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A STUDY OF ALKALINE PROTEASE PRODUCTION BY ALKALOPHILIC BACTERIAL ISOLATE

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ABSTRACT

13 different alkaline protease producing bacterial isolates were isolated from soil of salt pan, (Div) Saurashtra, Gujarat. India. Among them isolate Ksa7 was isolated at 10.0 and11.0 pH on casein agar medium on the basis of zone of clearance which gave maximum alkaline protease activity(84 U/mL) respectively in submerged condition. Wheat bran and Green gram husk induced the protease production of alkaline protease when used as substrate with different dilution with casein broth. The production of alkaline protease enzyme was carried out through solid state fermentation using green gram husk as a substrate. The crude enzyme was more active at pH-10, temperature 60° C and enhanced activity was found in presence of Mg^{+2} with 1% casein as well 0.5ml enzyme concentration, where as enzyme activity was inhibited in the presence of Cu^{+2}, Mn^{+2}, Ca^{+2} metal ions.

Keywords: ***Alkaline Protease, Submerged condition, Solid state fermentation***

I. INTRODUCTION

"Proteases are peptidyl-peptide hydrolyses and are industrially useful enzymes which catalyze the hydrolysis of peptide bond from protein molecule."[1].Proteases are one of the most important classes of biocatalyst from an industrial point of view, occupying a major share (60%) of total enzyme market. These biocatalysts hydrolyze peptide bond in protein and hence classified as hydrolases and categorized in the subclasses peptide hydrolases or peptidases [2].Proteases are found in a wide diversity of sources such as plants, animals and microorganisms.Alkaline proteases are of great value due to their wide use in detergent, tanning, textile, and dairy industries, organic synthesis, peptide synthesis instant recovery of silver from photographic plates and waste water treatment[3]). Alkaline protease constitutes 60 to 65% of the global industrial enzyme market [4].Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds and yeasts. In bacteria, this enzyme is produced mainly by many members belonging to genus *Bacillus* especially, *B.licheniformis; B. horikoshii, B. sphaericus, Bacillus furmis, Bacillus alcalophilus, Bacillus subtilis* [2].Alkaliphiles are defined as organisms which exhibit optimum growth in an alkaline pH environment, particularly in excess of pH 8, and generally in the range between pH 9 and 10. Alkaliphiles may also be found living in environments having a pH as high as 12. Obligate Alkaliphiles are incapable of growth at neutral pH. Nowadays Protease is very important enzyme in many industries and so there is a need for improved microbial alkaline protease production.

The aim of the study was to screen potent alkalophilic bacterial isolate from soil for enzyme production in submerged fermentation, effect of different parameters for maximum alkaline protease production under

solid state fermentation and Effect of pH, temp, and metal ion on enzyme activity

II. MATERIALS AND METHODS

Isolation and Characterization of Alkalophilic protease producing isolates:-

The soil samples were collected from salt pan soil containing high salt from Dist: Saurashtra, Gujarat, India. 1 gram of soil was dissolved in 10 ml of sterilized water in a test tube, and serially diluted to 10^{-5} dilution. Finally 0.1mL from 10^{-2} to 10^{-5} dilutions was spread on casein agar plates and incubated at 37°C for 24-72-h. Different colonies were picked from plates depending upon the growth and clearance zone of casein hydrolysis and transferred further on nutrient agar plates containing 1% peptone, 0.3% meat extract, 0.5% sodium chloride, 3% agar and pH 7.4. Plates were incubated at 37°C for 24h in incubator and streaked on slants for obtaining pure culture. Selected isolate was biochemically characterized by Gram's reaction, Motility Test, Spore Staining, carbohydrate fermentation, oxidase test, O-F test, H2S production, IMViC tests, NO2 reduction, starch hydrolysis, KOH Test , Vancomycin Test, as per the standard methods and was confirmed by Vitek 2 systems version:05.04

III. EFFECT OF NACL ON THE GROWTH OF THE ISOLATE

The isolate were screened for their salt tolerance level by growing them into nutrient broth tubes (containing varied NaCl) concentration i.e., 4%, 6%, 8%, and 10%. Each of this NaCl concentration containing 10 mL sterile nutrient broth was used for the experiment. All the sets were inoculated with actively growing culture which contain 10^6 Cells/mL, and incubated at 37°C for 24 h. After the incubation time optical density was measured at 660 nm.

