JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue

JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)

An International Scholarly Open Access, Peer-reviewed, Refereed Journal

ISOLATION AND CHARACTERIZATION OF PROTEASE PRODUCING BACTERIA FROM DAIRY WASTE SOIL

Priyanka Shirsath, Poonam Shinde, Pranoti Thorat

Department of microbiology, New Arts Commerce And Science College, Ahmednagar, Maharashtra, India

Abstract : Enzymes are basically known to be proteins which are biological catalysts. Proteases are also of the very common enzymes. Microbial Proteases generally are often found to be extracellular and are released in the growth medium by the producer microbes and hence the down streaming process is easy compared to other sources. In this research article a study has been carried out for isolating and characterizing protease producing bacteria from the best source of such bacteria that is dairy waste soil. Along with this the optimum conditions for maximum production of enzymes are studied and this study would contribute to promote microbial proteases economically and commercially.

IndexTerms **– Enzyme, Alkaline Protease, Bacteria, pH , Temperature, Nutrient Casesin, Carbon, Nitrogen, Time**

I. INTRODUCTION

Enzymes are biocatalyst highly specialized for the catalytic power that too of great specificity (17). Enzymes are derived from different sources from nature like animal, plants, microbes, etc. among which microbial enzymes are found to be of more applicable than that of those derived from animals and plants as microbial ones are more stable as well as convenient and importantly safer (23). Though microbial enzymes are useful but only about 2% of microbes in worldwide are the reservoir of enzymes (23). In the group of enzymes that are useful protease are one which occupy an important role and they were first enzyme to be produced in large account also called proteolytic enzyme or proteinases (13). They are enzymes which are classified on the basis of their optimum pH as acidic, basic and alkaline proteases while based on the ability to cleave "C" and "N" peptidase bond they are classified as endopeptidase and exopedtidase (18). Nowadays microbial proteases are considered one of the largest group of industrial enzymes and they contribute 60% of total industrial enzyme scale in the world (26).

Microbial Proteases generally are extracellular and are secreted directly into fermentation broth by the producer microbe and hence the downstreaming processing is simplified than that of plant and animal derived proteases (17). Extracellular proteases are naturally produced by microorganisms to degrade large polypeptides in the medium into peptides and amino acid before cellular uptake (21). The alkaline proteases are employed for various purposes in industries and the advantage of alkaline protease is that it is active over a broad pH (7-12) and temperature (35-80^{o}C) range (4).

Microbial proteases are produced from high yielding strains such as *Bacillus spp*., *Picaligenes faecalis*, *Pseudomonas flouroscens* and *Aeromonas hydrophylia* grown under submerged condition (28). In Japan 1994, alkaline proteases sale was estimated as 1500 million years (24). Despite that only few studies, on the protease from the protease from the *Bacillus spp.*, it is found that they are specific producers of extracellular producers are found to predominant (24, 11, and 28). The present research is based on optimization of growth condition of different species of *Bacillus spp.* are highly active and stable at different pH and temperature ranges, also are broad substrate specific and they can be purified at lower cost. The optimization of different fermentation parameter like nitrogen and carbon source, media, pH, incubation, temperature, agitation and incubation time can enhance yield of industrially useful Enzymes (8). The first method adopted to determine protease production by screening using bromocresol green (22). Protease use a water molecule for their proteolytic activity and thus are classified as 'hydrolases' is one of the major characteristics of these enzymes (9). Thermostable proteases among all proteases are now seen as an advantageous in the process where high temperature is an important parameter (11). Growth and development in each and every organism is an outcome balance between protein synthesis and proteolysis where protease plays an important part (3).

