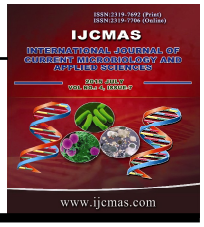
Int.J.Curr.Microbiol.App.Sci (2015) 4(8): 597-606

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 8 (2015) pp. 597-606**
http://www.ijcmas.com



Original Research Article

Screening and Isolation of Protease Producing Bacteria from Soil Collected from Different Areas of Burhanpur Region (MP) India

Dalal Rupali*

Department of Biotechnology, Prof. BrijMohan Mishra Institute Of Medical & Technical Sciences, Khandwa Road, Zhiri Burhanpur 450331 MP, India

**Corresponding author*

Keywords

Protease, Soil, Maximum activity, Bacteria, Fermentation time, Tyrosine standard curve, Detergent

A B S T R A C T

The objective of present study was to screen and isolate protease producing bacteria from soil samples collected from farm soil, garden soil of BIMTS college campus and oil spilled area of Burhanpur. Soil samples were serially diluted and 0.1ml of sample was spread on skim milk agar, at 37^{o} C for 48 hrs. Total six bacterial colonies from garden soil showed clear zone around the colony indicating protease activity. Among these, GS-P4 isolate produced highest protease activity and was identified as *Bacillus Sp*. by morphological and biochemical test. Moreover, various physiological characters were studied like pH, temperature, fermentation time. The protease showed maximum activity at pH 7.4 and the temperature for maximum protease activity was found to be 60^{o}C. The unknown concentration of crude protease was determined using tyrosine standard curve and found to be 0.14µmole. Maximum activity of protease enzyme was 0.0233U/ml. The isolated protease also give better result of washing with detergent. Hence it can be added in detergent. The above results indicate that these bacterial isolate can be use as biotechnological tool for industrial purpose.

Introduction

To produce environmental eco-friendly products and product out puts chemical process are being replaced by enzymes like proteases (Abebe et al., 2014). The production of enzymes is central to the modern biotechnology industry. The technology for producing and using commercially important enzyme products combines the discipline of microbiology, genetics, biochemistry and engineering (Rajni Hatti Kaul).Enzymes are biocatalysts produced by living cells to bring about specific biochemical reaction generally forming parts of the metabolic processes of cells (Mohammad B D et al.2013).Proteases, one among the three largest groups of industrial enzymes, account for about 60% of the worldwide sale of enzymes(Nurullal A et al 2011). Proteases which include proteinases, peptidases or proteolytic enzymes break peptide bonds between amino acids of proteins. They use a molecule of water for this and are thus classified as hydrolases.Proteases are of two

types exopeptidases and end peptidases (Grewal S et al 2010).

Based on their acid base behaviour, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by Fungi. Proteases having pH optima in the range of 7.0 or around are called neutral protesaes. Neutral proteases are mainly of plant origin while proteases having optimum activity at pH range 8 and above are classified as alkaline proteases (H. S.Alnahdi.2012).Alkaline protease in fact the first enzyme produced in bulk (M.Kalpana Devi et al 2008).Protease constitutes 59% of the global market of industrial enzymes. The major use of free proteases occur in dry cleaning,detergents,,meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds(Fekadu Alemu ,2015). Bacterial Proteases are preferred as they grow rapidly, needless space, can be easily maintained and are accessible for genetic manipulations (Odu. N.N et al .2012).

Currently, a large Proportion of commercially available proteases are derived from *Bacillus* strain (Sevine N et al.2011). In textile industry, Protease is being used to remove the stiff and dull gum layer of sericin from raw silk to achieved improved lustre and softness (Rashesh .D et al.2001).Since, Burhanpur is known for textile industry and Protease is one of the important enzymes used in textile industry. The presently available proteases are not sufficient to meet industrial demands. Hence; there is continuous search for new proteases with novel characteristics for industrial application from diverse bacteria isolates. Microbes from varied habitats have been examined by many researchers to obtain the industrially suitable proteases.

In the present study, soil was collected from different area of Burhanpur for screening of Protease producing bacteria and effect of physiological characters were studied.