IV. PROTEASE PRODUCTION ABILITY OF THE ISOLATES

4.1 Protease Production media and Culture Condition

The Cultures were grown in media containing yeast extract (4g/l), KH_2PO4 (0.5g/l). K_2HPO_4 (0.5g/l), NaCl (0.1g/l) ,casein (10g/l), pH 10. in 250 mL erlenmeyer flask. The flask was sterilized in autoclave at 121°C (15lb) for 15 minutes. After cooling the flask was inoculated one colony (approximately 10^6 cells/mL) in each flask. Flask were incubated on orbital shaker at 100 rpm at 37°C for 48 h .

4.2 Extraction of Enzyme

4.2.1 From Submerged Fermentation

After incubation, 1mL of broth from flask was removed under aseptic condition and mixed with 49 mL of (0.1M) sodium glycine buffer (pH 8.0). The contents were mixed properly and centrifuged at 8000rpm for 20 minutes. The supernatant was used as a crude enzyme source for enzyme assay.

4.2.2 From Solid Fermentation

After incubation time,1 gm solid media was taken from the flask in sterile condition and mix with 50 mL glycine sodium buffer (pH 8), mix it thoroughly for 5-10 minutes then centrifuged it at 8000rpm for 20 minutes using REMI- centrifuge.. The supernatant was used for the enzymatic assay.

4.3 Protease Enzyme Assay [5]:-

Protease is measured by determining the extent of hydrolysis of casein by the lowery's method that reacts with FCR to give blue colored complex. The color formed is due to the reaction of Na_2CO_3 with the protein and the reduction of phosphomolybdic and phosphotungstic components in the FCR by the amino acid tyrosine and tryptophan present in substrate. After performing enzymatic assay, one potent isolate was screened for further study from 13 different isolates which were selected on the basis of size of zone of clearance.

4.4 Selection of Potent Protease Producers:-

Total 13 protease producing strain were isolated from soil and their enzyme activity was calculated. Among them isolates ksa7 was selected on basis of its enzyme activity. The isolate were selected for further studies.

4.5 SSF Media Preparation:-

25g of desired solid substrate wheat bran, green gram husk was suspended in 52.5 mL of casein broth with or without casein used in a 250mL flask. Control was also taken with water and no other growth media. It was autoclaved at 15 lbs pressure, 121ºC for 15 minutes.

4.6 Fermentation process for Solid State Fermentation (SSF):-

Solid state fermentation may be defined as those in which microbial growth and product formation occur on the surface of solid substrate. Solid state fermentation holds tremendous potential for the production of enzyme.

4.7 Selection of Suitable Media for substrate for SSF

The unoptimized and optimized solid state fermentation was carried out in 250 mL cotton plugged Erlenmeyer flask. 25g of desired solid substrate wheat bran, green gram husk was suspended in 52.5 mL of casein broth with or without casein used in a 250mL flask. Control was also taken with water as media. The media was also used at different dilution. (1:2, 1:5, 1:10, 1:20, Neat control) It was then autoclaved at 15lbs pressure, 121ºC for 15minutes.The above-prepared medium was inoculated with (48hr grown) 2.5mL of inoculum. After thorough mixing, all the flasks were incubated at 37^0C temperature for 48 h in static condition in incubator (Nova). After a regular period, samples were drawn from each flask for extraction of enzyme and perform above enzyme assay method.

V. EFFECT OF DIFFERENT PARAMETERS ON ENZYME ACTIVITY

5.1 Effect of pH on Enzyme Activity

The enzyme was extracted from 1 g SSF in buffer of pH 7,8,9,10,11 keeping all the conditions of the assay same.

5.2 Effect of Temperature on Enzyme Activity

The enzyme was extracted from 1 g SSF in buffer. The influence of the temperature on the activity of protease was determined by incubating the enzyme reaction mixture at different temperature (40^0 C, 50^0 C, 60^0 C, 70^0 C) keeping all the other parameters of the assay same.