Now only in animal, but in plant these enzymes execute an important part in overall in all aspect of life (15). Protease produced from bacteria is advantageous as it is easy to manipulate them genetically in short span as compared to plants and animals (21). Various agricultural residues have been investigated as a possible substrate in the production of alkaline protease. As mentioned, *Bacillus spp.* are primary producers of protease several strains of bacteria of this species like *B.licheniformis, B.firmus, B.alcalophilus, B.amyloliquifaciens, B.proteolytiphillus, B.subtilis, B.thuringenesis,, B.cereus, B.stereothermophilus, B.megaterium* are reported to produce the same. (23). An interesting fact estimated about *Bacillus spp* producing protease enzyme is that exposure to magnetic field for 10 mins on Fe metal ion affect activity of these bacteria to protease enzyme. (25). For the screening of protease enzyme substrate have been incorporated in solid agar media (22). Alkaline protease and all other protease having various potential applications in different commercial industries. Extracellular Proteases have high commercial value and multiple applications in

detergent, food dairy, Pharmaceuticals, leather, diagnostics, waste, management , important in Leather industry for dehairing and bating of hide instead of chemical usage (20). Instead of synthetic biosurfactants which are amphiphilic biological compound in extracellular cell membrane by bacteria they are more applicable in terms of being biodegradable it is hence suited in bioremediation and dispersion of oil spills (4).Proteases when administered orally are effective for burn patient and also helps in healing wound (29). The silver and polyester sheet recovery from x-ray film waste is able by protease instead of burning and chemical treatment which creates unbearable smell and is pollutant (26). They are also used as an important agent in the product of biopharmaceuticals like contact lens cleanser and also modify the surface of wool as well as silk fibers so as to give an amazing finish (18). Protease is also used in baking, brewing, meat tenderization, peptide synthesis, cheese making, etc. (11). Enzymatic Debridement is primary technique in certain cases when surgical debridement is not possible (3). Alkaline Proteases are used and formulated in detergent for the removal of proteinaceous stains like blood, food, oil, grass stains (8, 20). Hide dehairing using enzyme is carried out at pH range in between 8-10 and proteases are applicable to deproteination of marine crustacean waste (23).

II. MATERIALS AND METHOD

1. Sample collection:-

Dairy waste soil sample was collected from dairy from MIDC, Ahmednagar, Maharashtra. The soil was collected using sterile spatula in a clean plastic bag.

2. Enrichment:-

Enrichment was performed using 250ml Erlenmeyer flask containing 100 ml of nutrient casein broth medium. The medium was sterilized by autoclaving 1 gm soil sample was inoculated in nutrient casein medium and incubated for 3 days at 37°C in shaking incubator.

3. Screening and Isolation for protease producing bacteria:-

After the incubation spot inoculation was done onto alkaline skimmed milk agar plate. Then those plates were incubated at 37°C for 48hrs to visualize the clear zone of proteolytic activity. To indicate the protease activity of organisms, the culture producing the largest clear zone was selected for further studies.

4. Characterization of isolate:-

Isolated bacterial culture was characterized morphologically and biochemically as per Bergey's Manual of Determinative Bacteriology 9th Edition. Gram staining and different biochemical tests were performed, which included catalase, oxidase and sugar fermentation test. Fresh culture was used for all the tests.

5. Cultural maintenance:-

The isolated proteolytic bacterial culture was preserved on nutrient agar plates for further studies. Screening activity for protease activity the isolate was plated on sterile skimmed milk agar plates and plates were incubated at 37°C for 24 hrs. A clear zone around colony on skimmed milk agar gave an indication of protease producing microorganisms.

6. Inoculation and production medium:-

Inoculums of isolate 1 was transferred to 100ml of sterile production medium separately and incubated in shaking incubator at 37^0C for 72 hrs. The composition of production medium was the (G/L) : Glucose 0.03 gm, Yeast extract 0.02 gm, Peptone 1 gm, MgSO4 0.1 gm, pH 8.5.

7. Extraction of protease from production medium:-

After incubation, the production media was harvested by centrifugation at 10000rpm for 10 mins at 40^0C. The supernatant was used for enzyme assay for protease.

8. Assay of protease:-µ

Materials:-

1. Tris buffer pH-8 at 37^0C
2. 2% (W/V) Casein Solution
3. Tricholoroacetic Acid Reagent (TCA)
4. Folin and Ceucalteu Phenol Reagent (FC Reagent)
5. 500 mM Sodium Carbonate solution (Na2CO3)
6. 1.1 mM L-Tyrosine (Standard Solution)
7. Standard Graph of tyrosine was prepared by using folin-lowry protein estimation method to calculate enzyme units.

