very common in the tanning process [1]. Further, the amylase producing organisms displaying heavy metal tolerance may be of potential use for the treatment of multimetal contaminated sludge generated during wastewater treatment. The increased load of antibiotics/disinfectants in health care and heavy metals in industries creates the selective pressure for the survival of bacteria in a contaminated environment. Thus, in a multiple stressed environment, bacterial cells acquire resistance/tolerances by alterations in genetic makeup either by mutation or transfer of resistant genes among the bacteria.

Table 4: Effect of heavy metals on amylase production (MIC determination)

S.N.	Concentration(µl)	Ni	Hg	Cr	Zn
1.	50	+	+	+	+
2.	100	+	+	+	+
3.	150	+	+	+	+
4.	200	+	+	+	+
5.	250	+	+	+	+
6.	300	+	+	+	+
7.	350	+	+	+	+
8.	400	+	+	+	+
9.	450	+	-	+	+
10.	500	+	-	+	+
11.	550	+	-	+	+
12.	600	+	-	+	+
13.	650	+	-	-	+
14.	700	+	-	-	+
15.	750	+	-	-	+
16.	800	-	-	-	-
17.	850	-	-	-	-
18.	900	-	-	-	-
19.	950	-	-	-	-
20.	1000	-	-	-	-
21.	1050	-	-	-	-
22.	1100	-	-	-	-
23.	1150	-	-	-	-

Table 5: Enzyme activity of organic solvent tolerant amylase producing bacteria

S.N.	Organic solvent	12h	24h	36h	48h	60h	72h	84h
1.	n-Dodecane	67.716	3035.59	1819.78	1788.31	1600.56	1316.70	1244.88
2.	Toluene	23.94	447.61	554.72	706.58	846.11	1316.70	2301.66
3.	Heptane	167.58	613.55	664.03	693.23	799.23	1460.34	1627.92
4.	Carbon tetrachloride	208.62	737.01	758.91	819.09	1434.34	2151.18	2287.98
5.	Iso-octane	345.42	819.09	1638.18	1712.05	2276.69	2965.14	2677.86
6.	n-octane	54.72	819.43	1486.33	1599.88	1977.10	3895.38	3464.46
7.	Hexanol	10.60	537.62	594.74	673.39	693.23	1368.00	1918.62
8.	Tetrachloro ethane	215.46	595.76	655.96	743.17	1023.26	1419.12	1891.62
9.	Benzene	164.16	485.98	659.03	716.15	819.09	2322.18	1956.24
10.	Acetone	23.59	790.97	646.38	636.46	160.39	134.67	127.91

3. Effect of Different Temperature: Effect of different temperature *viz.* 35°C to 65°C was evaluated for amylase production and stability by bacterium at different physico-chemical and nutritional levels. Bacterium showed higher amylase production and stability (2462.4 U/ml and 135.78%) at 35°C. This culture also sustains its stability 96.3% activity at 55°C. Further, increase in temperature could not affect the amylase production by bacterium (Figure 2). Similarly also reported that -amylase production by *B. cereus* [2] at 75°C is 90% activity was observed compared to the optimum enzyme activity at 55°C for liquefaction of starch. For -amylase produced by a laboratory *Bacillus* isolate AS-1, 88, 85 and 44% of activity has been reported at 60, 70 and 80°C, respectively [3].

4. Effect of Different Incubation: Just after optimization of temperature for amylase production in the liquid medium, incubation period was simultaneously optimized for enzyme production. The results clearly indicated that bacterium showed 2567 U/ml enzyme activity in 24 h of incubation. Further increase in the incubation period did not increase the enzyme

production (Figure 1). Therefore, 24 h of incubation was selected for evaluation of pH optima for enzyme production by bacteria. Incubation time depends on the characteristics of the culture, on growth rate and enzyme production [4]. Reported that -amylase production by *B. subtilis* was maximum (2902 U/mg) in 72 h, after which a gradual decrease was observed [5], similar results [6, 3]. It may be due to denaturation or decomposition of -amylase owing to interaction with other components in the medium, as it is reported elsewhere [7]. Moreover, the reaction for maximum enzyme production at 72 h could be due to the fact that the microorganism was in its exponential phase. At the later stage, when nutrients are depleted, it reaches its stationary phase and can start to produce secondary metabolites, thus resulting in a lower yield of enzyme [8]. Thus our strain produce -amylase production within 20h of incubation is better than reported by the other worker mention above.