5.3 Effect of Metals on Enzyme Activity

The enzyme was extracted from 1 g SSF in buffer and assay system as interacted with metals in fixed concentration. The effect of metal ions (Ca^{+2}, Cu^{+2}, Mn^{+2}, Mg^{+2}) at 50ppm, 100ppm, 150ppm in chloride form were added to reaction mixture. The enzyme activity was compared to control keeping all the other parameters of the assay same.

VI. RESULTS AND DISCUSSION

6.1. Isolation and Selection of Alkaline Protease producers:-

In the present study 13 isolates were obtained in pure culture after subculturing on casein medium and nutrient agar medium. One bacterial isolateKsa7 was selected for protease production using SSF on the basis of enzyme activity it produced 84.0 $UmL^{-1}min^{-1}$. The isolated bacterial strains were screened for protease producing ability on skim milk agar which formed zone due to casein. **Fig.1.**

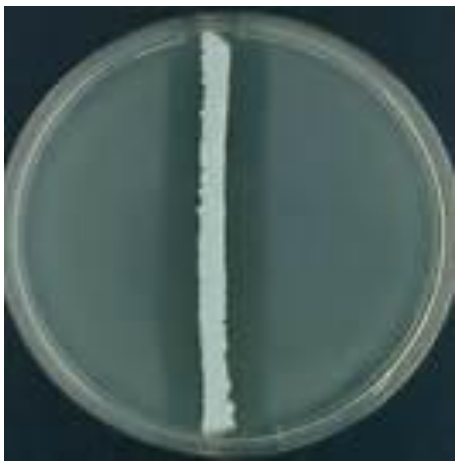

Fig 1:Zone of clearance observed during growth of Ksa7

6.2. Identification of Alkaline Protease producers:-

The bacterial isolate were found to be Gram positive, motile, spore forming medium rod shaped bacteria. The above results were confirmed by KOH and Vancomycin test. The isolate Ksa7 was identified on the basis of different biochemical tests performed. For further conformation the isolate Ksa7 was sent to Supra Tech laboratory where it was identified as ***Bacillus lentus*** using Installed VITEK 2 Systems Version: 05.04 . The results of the biochemical characterization of the selected isolate are shown in **Table1** .

Table1: Biochemical test on basis of VITEK 2 system *Bacillus lentus* (Ksa7)

No.	Test	Result
1	BETA-XYLOSIDASE	-
2	L-LYSINE-ARYLAMIDASE	+
3	L-ASPARTATE ARYLAMIDASE	-

4	LEUCINE ARYLAMIDASE	+
5	PHENYL ALANINE	+
6	L-PROLINE ARYLAMIDASE	-
7	BETA-GALACTOSIDASE	+
8	L-PYRROLIDONYL- ARYLAMIDASE	-
9	ALPHA-GALACTOSIDASE	-
10	ALANINE ARYLAMIDASE	+
11	TYROSINE ARYLAMIDASE	+
12	BETA-N-ACETYL GLUCOSAMINE	-
13	ALA-PHE-PRO- ARYLAMIDASE	+
14	CYCLODEXTRIN	-
15	D-GALACTOSE	-
16	GLYG	-
17	INO	-
18	METHYL-D-GLUCOPYRANOSIDE	-
19	ELLMAN	+
20	MDX	-
21	ALPHA-MANNOSODASE	+
22	MTE	-
23	GYLA	-
24	D-MANNITOL	-
25	D-MANNOSE	-
26	D-MLZ	-
27	N-ACETYL-D-GLUCOSAMINE	-
28	PLEATINOSE	-
29	IRHA	-
30	BETE-GLUCOSIDASE	+
31	B-MAN	-
32	PHC	-
33	PVATE	-
34	ALPHA-GLUCOSIDASE	+
35	DTAG	-
36	D-TREHALOSE	-
37	INU	-
38	D- GLU	-
39	D-RIBOSE	-
40	PSCNA	-