Method:-

The amino acid released from casein per ml per min at 750 nm was determined by Folin Lowry method. Total protease activity was determined by measuring the amount of amino acid formed from casein by incubating 0.1 ml of crude enzyme with 0.5 ml of 1% casein in 0.1 M sodium phosphate buffer (pH 8) and incubated at room temperature for 10 min. After incubation, reaction was stopped by the addition of 1 ml of 10% TCA reagent. Then the mixture was incubated at 37°C for 30min. After incubation to the mixture 5 ml of Na2CO3 and 1 ml FC reagent was added. This mixture was again incubated at 37°C for 30min. After incubation absorbance was measured at 750nm. Amino acid liberated was estimated by measuring absorbance spectrophotometrically at 750 nm. Protease production was estimated by using tyrosine as standard. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release l µmol amino acid (measured as tyrosine) per ml per minute.

9. Optimization of protease production:-

Various parameters were tested for the production of proteases. Only one parameter was changed at a time and all other conditions were maintained as described as above.

1. Optimization of time period for protease production:

The bacteria were inoculated into 250 ml Erlenmeyer flanks containing 100 ml of nutrient casein broth (pH 12) and incubated at 37 ^{0}C for different time intervals. Then the enzyme assay was performed and readings were recorded at 250 nm after 24h, 48h, 72h and 96h.

2. Optimization of temperature for protease production:

The bacteria were inoculated into the 250 ml of Erlenmeyer flask containing 100 ml nutrient casein broth pH 12 and incubated at different temperature 30°C 37°C, 52°C, and 60°C. After 48hrs of incubation the enzyme assay was performed and readings were recorded at 750 nm.

3. Optimization of pH for protease production:

The bacteria were inoculated into the 250 ml of Erlenmeyer flask containing 100 ml nutrient casein broth having different pH value i.e. 5, 7, 9, 11 and 12 and were incubated at 37°C for 48 hrs. After that enzyme assay was performed and readings were recorded at 750 nm.

4. Optimization of carbon source for protease production:

The bacteria were inoculated into the 250 ml of Erlenmeyer flask containing 100 ml nutrient casein broth of pH 12. To study the efficiency of various carbon sources, the medium was supplemented independently with 1% glucose, fructose, sucrose, and galactose. The broths were incubated at 37°C for 48 hrs. After 48 hrs of incubation the enzyme assay was performed and readings were recorded at 750 nm.