Materials and Methods

Source of sample collection:

Soil samples were collected from different areas of Burhanpur that is garden soil of BIMTS college campus, farm soil, oil spilled soil. Soil Sample were collected below 5-6 cm depth and stored in sterile plastic bags at 4^{o}C.Date and time were noted on sample collecting bags.

Isolation of protease producing bacteria:

The techniques used for isolation of bacteria were serial dilution and spread plate method.1 gm of soil sample was weighed and serial dilution (10^{-1} to 10^{-6}) of each soil sample were carried out.0.1 ml of each aliquot was spread on skim milk agar (1%) plate at temperature 37^{o} C for 48 hr.The zone of hydrolysis was noted for each sample. The colony showing highest zone of inhibition was selected for further study. The colony was grown on nutrient agar plate repeatedly and preserved on nutrient agar slant at 4^{o}C.Based on the morphological and biochemical tests the bacterial isolate was identified (Sneath HAP et al, 1986).

Identification of bacteria: The identification of bacteria was carried out by morphological studies i.e. staining including Gram staining, motility test Acid Fast test, Endospore staining. Cultural characterization on agar plates like colony morphology that is shape, size, margin, elevation, opacity, texture and pigmentation

and also growth in broth and biochemical test includes catalase test,oxidase test, carbohydrate fermentation test,indole,methyl red, citrate utilization test, Voges Proskauer test,H_2S production test, Starch hydrolysis test,urease production test, nitrate reduction test (Aneja K R).

Qualitative test for protein: To identify crude sample as protein some test were carried out as biuret test, Ninhydrin test, Millon's test, Xanthoproteic test and Sulphur test. This test gives colour reactions on the basis amino acid present (Deb A C, 1996).

Quantitative assay of protein: The total protein content of the samples were determined by Lowry's method.(Nighojkar A, 2007).The protein standard used was Bovine Serum Albumin(BSA) (1mg/ml).

Preparation of casein solution: Casein was used as substrate. It was prepared from alkali soluble casein which was dissolved in 10 ml distilled water. The insoluble portion was dissolved by addition of the alkali. The pH was adjusted to 8.0 with 0.1 M sodium hydroxide.

Crude enzyme preparation: The protease producing bacterial colony was inoculated in casein broth medium.It was incubated at 37^{o}C for 48 hrs.Using Whatmann No.1 filter paper cultured medium was filtered aseptically in laminar air flow.The filtrate was subjected to centrifugation at 10,000rpm for 10 minutes to remove unwanted particles.The supernatant was used as crude enzyme preparation for further studies.

Protease activity assay: To study proteoloytic activity, supernatant was used as enzyme source.1%casein in 0.1 M phosphate buffer and pH 7.0) was used as substrate.1ml enzyme and substrate was incubated at 50^{o}C for 60 min.To stop the reaction 3ml Trichloroacetic acid was used. One unit of protease activity was defined as the increase of 0.1 unit optical density at 1 hr incubation period. Then it was centrifuged at 5000 rpm for 15 min.From this ,0.5ml of supernatant was taken, to this 2.5ml of 0.5 M sodium carbonate was added, mixed well and incubated 20 min.Then it was added with 0.5ml of folin phenol reagent and the absorbance was read at 660 nm using Spectrophotometer(Bharat Pokhrel et al,2014.).The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity.

Effect of pH on enzyme activity: To study the effect of pH culture media pH was adjusted using different pH buffer ranging from 5.8 -8.0. It was incubated at 37^{o}C for 48 hrs.Enzyme activities was determined by standard enzyme assay.

Effect of temperature on enzyme activity: To study the optimum temperature where an enzyme shows its maximum activity the substrate with crude enzyme were exposed to different temperatures between 10^{o}C to 100^{o}C. Enzyme activities were determined by standard enzyme assay.

International unit: One protease unit was defined as the amount of enzyme that released 1µg of tyrosine per ml per minute under above assay conditions.

