Isolation, identification and production of alkaline protease enzyme producing organism for the industrial application

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Abstract
Alkaline Protease is a protein hydrolyzing enzyme which is most important among industrial enzymes and research widely. Proteases constitute 7ne of the third largest groups of enzymes in industry. They all used in all kinds of industrial situations such as pharmaceuticals, drug manufacturing, detergents, surface cleaning formulations, waste treatment, silver recovery, digestive supplement, leather industry, textile industry, agrochemical additives and diagnostic reagents. In present study of alkaline protease enzyme producing bacteria were isolated from 6 alkaline soil samples by screening on skimmed milk agar plates. Different biochemical tests are performed with reference to –Bergey's manual of determinative bacteriology‖ all isolates belong to genus *Bacillus*. The enzyme was partially purified and different parameters for enzyme activity (Optimization temperature, pH and effect of ion concentration) were determined. This study also focuses on optimization of environmental parameters (temperature, pH, carbon source, nitrogen source etc.) for enzyme production from isolate.

Keywords: Alkaline Protease, *Bacillus*, skimmed milk agar, Bergey's manual, Optimization temperature, environmental parameter.

Introduction

Proteases are one of the most important classes of enzymes, are also termed as proteinases or peptidases are enzymes occurring everywhere in nature, be it inside or on the surface of living organisms such as plants, animals and microbes. Proteases have been purified and characterized because of their significant proteolytic activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification and low cost (Maurer, 2004 & Haddar *et al.*, 2009).

The choice of a protease for an application depends in part on its specificity for peptides bonds, and pH and temperature activity and stability, response to inhibitors and metal ions, cost and availability of the enzyme (Rai *et al.*, 2010). Alkaline proteases are particularly important because they are both stable and active under harsh conditions, such as temperature ranging from 50 to 90 ºC, high pH, and the presence of surfactants (Chen *et al.*, 2006). Proteases have optimum pH in the range of 7.0 or around are neutral and they are mainly originated from plants however some bacteria and fungi are also able to produce neutral proteases. While those which work in the pH range of 8.0-11.0 are alkaline proteases. Some of the important alkaline proteases are Solanain, Hurain and Proteolytic enzymes of *Bacillus* and *Streptomyces* species (Hameed, A. *et al.*, 1996, Lee, J.K. *et al.*, 2002). Screening of alkaline proteases producing *Bacillus* spp. from different ecological environments can result in isolation of new alkaline proteases with unique physio-chemical characteristics (Shumi *et al.*, 2004; Singh *et al.*, 1999). One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity.

Materials and methods

The materials and methods employed in the present study are described under the following headings:

1) Isolation & Identification of bacteria

a) Collection of samples

Soil samples were collected from various regions Niphad tehsil, Nashik District.1 g of the sample was suspended in 100 ml sterile distilled water, agitated for .45 min on a shaker at 50°C and 0.1 ml was spread on casein agar plates (nutrient agar with 1% casein) and incubated at 30°C for 48hours .After incubation for 24 h, Colonies showing clear zone were picked and purified.

b) Screening of protease producers

The isolated colonies were screened for protease production using skim milk agar medium. All the isolates were streaked on to skim milk agar plates and the plates were incubated for 48 h at room temperature. The clear zones around the colonies were evaluated as protease producers. (Prabakaran *et al.*, 2015).

c) Identification & Characterization of the microorganism using different tests

The bacterial isolate with prominent zone of clearance around the streak of bacterial isolate and showing efficient enzyme production was processed for the determination of colony morphology, Gram staining, starch hydrolysis, Catalase, IMViC, Nitrate and biochemical tests and then identified in accordance with the Bergey‘s Manual of Determinative Bacteriology.(Hala Ezzat and Samaa, 2016).

d) Fermentations and Crude Enzyme Preparation

A loopful of culture was inoculated into 10 ml of Luria Bertani broth medium (HiMedia, India) and incubated at 37°C for 24 hours. After this, 100 µl of the bacterial culture was inoculated to 20 ml of production medium (production medium composed of glucose 0.5 gm, peptone 0.75 gm, $MgSO_4$ 0.5 gm, KH_2PO_4 0.5 gm, $FeSO_4$ 0.01 gm in 500 ml distilled water) and kept in a refrigerated incubator shaker at 140 rpm for 24 hours at 37°C. At the end of the fermentation process, the fermented broth was centrifuged at 10,000 rpm in a centrifuge for 10 minutes and the supernatant was separated and used as crude enzyme source for the assay of protease production.