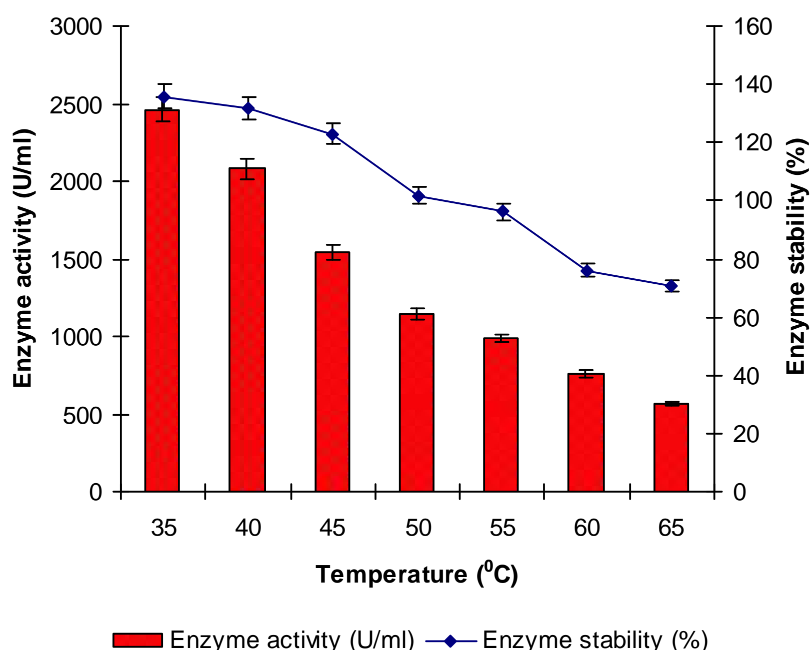

Figure1. Effect of different temperature on amylase activity and stability

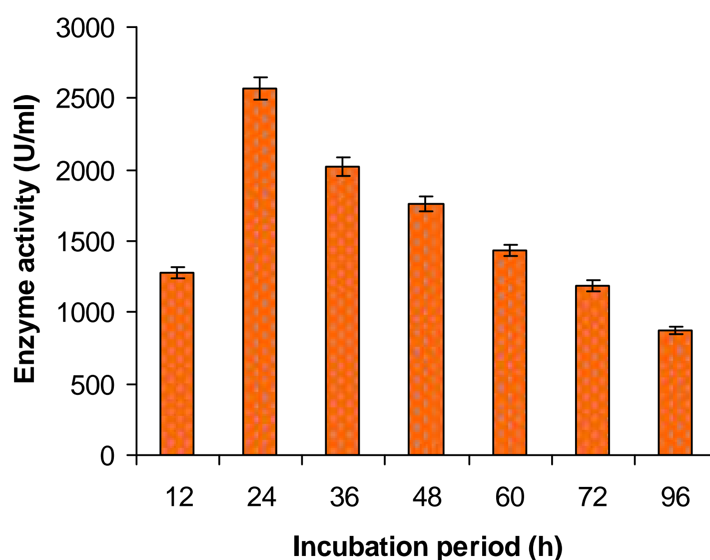

Figure2. Effect of incubation period on amylase production

5. Effect of Different pH: Different pH *viz.* 6.0 to 10.0 in the medium was evaluated for amylase production and stability by bacterium at their optimal temperature and incubation period.