Effect of fermentation period on enzyme activity: The test organism was grown in nutrient broth containing 1% casein and 3% NaCl.It was incubated at 37^{o}C for 24,48,72,96 and 120 hr in an orbital shaker at 150rpm.the contents were then centrifuged at 10,000rpm at 4^{o}Cfor 10 min and protease activity was checked in the cell free extract(Balakrishnan Padmapriya.et.al 2012).

Washing test: Application of protease enzyme by isolated organism as a detergent additive was studied as per (Sidra Aftab et. al 2006). For this three white cotton clothes (5*5cm) were stained with blood and grass separately and following sets were then prepared and studied:

- Blood or grass stained cloth dipped in flask with distilled water (100ml).
- Blood or grass stained cloth dipped in flask with distilled water(100ml)+1ml detergent(7mg/ml)
- Blood or grass stained cloth dipped in flask with distilled water (100ml)+2mlenzyme solution.
- Blood or grass stained cloth dipped in flask with distilled water (100ml) +1ml detergent (7mg/ml) +2ml enzyme solution.

All four flasks were incubated at 60^{o}C for 15 min.After incubation, cloth pieces were taken out, rinsed with water and dried. Visual examination of cloth pieces exhibited the effect of enzymes in removal of stains. Untreated cloth pieces stained with blood and grass were taken as control.

Results and Discussion

Screening of Proteolytic activity: Various isolates were screened for protease activity on the casein agar plates. Protease activity was observed from the zone of hydrolysis observed on agar surface mentioned in table1. For further study the strain showing largest zone of hydrolysis was considered and designated as GS-P4 and maintained by repeated sub culturing.

Qualitative test for protein: To identify crude sample as protein some test were carried out as biuret test, Ninhydrin test, Millon's test, Xanthoproteic test and Sulphur test. This test gives colour reactions on the basis amino acid present (Deb A C, 1996).

Quantitative assay of protein: The total protein content of the samples were determined by Lowry's method.(Nighojkar A,2007).The protein standard used was Bovine Serum Albumin(BSA) (1mg/ml).

Protease assay:

Tyrosine standard curve: Tyrosine standard curve was plotted to measure the enzyme
Activities of protease .A standard solution was prepared by using 0.5 M of Na_2CO_3,50mM of glycine buffer,pH 10.00,1:1 diluted Folin reagent and 200 µg/ml of Tyrosine stock solution.A required amount test tube buffer and tyrosine were added to each test tube.2.5 ml 0.5 M Na_2CO_3 was added in each test tube subsequently, the mixtures were kept at room temperature for 10 min.After incubation 500µl of Folin reagent was added and further incubated for 30min at room temperature.A Solution of glycine buffer (pH-10),2.5ml of Na_2CO_3 and 0.5ml of Folin reagent were added in a test tube which served as a blank. The optical density was measured at 660nm using spectrophotometer and the standard curve was plotted (Bharat Pokhrel et al.2014).

Assay of protease enzyme activity:

Effect of pH on enzyme activity: To study the effect of pH culture media pH was adjusted using different pH buffer ranging from 5.8 -8.0. It was incubated at 37^{o}C for 48 hrs.Enzyme activities was determined by standard enzyme assay. The maximum activity of enzyme was found at pH 7.4.

Effect of temperature on enzyme activity: The protease activity is relatively stable in the temperature range 60-65^{o}C and retains 85.2% of its activity at 70^{o}C(.Balakrishnan

Padmapriya et al. 2012). In present study the maximum activity was found at 60°C.

Effect of Fermentation Period on Enzyme activity: The maximum protease production by bacteria was found at 48 to 72 hrs of incubation period. The present study shows maximum activity of Protease at 48 hrs of fermentation period.