e) Protease assay

The alkaline protease activity was assayed by Kuntiz (1947) method using casein as substrate at pH 9.0 and 37ºC. After incubation for 30 min, the reaction was stopped using trichloroacetic acid and the amount of liberated amino acid was quantified by measuring the absorbance at 280 nm. Protease activity was estimated using tyrosine as standard. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of tyrosine from the substrate per minute per gram of substrate under the assay conditions (Zareena *et al.* 2014). Casein agar plates were also used as qualitative assay for protease activity. Diameter of zones was measured using scale. The total protein was estimated using Lowry‘s method (Lowry et al. 1951) using BSA as standard.

2) Process for maximum protease production

a) Submerged fermentation:

Four different types of media were screened for protease production in submerged fermentation. Various process parameters influencing protease production such as fermentation time (24-96 h), carbon source and nitrogen source were optimized for enhanced extracellular alkaline protease production by isolate in submerged fermentation. (Zareena *et al.* 2014).

The composition media for 1 liter

PM1: (Sucrose-50g, Soybean meal-10g, $(NH_4)_2SO_4$-3g, K_2HPO_4-1g, $CaCl_2$-0.5g, pH-9)

PM2: (Glucose-10g, Peptone-5g, Yeast extract-5g, K_2HPO_4-1g, MgSO4.7H2O-0.2g, Na_2CO_3-5g, pH-9)

PM3: (Glucose-10g, Soybean meal-10g, K2HPO4-3g, MgSO4.7H2O-0.5g, Nacl-0.5g, $CaCl_2.2H_2O$-0.5g, pH-9)

PM4: (Starch-10g, Beef extract-7.5g, Nacl-5g, $MgSO_4.7H_2O$-0.5g, $FeSo_4.7H_2O$-0.1g) (O P Varma *et al.* 2011, pH-9) (Varma et. al. 2011)

Fig: Production media

b) Enzyme production at different time period

Production medium at pH 9.0 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different time period of 24, 48, 72 and 96 hours. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source. (Onkar Nath *et al.* 2015).

3) Characterization of crude protease

a) Effect of pH on enzyme activity

Effect of pH on alkaline protease activity was determine by incubating enzyme solution with 1% substrate (Casein) prepared in buffers (0.1M) of different pH such as KH_2PO_4-K_2HPO_4 (6.0-7.5), Tris –HCl (8.0 -9.0), Glycine-NaOH (9.0 –13.0) and Na_2HPO_4-NaOH (11.0-12.0).

b) Effect of temperature on protease activity

To check the optimum temperature of alkaline protease, the enzyme with substrate was incubated at different temperatures (30, 35, 37, 40, 45 and 50oC). (Zareena et al. 2014).

c) Effect of metal ions on protease activity

To detect the effect of metal ions (Ca2+, Mg2+, Mn2+, Cu2+ and Hg2+) on crude enzymes, metal salt solutions were prepared in a concentration of 10 mM, and 1.0 ml of metal solution was mixed with 5.0 ml of crude enzymes and was incubated for 2 hr. Initial and final enzyme activities were measured.

4) Lab scale fermentation

The fermentation experiments for the production of extracellular alkaline protease by *Bacillus* which we isolate were carried out in a laboratory scale 5.0 L fermenter (Jai Lab Fermentation System, Nashik MIDC, India) with a working volume of 3.0 L. The fermenter was equipped with monitors, which were used to measure and control foam, temperature, pH, stirring rate and dissolved oxygen. A peristaltic pump was used to control the foam by automatic addition of an antifoam silicon agent. For tests with automatic pH control, a system operating with an in gold sterilizable electrode and automatic addition of oxalic acid/KOH peristaltic solutions through pump was used. Controls were performed at different levels of different parameters. The vegetative bacterial inoculum was transferred to the basal medium at a level of 2 % (v/v) based on total working volume of the fermentation medium. The batch was carried out at an incubation temperature of 37°C, agitation speed of 300 rpm and aeration rate of 1.0 vvm. (Hamid Mukhtar and Ikram-Ul-Haq, (2008))(79)

The kinetics of growth rate and enzyme production were followed at different time intervals. Culture samples were withdrawn aseptically every 30 minutes, and cell density, along with enzyme activity, was monitored. This study was conducted in the laboratories of Jai Biotech Industries, Department of science & industrial research (DSIR) recognized R & D center, MIDC Satpur, Nashik District, Maharashtra, India.