Bacterium showed 2128.95 U/ml enzyme productions and 98.6% stability at pH 7.5. Further, increase and decrease in the medium pH reduced the enzyme production and stability (Figure 3). Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion [9, 10]. The pH change observed during the growth of the organism also affects product stability in the medium. Most of the *Bacillus* strains used commercially for the production of bacterial - amylases have an optimum pH between 6.0 and 7.0 for growth and enzyme production [3,11]. Reported pH 7.5–8.0 to be the best for the production of alpha amylase by *Bacillus subtilis* [12]. Results show that enzyme production was generally stable from pH 4.0-9.0, which indicates excellent buffering property. The pH values also serve as a valuable indicator of the initiation and end of enzyme synthesis [13].

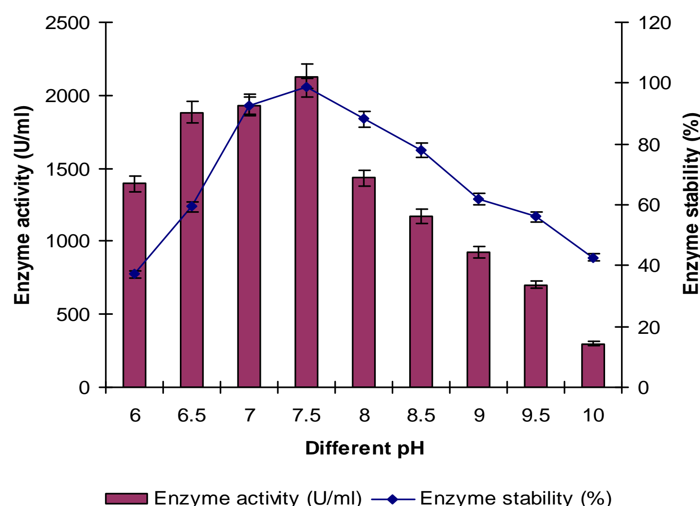

Figure.3 Effect of different pH on amylase activity and stability

6. Effect of Different Carbon Sources: Various carbon sources *viz.* Starch, wheat bran, rice bran, rice husk, potato starch, glucose, fructose, lactose, maltose and sucrose at a concentration of 2.0% (w/v) were individually tested in the basal medium at their optimal temperature, incubation period and pH to observe the effect on enzyme production by the bacterium. Out of these carbon sources, starch (soluble) was found best for amylase production by the bacteria followed by potato starch. Higher enzyme production 2354.77 U/ml was reported by bacteria. Bacterium was least affected and showed less affinity regarding (Figure 4).

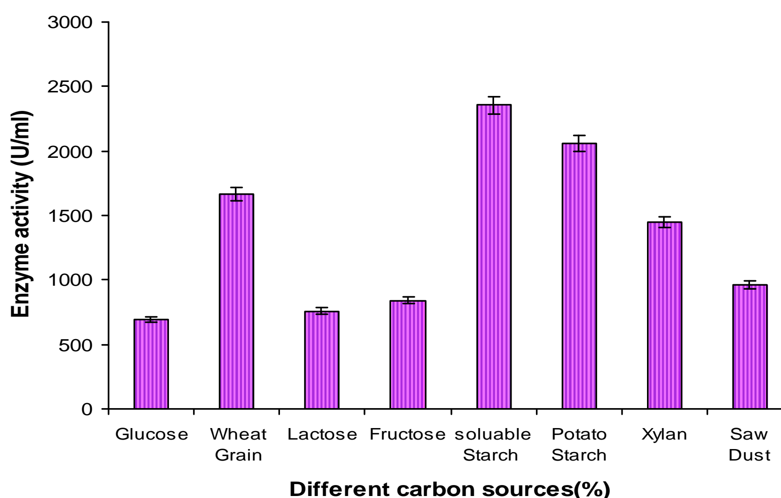

Figure 4: Effect of Different carbon sources on amylase production

In another set of the experiment, different concentrations of starch (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0%, w/v) in the medium were tested for amylase production at the same growth

conditions at which carbon sources were evaluated. Bacteria showed 2586.53 U/ml amylase production at 4% starch (soluble) concentration, above this concentration enzyme production was slightly increased.