Table.1 Showing zone of inhibition (mm)

Sr.No	Isolates	Zone of Inhibition(mm)
1.	GS-P1	15
2.	GS-P2	19
3.	GS-P3	21
4.	GS-P4	29
5.	GS-P5	12
6.	GS-P6	11

Table.2 Colony characters on nutrient agar plate

Size	Shape	Edge	Elevation	Opacity	Texture	Pigmentation
Big	**irregular**	**lobate**	**flat**	**opaque**	**Rough**	**white**

Table.3 Result of Staining of GS-P4

Sr.no	Staining	Result
1.	Gram Staining	Positive
3.	Endospore Staining	positive
4.	Motility	motile

Table.4 Biochemical characterization: various biochemical tests

Sr.no	Biochemical Test	Result
1.	Indole Production Test	Negative
2.	Methyl red Test	Negative
3.	Voges Proskauer Test	Positive
4.	Citrate utilization Test	Positive
5.	H_2S Production Test	Negative
6.	Urease Test	Positive
7.	Catalase Test	Positive
8.	Oxidase Test	Negative
9.	Starch Hydrolysis Test	Positive
10.	Nitrate reduction Test	Positive
11.	Gas Production from glucose	Negative

Table.5 Washing test of protease GS-P4

Stain	Water	Water+ Detergent	Water +Protease GS-P4	Water Detergent+ Protease GS-P4	Control
Grass	+	++	++	+++	**Untreated**

+ Poor removal of stain, ++Good removal of stains, +++ Very good removal of stain