Results and Discussion:

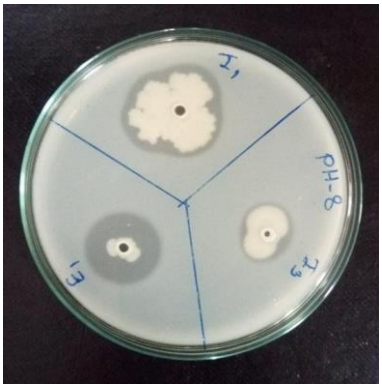
1) Isolation of bacteria:

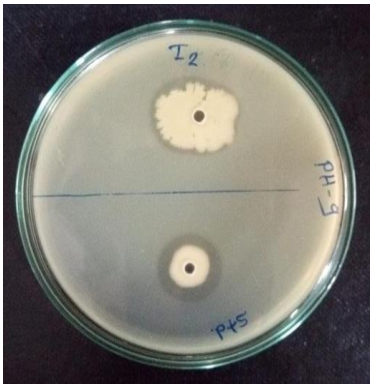
In the present study, different bacterial isolation were done from soil sample by serial dilution method (10^{-6} dilution) sample was spread onto Nutrient agar media with alkaline pH 8,9 and 10 respectively and incubated for 24 hours at 37ºC. About five dominant morphologically distinct colonies namely I1, I2, I3, E1 & E2 were selected and pure cultured by repeated streaking on the nutrient agar plates.

2) Screening for Protease Enzyme

The isolated five bacterial strains were screened for protease producing ability on skim milk agar. The zone formation around the bacterial growth was identified as the positive protease producers which may be due to hydrolysis of casein.

Plate No. 1 **Plate No. 2**





Among the five isolates screened, only two isolates showed maximum proteolytic activity with a zone of about 27 mm and 21 mm whereas the other three isolates exhibited poor proteolytic activity. The results were noted in (Plate No:1 & 2). Therefore these efficient protease producing strains I1, I2 & E1 were selected for further experimental studies and biochemical tests.

3) Characterization and of Protease Producing Isolates

The bacterial isolates with prominent zone of clearance and showing efficient enzyme production were processed for the determination of colony morphology(Table No. 1), Gram staining, spore staining and biochemical tests(Table No. 2) then identified in according to the Bergey‘s Manual of Determinative Bacteriology.

Table No. 1

Isolate	Size (mm)	Shape	Color	Margin	Opacity	Elevation	Consistency
I1	2	Circular irregular	Pell white	Irregular	Non Transparent	Convex	Sticky
I2	2	Circular irregular	Dirty white	Irregular	Non Transparent	Flat	Smooth
I3	3	Circular	Milky white	Regular	Non Transparent	Convex	Smooth
E1	1	Circular	Dull white	Regular	Semi Transparent	Flat	Rough
E2	2	Circular	Dull white	Regular	Semi Transparent	Flat	Smooth

Table No. 2

Sr. No.	Biochemical Tests		Isolates				
			I1	I2	I3	E1	E2
1	Gram Character		+ve	+ve	+ve	+ve	+ve
2	Morphology		R	R	R	R	R
3	Spore staining		NS	S	NS	S	S
4	Amylase test		-ve	+ve	-ve	-ve	+ve
5	Catalase test		+ve	+ve	+ve	+ve	+ve
6	Sugar fermentation	Glucose	+ve	+ve	-ve	-ve	-ve
		Sucrose	+ve	+ve	+ve	+ve	-ve

	Mannitol	+ve	+ve	-ve	-ve	-ve
7	Indole test	-ve	-ve	-ve	-ve	-ve
8	Methyl Red test	-ve	-ve	-ve	+ve	+ve
9	Vogurs Prosker test	-ve	-ve	-ve	-ve	-ve
10	Citrate test	-ve	-ve	-ve	-ve	-ve
11	65% NaCl	+ve	+ve	+ve	+ve	+ve

(*R= Rod shaped; *NS = Non Spore forming; *S = Spore forming; +ve= Positive; -ve= Negative)

The isolates were isolated i.e. I1, I2, I3, E1, & E2 they were may be *Bacillus insolitus, Bacillus brevis, Bacillus marinus, Bacillus pasteurii*, and *Bacillus macquariensis* respectively.