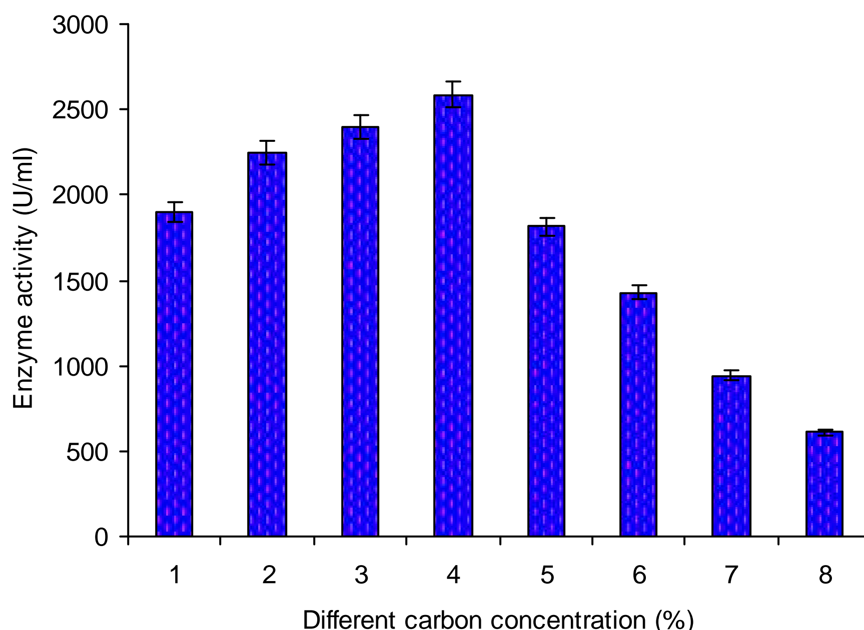

Figure 5: Effect of Different concentration of starch on amylase production

Supplementation of carbon sources in the form of monosaccharides, disaccharides and polysaccharides resulted in marginal increase in -amylase production. Reported that *B. cereus* using wheat bran showed highest production (122±5 U/g) with glucose [2]. *Bacillus thermooleovorans* is reported to prefer starch, glucose, lactose, maltose and maltodextrins as carbon sources for -amylase secretion [14]. Alpha amylase is an inducible enzyme, which is generally induced in the presence of starch or its hydrolytic product maltose [15]. Similarly reported that in enzyme production addition of soluble starch (1% by mass) gave the highest enzyme yield (62470 U/g), followed by maltose (58499 U/g) [6]. Hydrolyzed starch and glucose were found to repress the enzyme yield, which may be due to feedback inhibition caused by the presence of reducing sugars. Easily metabolizable carbohydrates may result in the better growth of the bacteria along with reduction in the enzyme formation [16, 15]. Since soluble starch was found to induce enzyme production, experiment was conducted to find out the appropriate concentrations for enhanced enzyme production. Using 1.5 %, the yield rose to 65 275 U/g and it was found to be the optimum since further increase resulted in gradual decrease in enzyme titre [6]. *B. licheniformis* SPT 27 gave higher yields of amylase in presence of starch. The bacteria can produce the enzyme even in the absence of starch [17]. Reported that starch increased -amylase production (4857 U/mg), [5] followed by sucrose (3804 U/mg), which was similar to the findings of starch was also known to increase enzyme production in *Bacillus* sp. PS-7, [18] *Bacillus subtilis* CBTK 106, *B. subtilis* IMG22 and *Bacillus* sp. I-3 [19, 20, 21]. Some worker reported that reported that supplemantion of glucose caused a negative effect on amylase production [22, 5]. It is well known that the synthesis of carbohydrate degrading enzymes in most species of genus *Bacillus* is subject to catabolic repression by glucose. Other three compounds, *i.e.* mannose, arabinose and galactose, did not have an effect on the enzyme synthesis. Similar results have been reported [23]. In this experiment our stain utilized 6% starch for maximum amylase production is higher than other.