Figure.1 Primary screening of protease producing bacteria

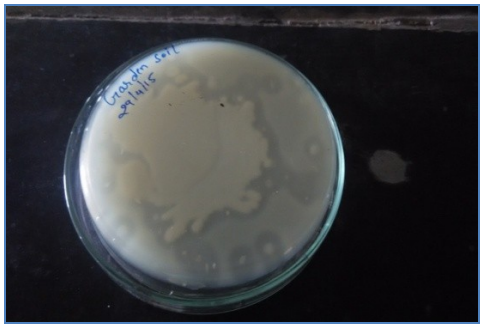


Fig.2 Zone of Inhibition on casein agar

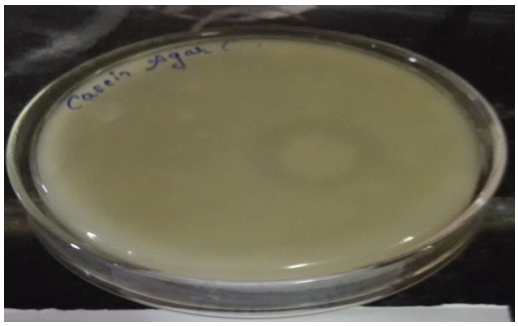


Fig.3 Isolated colony on nutrient agar

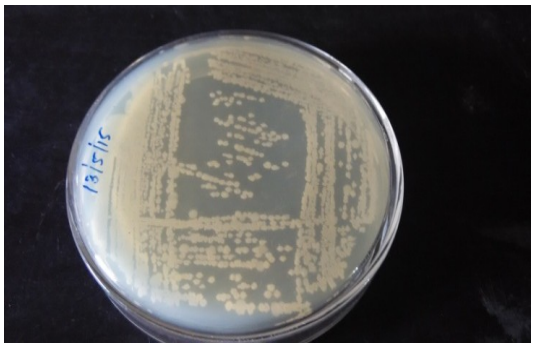


Figure.3 Qualitative test for Std.Protein and Test Sample

Figure.4 Lowry's method for quantitative test of protein

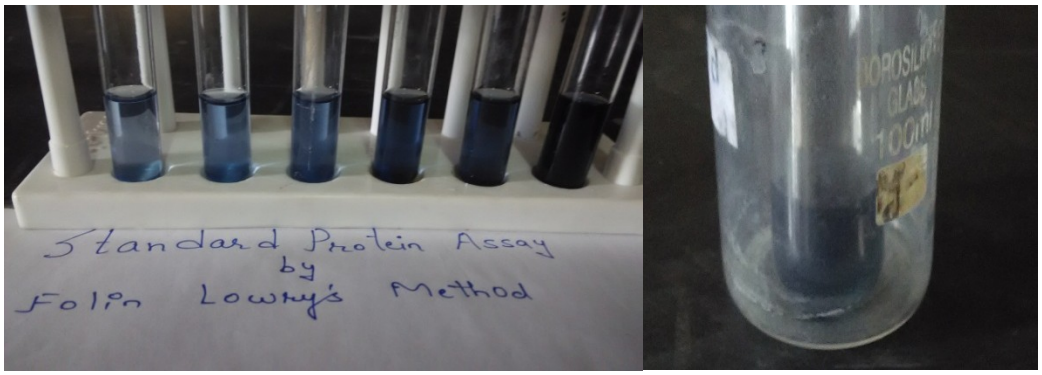


Figure.5 Tyrosine standard curve

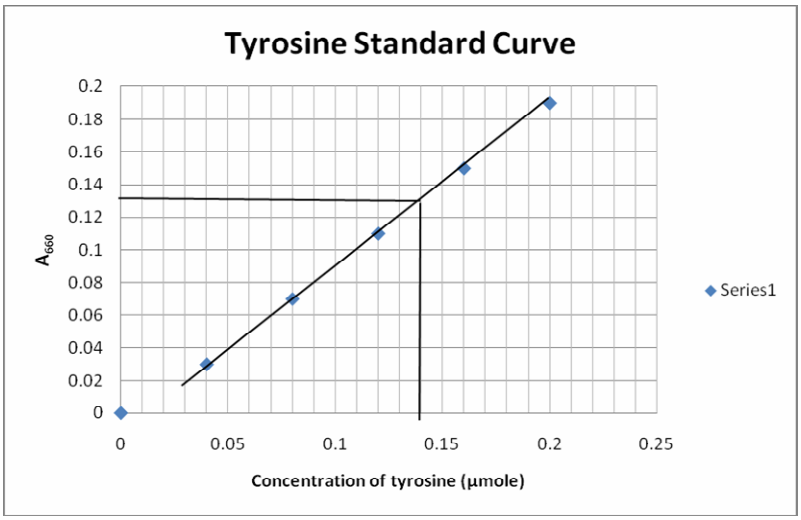


Figure.6 Effect of pH on Protease activity

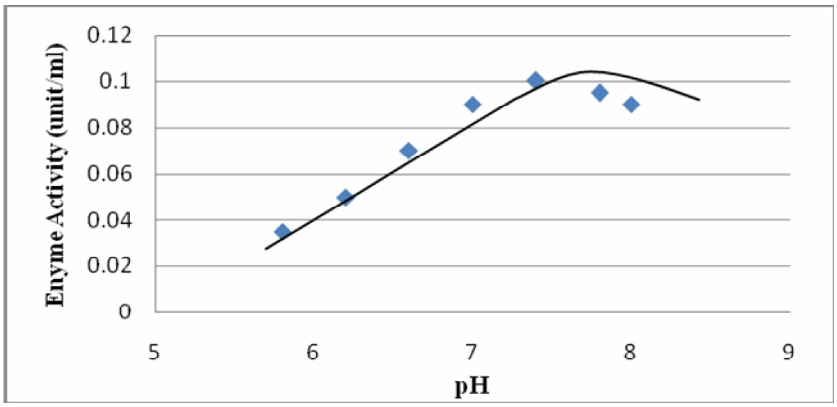
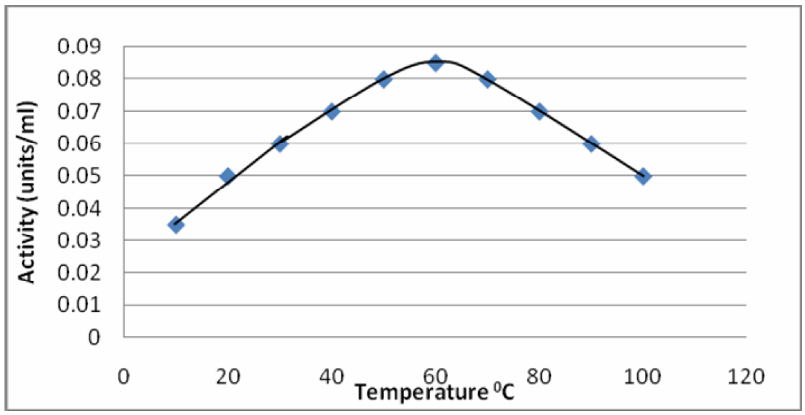