5. Optimization of nitrogen source for protease production:

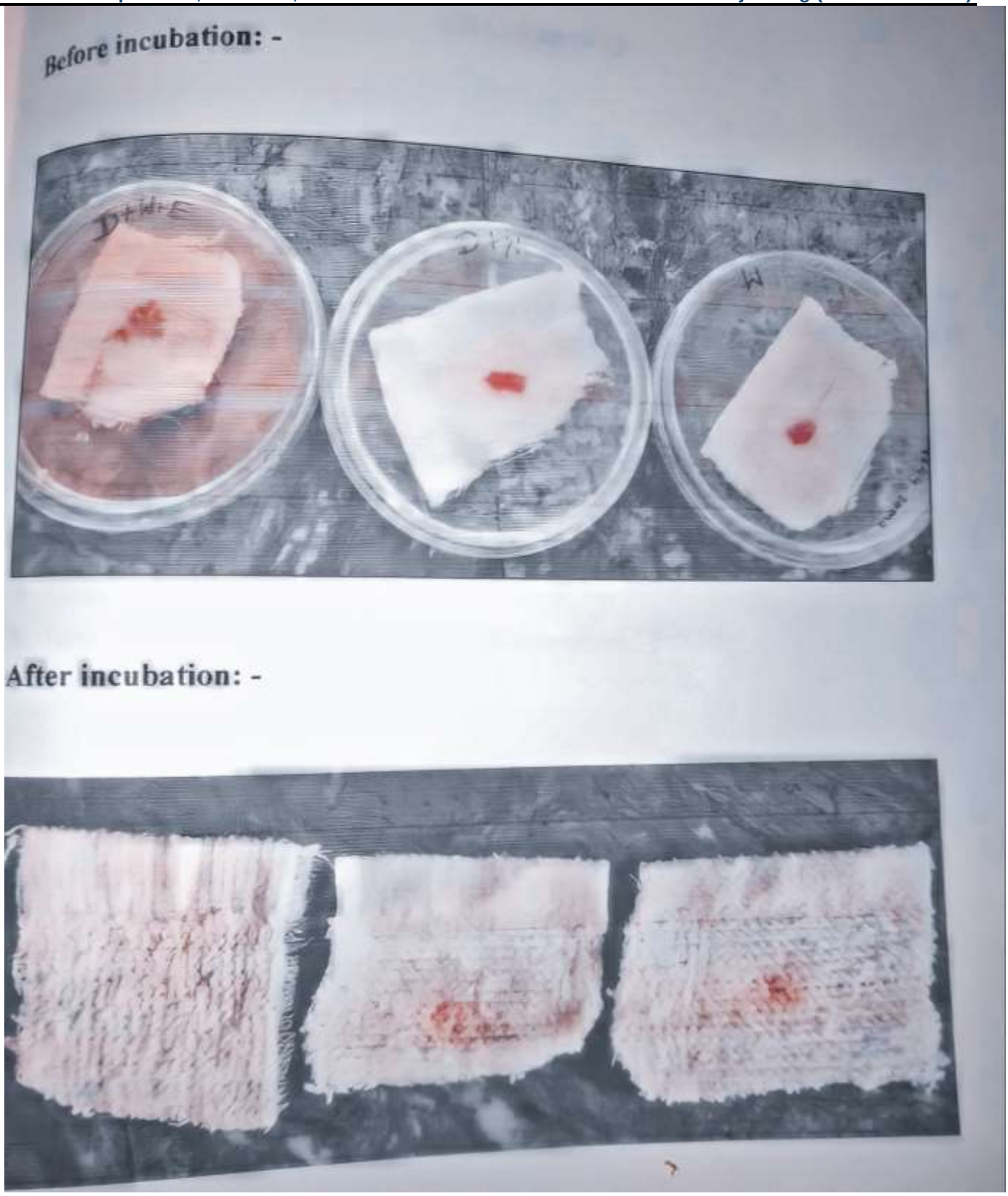
The bacteria were inoculated into the 250 ml of Erlenmeyer flask containing 100 ml nutrient casein broth of pH 12. To study the efficiency of various nitrogen sources, the medium was supplemented independently with 19% ammonium nitrate, ammonium sulfate, ammonium chloride, and tryptophan. The broths were incubated at 37°C for 48 hrs. After 45 hrs of incubation the enzyme assay was performed and readings were recorded at 750 m.

10. Destaining efficiency of enzyme:-

For commercial purpose of enzymes, the isolated protease was determined for its destaining efficiency with detergent by incubating with locally available detergents for 10 min. Pieces of white cotton cloths (5 cm x 3 cm) were stained with blood spot separately and taken in separate petri plates as describe below. Then they were washed with commercial detergent and isolated alkaline protease and examined for Destaining from cloth.

1. Distilled water (10 ml) + stained clot
2. Distilled water (10 ml) + stained cloth + 0.1 gm detergent
3. Distilled water (10 ml) + stained cloth + 0.1 gm detergent + 0.5 ml crude enzyme solution

All sets of plates were kept for 10 min of incubation. After incubation, cloth pieces were taken out and dried. Visual examination of the cloth pieces exhibited the effect of enzymes in the removal of stains. Untreated cloth pieces stained with spot was takes as control.



III. RESULT AND DISCUSSION:-

1. Isolation and screening of protease producing bacteria:-

The screening was carried out by using skimmed milk agar. After incubation at 37°C for 72 hrs. The zone of proteolytic activity was seen on skimmed milk agar plates. Two isolate showed the clear zone of proteolytic activity. The isolate which showed highest zone of proteolytic activity was selected for further studies.

2. Morphological and biochemical characteristics of isolates:-

1. Morphological characters of isolate: -

After incubation at 37°C for 48 hrs on nutrient casein agar plate the following colony characters were observed.