7. Effect of Different Nitrogen Sources: Inorganic and organic nitrogen *viz.* peptone, beef extract, yeast extract, peptone, ammonium sulphate, Ammonium chloride and Ammonium hydrogen phosphate, at the rate of 0.5% (w/v) were used in the basal medium for amylase production by the bacterium (Figure 6). Best result was reported by bacteria at pH 7.5 and its optimum physico-chemical levels. The enzyme production by the isolate was almost similar in

potassium nitrate and yeast extract amended medium (2688.59 U/ml), while other nitrogen sources, did not increase in enzyme production.

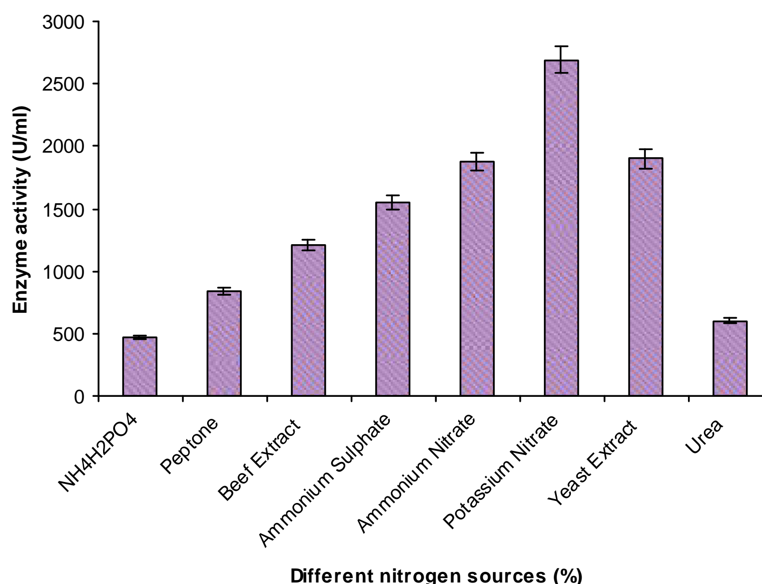

Figure 6: Effect of Different nitrogen on amylase production

Different concentrations of potassium nitrate (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%, w/v) in the medium were also tested for amylase production at the same growth condition at which nitrogen sources were evaluated. Bacteria showed higher enzyme production (2734.29 U/ml) at 0.2% beef extract concentration, further increasing in concentration, enzyme production reduced (Figure 7).

Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes including alpha amylase [10, 12]. Various organic and inorganic nitrogen sources have also been reported to support maximum -amylase production by various bacteria and fungi. However, organic and inorganic nitrogen sources viz. beef extract, peptone, yeast extract, ammonium sulphate [24, 25], ammonium chloride and ammonium hydrogen phosphate [14] and com steep liquo favoured growth and enzyme secretion by bacterial strains [26, 27, 28, 29]. Already reported that supplementation of urea at 1% concentration resulted in a decrease in amylase production [30]. A marginal increase was noted with the addition of peptone (53624 U/g) or tryptone (53 691 U/g) [2]. Presence of organic nitrogen sources, urea and peptone, has been reported to enhance a-amylase enzyme production by *Aspergillus niger* in wheat bran containing solid substrate medium, but inorganic nitrogen source, ammonium chloride, repressed enzyme production [31].

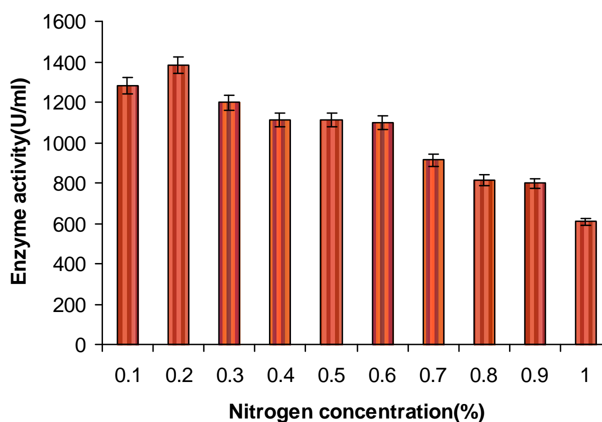

Figure 7: Effect of Different concentration of potassium nitrate on amylase production