4) Enzyme activity assay

In present studies isolate I1, I2 & E2 were gives larger protein hydrolysis zone in skimmed milk agar plate. Proceed to check the enzyme activity standard graph of tyrosine will prepared to calculate further enzyme activities.

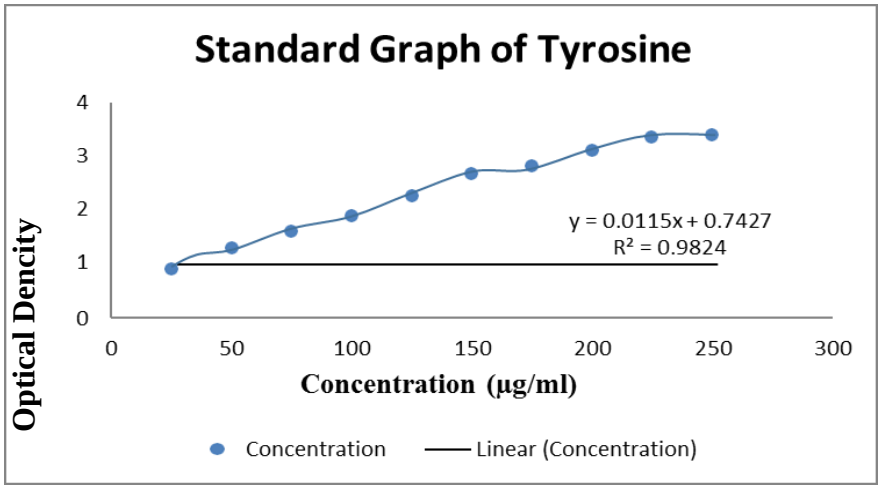


Fig: Standard Graph of Tyrosine

5) Process for maximum protease production

5.1. Protease assay for isolate which gives maximum enzyme activity

The maximum enzyme activity were given by I2 i.e. 1476.23 U/gm. Therefore I2 proceed for the further experiments.

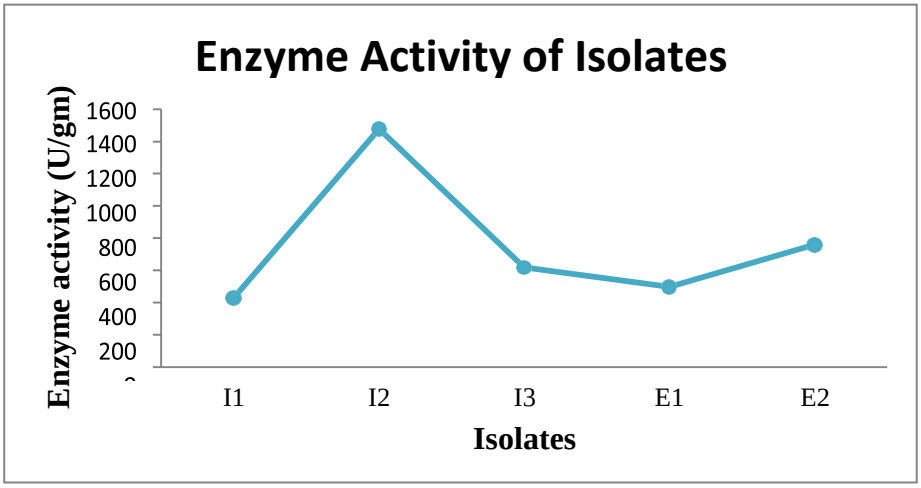


Fig: Enzyme Activity of Isolates

5.2. Maximum protease production in different media:

The maximum enzyme production was found 1896.23 U/gm. in medium PM3. Production media contains 1% Glucose as carbon source, 1% soybean meal as nitrogen source and MgSo4, CaCl2 like metal ions.