41	GROWTH IN 6.5% NACL	-
42	KANAMYCINE RESISTANCE	-
43	OLD	-
44	ESCULIN HYDROLASE	+
45	TTZ	-
46	POLYMIXIN-B RESISTANCE	-

6.3. Effect of NaCl on the Bacterial Growth:-

The isolate was grown in a range of 4 to 10% NaCl concentration and optical densities were measured at 660 nm. Isolate Ksa_7 showed optimal growth at NaCl concentration of 8% respectively Fig 2. Similar results were obtained by [6], who reported that *Bacilli spp* C3 and S5 obtained showed optimum growth at 0.2M NaCl .[7] also reported that *Alkali bacillus* HSD 20 and *Virgibacillus pathothetics spp.* isolated from solar salt pan of Ribander, Goa showed optimum growth value in the range of 5%-15% NaCl, and thus can be classified as moderately halophilic bacteria.

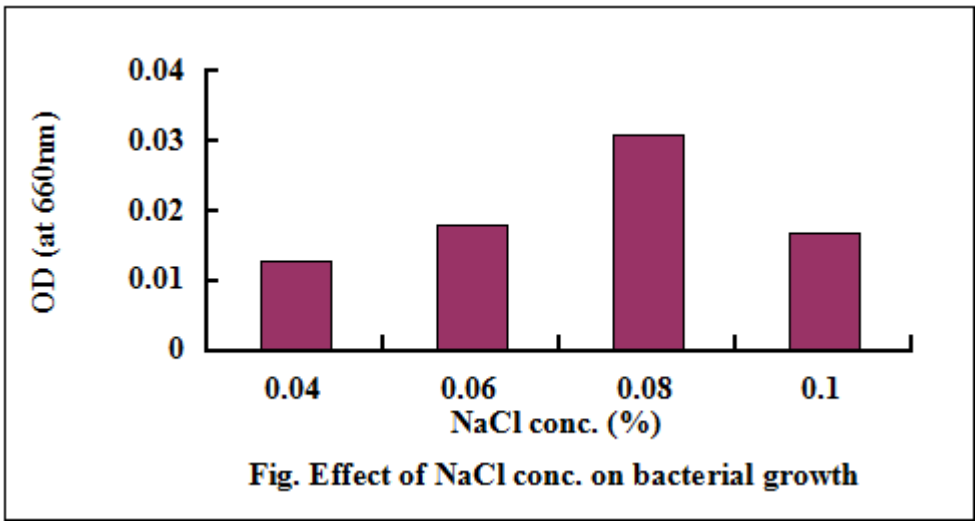

Fig2: Effect of NaCl on Ksa7

6.4. Solid State Fermentation:-

Fermentation condition plays most important role in the production of any metabolites. In present study, solid state fermentation was carried out for production of protease using the potent isolates. The isolate Ksa7 was studied for protease production using SSF. using various substrate were studied i.e., Wheat bran and Green Gram Husk. Green gram husk was found to be a better substrate for protease production.

6.4.1Selection of Substrate for Protease Production:-

In solid state fermentation process, the selection of suitable substrate is critical factor. To study the selection of substrate for protease production, a biomass source would be economically viable and its availability is also important for production of valuable enzyme. Here two substrate i.e., wheat bran, green gram husk with or without casein containing growth media in different dilution were used for protease production.

6.4.1.1Protease Production on Wheat Bran. (Isolate Ksa7)

In medium dilution 1:2, 1:5, 1:10, 1:20, neat, control the protease activity for the isolate Ksa7 were 1236U/gds, 789.06U/gds, 140.6 U/gds, 145U/gds, 168 U/gds, 84.36 U/gds respectively after 120 h**.** In our study medium dilution 1:2 and 1:5 gave highest protease activity than other dilutions Fig 3**.**