Figure.7 Effect of temperature on protease activity



It has been reported that the production of extracellular protease by different micro organisms can be strongly influenced by the culture conditions. *Bacillus sp*, was found to be predominant in soil. Hence 2% found to be *B.coagulans*, *B.stearothermophilus* and *B.lichenformis*, 4%were *B.cereus* and *B.circulans*,6% were ,8% were *B.*laterosporus,10% were *B.pumilis*,20% were *B.brevis* and 21% were *B.sphaericus* and *B.macerans* (Sidra A et al. 2006).In the present study six bacterial isolates shown proteolytic activity. Out of this, bacterial colony showing maximum proteolytic activity was studied further. The protease producer was identified as genus Bacillus *Sp.*Various physiological factors were studied like effect of pH,temp ,fermentation media, washing test. It is Gram positive, motile, catalase positive, spore former indole negative, VP positive, methyl red negative.

The optimum temperature was 60^{o}C as also reported by Sidra A et al. (2006).The maximum activity of enzyme shows as thermo stable and this property can be exploited in detergent application. It also shows better result of washing with detergent Sidra A et al.(2006). It was found that enzyme activity increases with rise in temperature reaches to maximum at 60^{o}C.Enzyme activity was stable with temperature within range of range of 40^{o}C to 70^{o}C.Enzymes was also still active at 80^{o}C. In literature, optima temperature have been reported between 30-70^{o}C for *Bacillus* sp. protease in Sevine N et al.(2011).Optimum

temperature for protease of *B.lichenformis* UV-9 was found to be 60^{o}C by Muhammad N et.al(2013). Abebe Bizuye et al (2014) reported optimum temperature for proteolytic activity of protease producing bacteria was 37^{o}C – 50^{o}C.Abebe Bizuye et al (2014) reported optimum temperature for proteolytic activity of protease producing bacteria was 37^{o}C -50^{o}C.

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Increase in pH shows increase in enzyme activity. Maximum activity at alkaline pH 9 was reported by Odu .N.N et al. (2012). In literature it was shown that the enzyme also gave high activity in the alkaline pH range 6.0-9.0(Sevine N et al. 2011). Abebe Bizuye et al (2014) reported optimum pH for Protease producer was pH 8-10.

In this present study soil of Burhanpur region shows presence of protease producers. The bacteria was screened and identified as *Bacillus* Sp.Qualitative and qualitative estimation were also studied.Various physiological factors were studied like pH, temperature, fermentation time, washing test. Keeping in view about use of Protease enzyme it can be harnessed for biotechnological processes. This organism can be very useful in textile industries Since Burhanpur is known for textile industry. Further experiments were carried out to enhance enzyme production for commercialized process is needed.

Acknowledgement

The author is very thankful to the President Mr.Amit Mishra, Vice President Mrs.Rakhi Mishra and Secretary Mr Anil Jain of Prof.BrijMohan Institute of Medical and Technical Sciences, Burhanpur (MP) who has given the opportunity to perform this set of work in the laboratory of Biotechnology department. Also express my thanks to Dr.Varsha Sharma and Mr.RamGopal Prajapati who has helped me in my research work.

References

Abebe,Bizuye.,A,sago.,G,Admasu.,H,Getache w.,P,KassaandM,Amsaya.2014.Isolation , Optimization and characterization of protease producing bacteria from soil and water in Gondar town, Northwest Ethiopia. International Journal of Bacteriology, Virology and Immunology.Vol 1(3):020-024.

Balakrishnan,padmapriya.,T,Rajeswari.,R,Nan ditaandF,Raj.2012.Production and Purificationof Alkaline Serine Protease from Marine Bacillus sp and its Application in Detergent Industry. European Journal of Applied Sciences.Vol 4(1):21-26.

Bharat,Pokhrel.,A,Pandeya.,S,Gurung., G, Bista., S ,Kandel.,R C,Kande and R T,Magar 2014.screening and optimization of extra cellular protease from bacteria isolated fromsewage.European Journal of Biotechnology and Biosciences.vol 2(1):46-49.