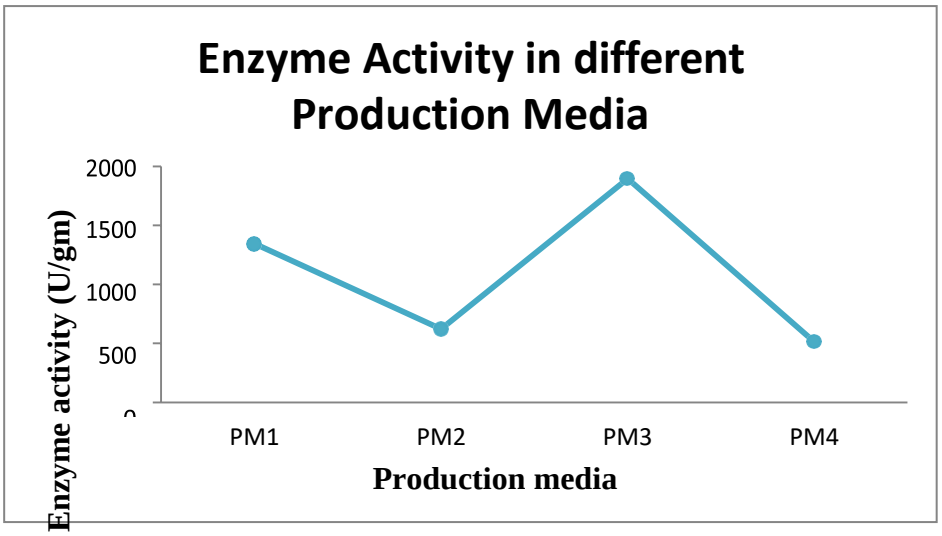


Fig: Enzyme Activity in different Production Media

5.3. Enzyme production at different time periods:

Enzyme synthesis is related to cell growth and therefore there is a co-relation between incubation period and enzyme production. The graph of protease production proceeded gradually reaching a maximum value at 48 hours for all the isolates.

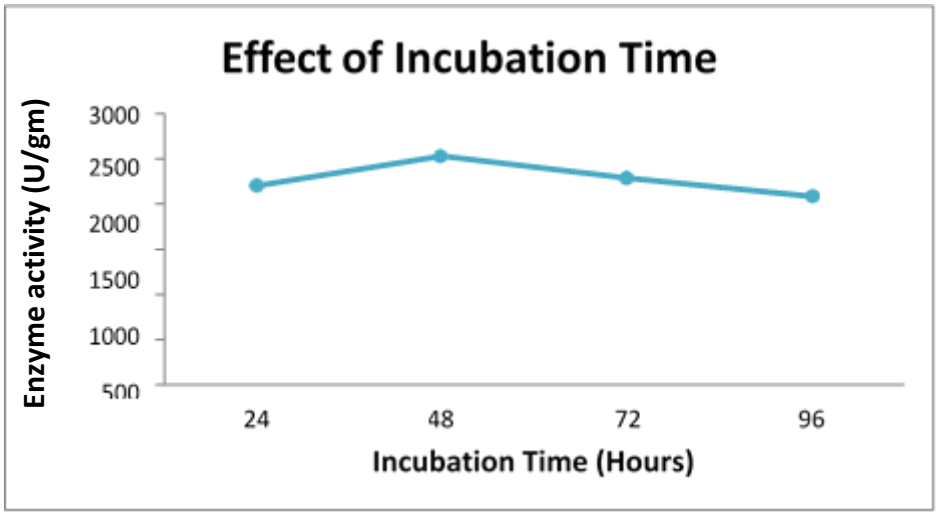


Fig: Effect of Incubation Time

6) Characterization of crude protease

6.1. Effect of pH on enzyme activity

From the pH optimization study, shown in Fig., it was clearly observed that pH 9.0 were shows maximum enzyme activity i.e. 1791.88 U/gm.