8. Effect of Different Metal Salts: Various metal ions *viz.* NaCl, $CaCl_2$, $MgSO_4$, $HgCl_2$, $FeSO_4$, $NiCl_2$, $CuSO_4$, $CoCl_2$ and $ZnCl_2$ at a concentration of 0.05% (w/v) were individually

tested in the basal medium at their optimal temperature, incubation period and pH to observe the effect on enzyme production by the bacterium. Out of these metal ions, sodium and calcium ion was found best for amylase production. Bacterium also tolerates 0.05% $HgCl_2$, a noval finding of this strain. Higher enzyme production 2722.71 U/ml was reported by bacteria (Figure 8).

K^+, Na^+, Fe^{+2}, Mn^{+2}, Mo^{+2}, Cl, Ca^{+2} $SO4^{+2}$ had no effect while was inhibitory to amylase production by A. oryzae EI 212 [32]. Mg^{+2} played an important role and production was reduced to 50% when Mg^{+2} was omitted from the medium. Na^+ and Mg^{+2} show coordinated stimulation of enzyme production by *Bacillus* sp. CRP strain [33]. Addition of zeolites to control ammonium ions in B. amyloliquefaciens resulted in increased yield of -amylase [34]. An inverse relationship between -amylase production and growth rate was observed for Streptomyces sp. in the presence and absence of Co^{+2} [35], the presence of Co^{+2} enhancing the final biomass levels by 13-fold, albeit with a reduction in enzyme yield.

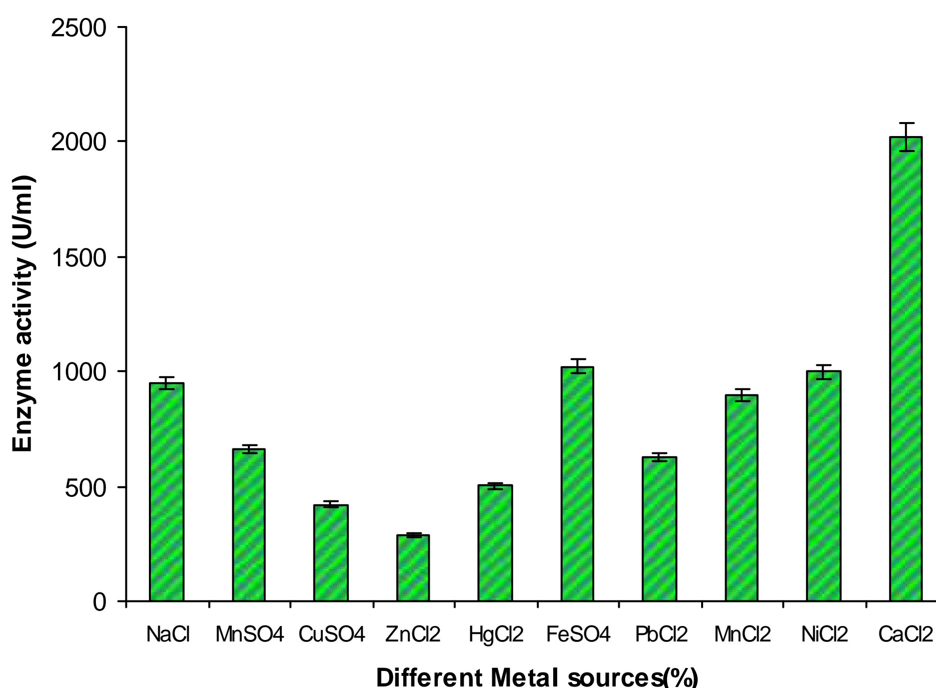

Figure 8: Effect of Different metal ions on amylase production

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