Bhavesh Patel and Nandini phanse .2000. Microbiology a practical approach. Print Care: edition I.61-64

Fekadu, Alemu.2015.Isolation and Screening of Protease Enzyme Producing Bacteria from Cheese at Dilla University,Ethiopia. International Journal Of Nutrition And Food Sciences.Vol 4(2):163-168.

F,Soundra Josephine.,Ramya,V S.,N,Devi., Suresh B G.,Siddalingeshwar KG., Venugopal, N and Vishwanatha T.2012. Isolation, Production and Characterization of Protease from Bacillus Sp. isolated from Soil sample.Journal of Microbiology and Biotechnology Research.Vol 2(1):163-

168.

Grewal, S and Mutha, P.2010.Enzyme technology. Published by Agrobios (India):145-148

G.Prabhavathy.,Pandian R M and Senthilkumar B.2012.Optimization and Production of extracellular Alkaline protease by Solid State Fermentation using *Bacillus subtilis*. Journal of Academic Industrial Research.Vol 1(7):427-430.

G.SaiSmita., Ray, P.Mohapatra S.2012.Quantification and Optimisation of Bacterial Isolates for production of Alkaline Proteases. Asian Journal of Experimental Biological Sciences .Vol 3 (1):180-186.

Hanan S Alnandi. 2012. Isolation and Screening of Extraceullar Proteases Produced by new isolated *Bacillus*Sp.Journal of Applied Pharmaceuticals Sciences.Vol 2(9):071-074.

L,SrinivasNaik.,Dr.K,Aruna.,P,Sreevennela.,Prof.Ch.VenkataRamanaDevi.2013.Isolation and biochemical characterization of protease isolated from *Bacillus* sp SVN12.International journal of research in Pure and Applied Microbiology.Vol 3(3):94-101.

Mohammad B.D and Mastan S A.2013.Isolation, Characterization and Screening of enzyme producing Bacteria from different Soil Samples.International Journal of Pharma and Biosciences. Vol 4(3):813-824.

Muhammad N,Qazi J I,Syed Q and Muhammad G.2013Purification and characterization of an alkaline protease from *Bacillus lichenformis*UV-9 detergent formulations. Songklanakarin J.of Science and Technology.Vol 35(2),187-195.

M.Raja Rani.,B.Lalithakumari,M.Hanuma Sri and Prasad Siva D.2012.Isolation and Screening of alkaline protease producing bacteria and induction of overproducing *Bacillus lichenformis* mutants through UV_irradiation Journal of Pharmacy Vol 1(1):001-014

Nihan Sevine and E,Demirkan. 2011. Production of Protease by *Bacillus* sp.N-40 Isolated from soil and its enzymatic properties.Journal of Biol.Environmental Sciences.Vol 5(14),95-103.

Nurullah Akcan and Fikret Uyar.2011.Production of extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation.EuAsian Journal of Biosciences.Vol 5:64-72.

Odu, N N.,Akujobi,C O.2012.Protease Production Capabilities of *Micrococcus Luteus* and *Bacillus* Species isolated from Abattoir Environment. Journal of Microbiology Research.Vol 2 (5):127-132.

Rajni, Hatti Kaul. Enzyme Production. Biotechnology. Vol V

Rashesh, Doshi and Vinod, Shelke.2001. Enzymes in textile industry.Indian Journal of Fibre and textile research.Vol 26:202-205

Sidra, Aftab.,Samia, A.,Sadia, Saeed and Sheik,h A R.2006.Screening ,Isolation and Characterization of Alkaline Protease Producing Bacteria From Soil.Pakistan Journal of Biological Sciences Vol 9(11):2122-2126.

Sneha, S., Merine,Paul Das and L.Jeyathi Rebecca.2014.Isolation and screening of protease producing bacteria from marine waste.Journal of chemical and Pharmaceutical Research.Vol 6(5):1157-1159.

Sneath, HAP., Halt, G J.1986 .eds.Bergey's Manual of Systematic Bacteriology..Vol 2

Tuhina Verma and Vandana Baiswar.2013. Isolation and Characterization of Extracellular Thermoalkaline Protease *Bacillus cereus* isolated from Tannery Effluent.The International Journal of Engineering and Sciences.Vol 2 (7):23-29.