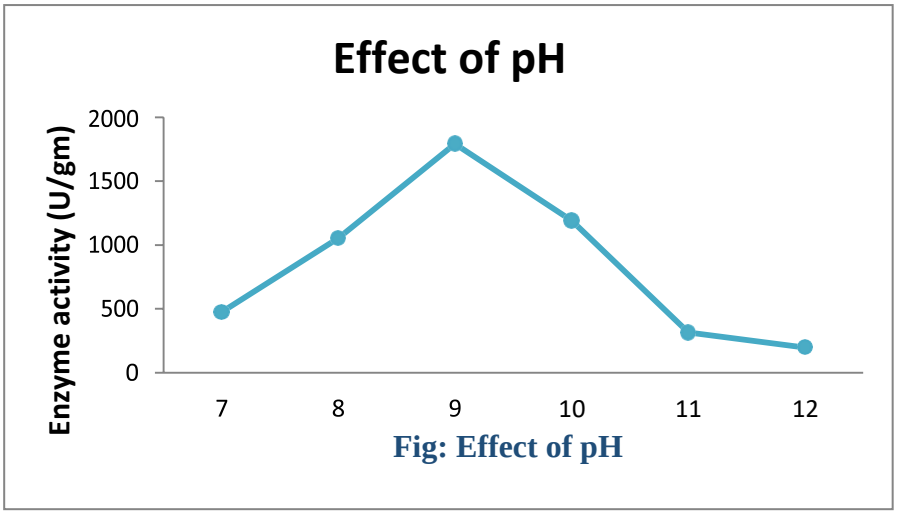


Fig: Effect of pH

6.2. Effect of temperature on protease activity

Temperature also played an important role in activating and inactivation of enzymes. In the present study, the effect of temperature on enzyme activity was studied with various temperatures ranging from 30-50°C. The protease activity is relatively stable at 37°C temperature (883.1 U/gm).

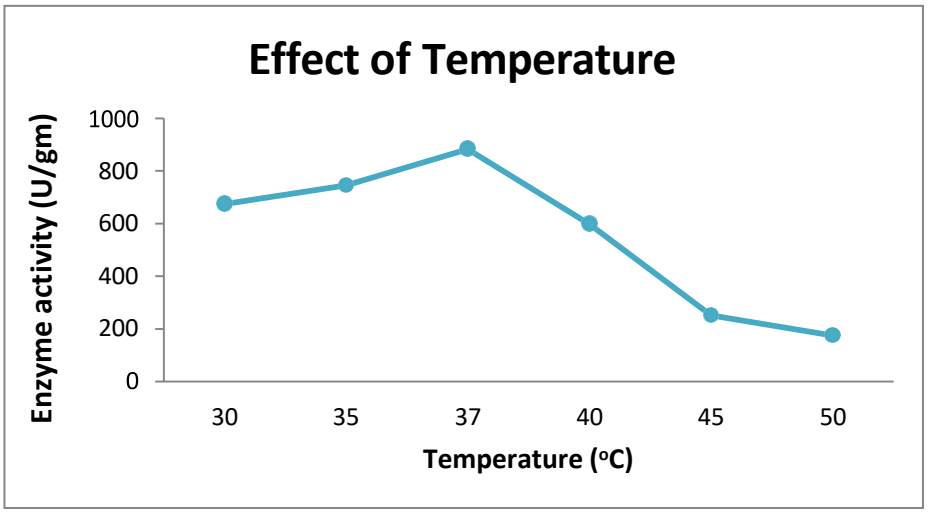


Fig: Effect of temperature

6.3. Effect of metal ions on protease activity

Storage stability is a prominent factor for commercialization of an enzyme. With the view of increasing the storage stability, the effect of various metal ions on crude protease was investigated. Fig. shows that the enzyme activity increased by 30.21%, 27.8%, 24.4%, 27.34% with Ca2+, Mg2+, Mn2+, Cu2+ respectively, and decreased by 19.12% with Hg2+. This phenomenon indicates that the enzyme requires metal ions as cofactors.

These results correlate with the observations of Kunamneni *et al.*, 2003 who found that Mg2+, Ca2+ and Mn2+ increased the enzyme activity by 16%, 35% and 8%, respectively, while Hg2+ reduced the activity by 7% and has also been reported by Takeda *et al.*, 2000.