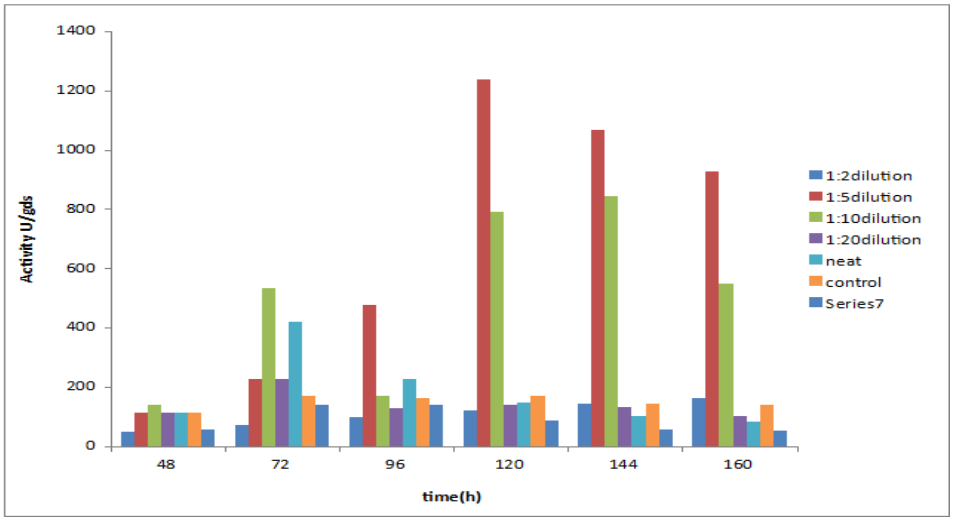

Fig3: Protease Production on Wheat Bran. (Isolate Ksa7)

6.4.1.2.Protease production on Green Gram Husk (Isolate Ksa7)

The enzyme productionfor isolate Ksa7 was 2193.33 U/gds and 2025 U/gds with same media dilution after 120 h.

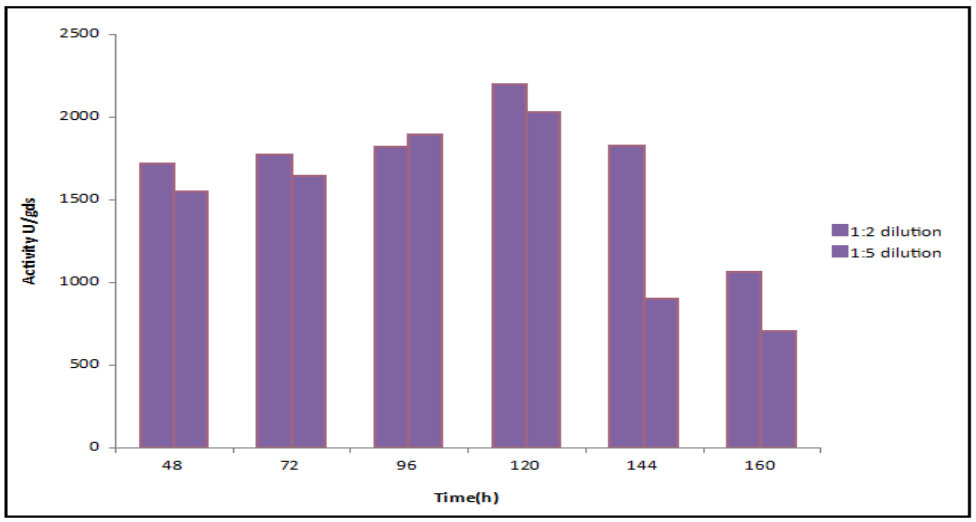

Fig 4: Protease Production on Green Gram Husk (Isolate Ksa7)

Kuberan *et al.,* (2011) maximum protease production with green gram husk (2702.401U/mL) for *Bacillus spp. Tk1* and *Bacillus spp. Tk2* [8]. They obtained less protease production in wheat bran (860.904U/mL) for *Tk1* and cotton seed (907.099) U/mL) for *Tk2.M*aximum enzyme production (9550U/g biomass) was obtained with green gram husk, while minimum protease production (350U/g biomass) with red gram husk [9].Enzyme production

(4059.9U/mg) was reported with lentil husk after 120 h of fermentation for this substrate[10].Studying different solid substrates used for the production of alkaline protease by *Bacillus subtilis* KHS-1. maximum protease production was observed with green gram husk (4764 U/g Biomass) followed by soya bean meal (4514 U/g Biomass), wheat bran (4416 U/g Biomass), rice bran (4268 U/g Biomass), ground cake (3845 U/g Biomass), coconut nut cake (2756 U/g Biomass), rice husk (3625 U/g Biomass) and maize bran (3348 U/g Biomass) [11]. The reason may be that the medium with green gram husk might contain the protein components and mineral nutrients required for the growth of the bacterium, thereby enhanced protease production was observed in comparison to other solid substrates.