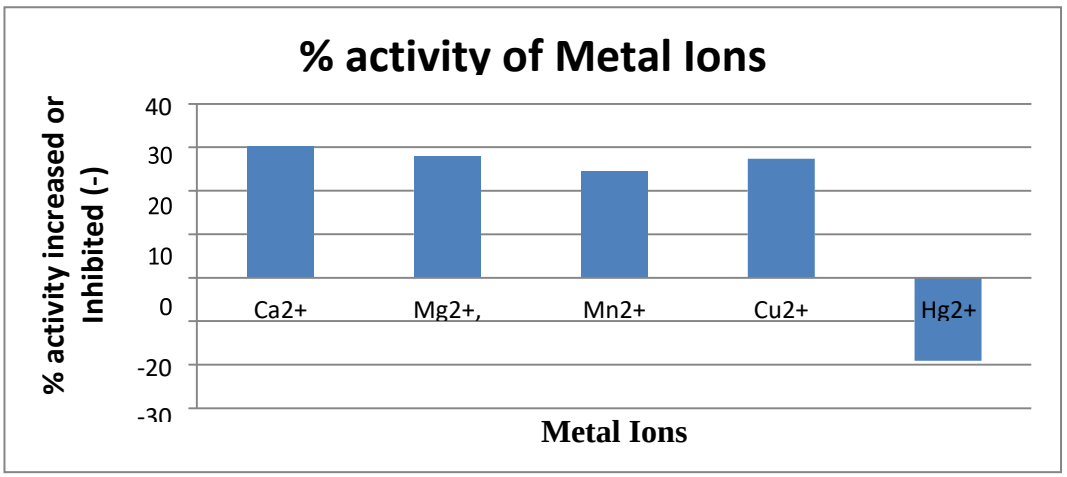


Fig: Percent activity of metal ions

6.4. Enzyme activity and growth rate

The enzyme activity of isolate I2 i.e. *Bacillus brevis* bacteria and cell growth are exhibited. The enzyme activity increased as the incubation times increased. Growth rate increased sharply with increasing incubation times. The highest absorbance of growth was achieved after 240 minute incubation. The proteolytic activity increased with cultivation time, and reached the highest enzyme activity (1320 U/gm) after 180 minutes of incubation. After the highest absorbance the constant absorbance recorded at that time enzyme activity get decrease.

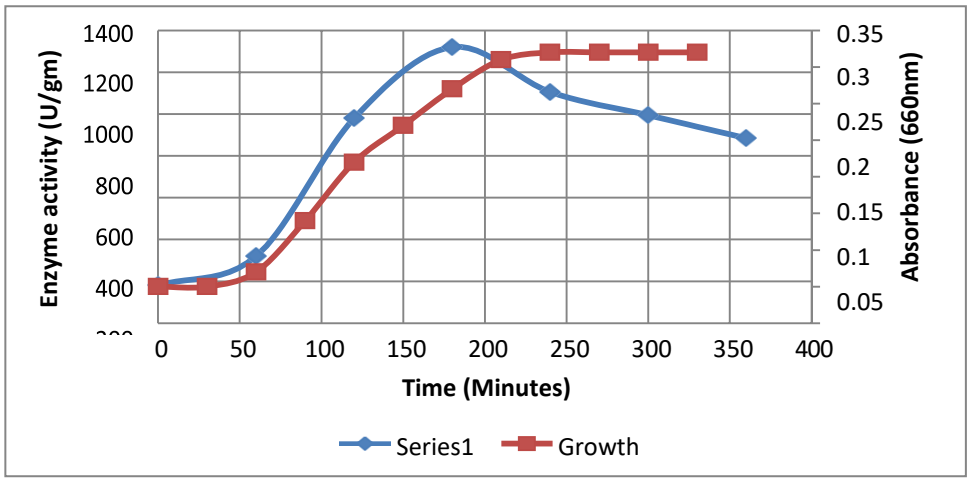


Fig: Graph of enzyme activity v/s growth

Conclusion:

Isolated species of *Bacillus* was identified by morphological and biochemical testes, they were *Bacillus insolitus, Bacillus brevis, Bacillus marinus, Bacillus pasteurii,* and *Bacillus macquariensis* respectively.

1. Alkaline protease produced from isolate I2 i.e. *Bacillus brevis by* using submerged fermentation.
2. The media number 3 (PM3) were the best media for all *Bacillus brevis* for Alkaline protease production.
3. Characterization of crude alkaline protease
   - Optimum enzyme activity at pH 9.
   - Optimum enzyme activity at temperature 37ºC.
   - Enzyme activity rise in the presence of Ca2+, Mg2+, Mn2+, Cu2+ metal ions and decrease in the presence of Hg2+.

4. In laboratory scale fermenter study shows the relationship between growth rates V/S enzyme activity. The enzyme activity increased as the incubation times increased. Growth rate increased sharply with increasing incubation times.

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