6.5. Effect of Different Parameters on Enzyme Activity:-

6.5.1.Effect of pH on crude enzyme activity:

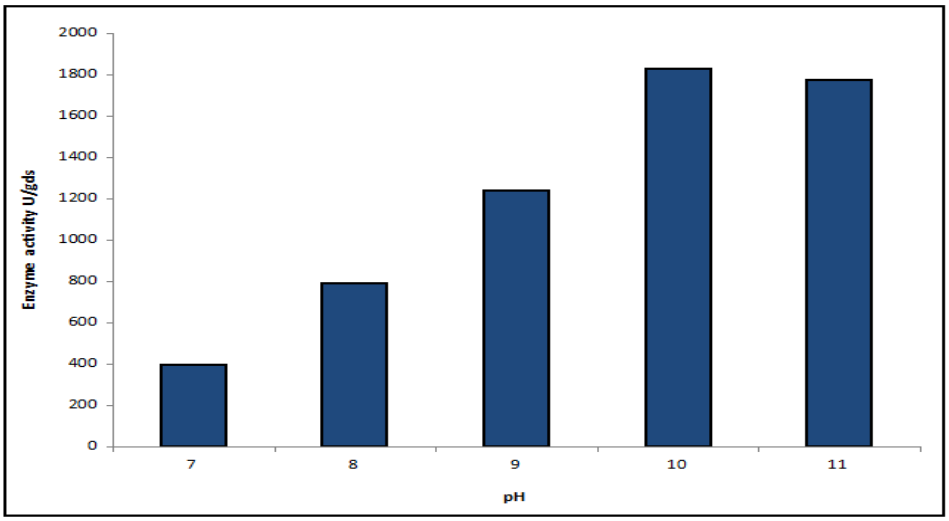

Fig 5: Effect of pH on enzyme activity

The Effect of pH on alkaline protease enzyme activity was characterized by pre- incubating crude alkaline protease enzyme in the pH range of 7.0 to 11.0 for (20min). The result showed that crude alkaline protease enzyme was more active in alkaline pH and showed maximum activity at pH 10.0.maximum protease activity was obtained at 10 pH with *Bacillus odysseyi* and maximum activity of protease at 9.5 with Cohnella *thermotolerans*[12]. Alkaline protease was found to be active at pH 9.5 with casein as substrate in *Bacillus subtilis AKRS3*[13]. Our study showed that maximum protease activity was obtained at 9.0 to11.0 pH and optimal was at 10.0 to extreme alkaline pH (Fig5).

6.5.2.Effect of temperature on enzyme activity:

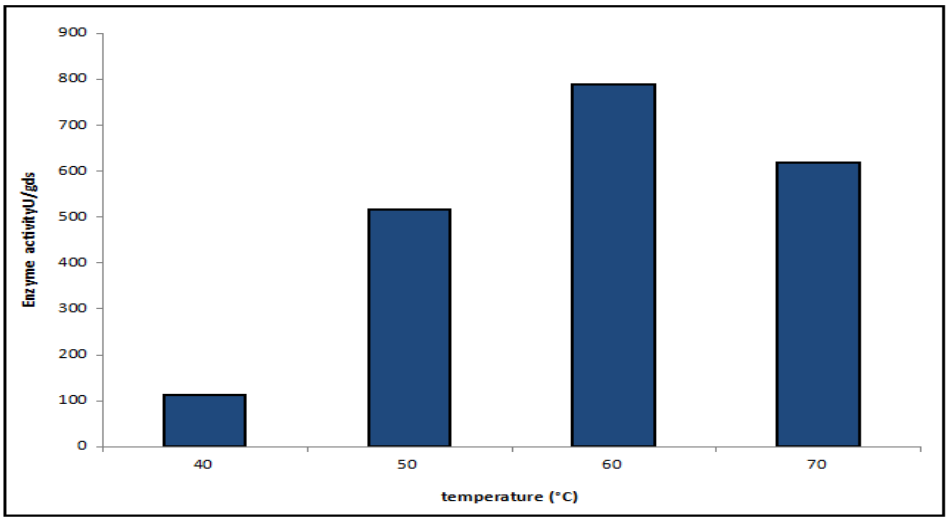

Fig 6: Effect of temperature on enzyme activity

The crude enzyme was pre incubated at the temperature range (40-70°C) for 20 min. The obtained result indicated that protease gave highest activity (787.5U/gds) at 60°C(Fig 6). An increase in enzyme activity was observed up to 60°C. Maximum protease activity has been reported at 60°C[14]. Enzyme from *B. subtilis* NCIM 2724 and *Neisseria spp.* was found to be stable up to 65°C whereas enzyme from *P. aeruginosa* was stable up to 55°C[15]. Optimum temperature for protease activityhas also been reported as 60°C[16,17,18].

6.5.3.Effect of metal ions on enzyme activity:

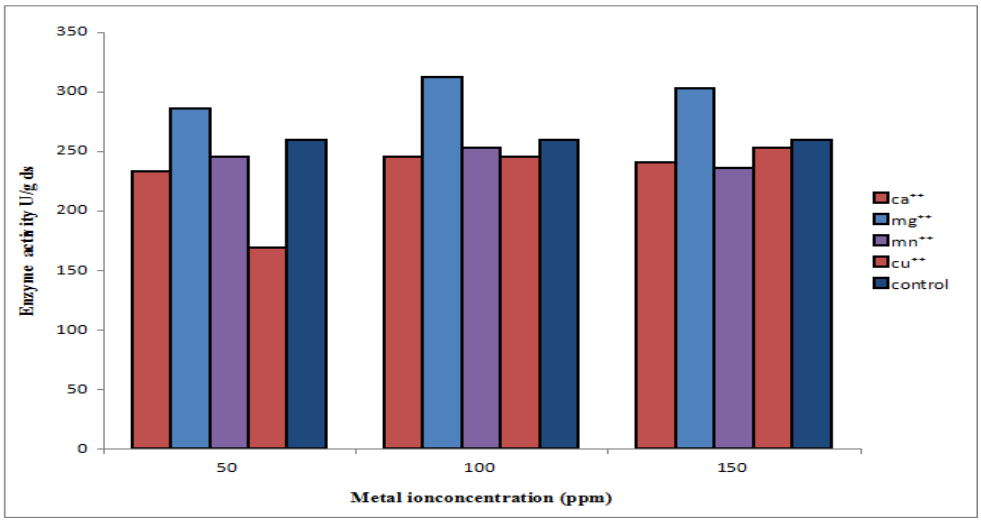

Fig7: Effect of metal ions on enzyme activity

Crude protease enzyme was pre incubated with different metal ion of different concentration for (20mins). Obtained result showed that Magnesium showed enhanced enzyme activity while others Copper, Calcium, and Manganese show inhibitory effect on enzyme activity. In the present study the Mg^{+2} ion was shown to play the major role in increasing the enzyme activity on 100 ppm concentration of metal while reduced activity above 100 ppm concentration (Fig 7). The Protease activity was inhibited by Zn^{+2}, Ni^{+2} and Sn^{+2} and increased by Ca^{+2}, Mg^{+2} and Mn^{+2}.[19] Mg^{+2} and Ca^{+2} ions enhanced protease activity up to 128%.[20]

VII. CONCLUSION

The finding of optimization concluded that physical and nutritional parameters also play an important role in protease production. . The further investigation is required to make use of the full potential of this (Ksa7) isolate for protease production by employing molecular biology and genetic engineering.techniques.

VIII. ACKNOWLEDGEMENTS

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