Table No.1 – Characterization of isolate

Sr.No.	Characters	Observation
1	Size	1 mm
2	Shape	circular
3	Color	White
4	Margin	Irregular
5	Consistency	Sticky
6	Elevation	Convex
7	Opacity	Translucent
8	Gram character	Gram positive short rod
9	Motility	Motile

2. Biochemical Characters of isolates: -

Table No.2- Biochemical Characters		
Sr.No.	Test	Result
1	Oxidase	+
2	Catalase	-

3. Optimization of protease production by isolate- 1:-

Various factors mentioned below were optimized for the production of protease by isolate-1. The protease activity was determined. The protein contents of the enzyme preparation were determined by Folin-Lowry method. One unit of protease activity was defined as the number of micromoles of Tyrosine liberated by 1 mL of enzyme solution /min. For this standard graph of Tyrosine was prepared using stock of 1.1mM.

Table-Standard graph of tyrosine (stock concentration -1.1mM)

Standard stock	Distilled Water (ml)	Na2CO3(ml)	FC Reagent(ml)	O.D.at 750nm
0.1	0.9	5ml	1ml	0.253
0.2	0.8	5ml	1ml	0.338
0.3	0.7	5ml	1ml	0.491
0.4	0.6	5ml	1ml	0.850
0.5	0.5	5ml	1ml	0.757
o.6	0.4	5ml	1ml	1.000
0.7	0.3	5ml	1ml	1.057
0.8	0.2	5ml	1ml	1.253
0.9	0.1	5ml	1ml	1.150
1.0	0	5ml	1ml	1.902
Blank	1	5ml	1ml	-

.

Formula:

Enzyme units= µM of product formed x volume of enzyme / Reaction time (min)

4. Optimization of the different parameters on protease production:-

1. Time of incubation: -

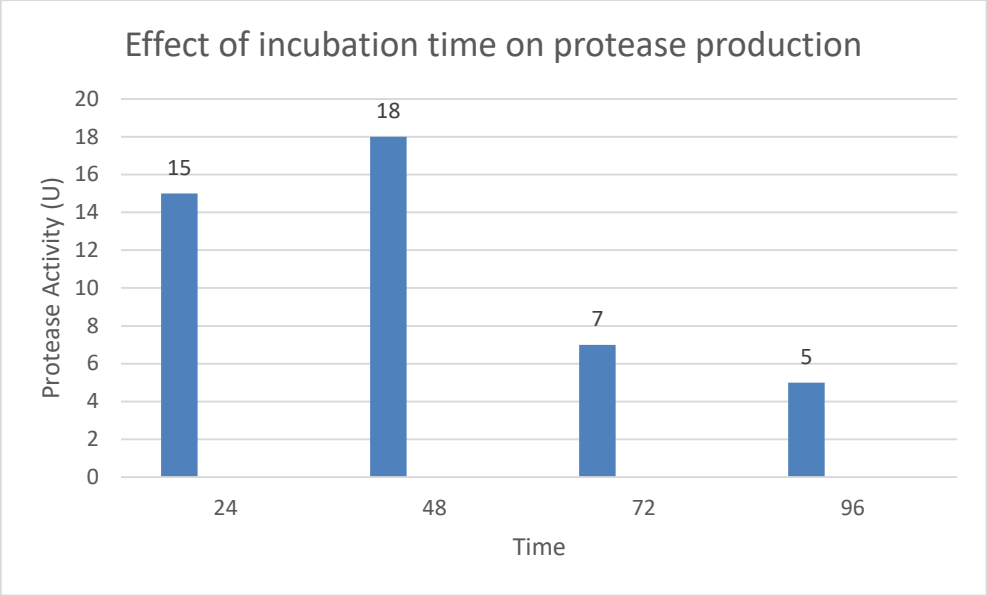
The effect of incubation time on protease production by the isolate was studied. It was found that the concentration of protease production increases with increase in incubation time until 48 hrs. The maximum protease production was obtained after 48hrs, which was 18 U/ ML. When incubation time was further increased, of protease was reduced.

B. Asha, M. Palaniswamy (2018) found that the B. cereus FF 1 showed protease production of about 148U/mL at 48h of incubation.

R.C. Patil & B.L. Jadhav (2017) found that the isolated *Bacillus cereus* (SP3) has maximum protease enzyme production was observed at 72 hrs.

Table no.3-Effect of incubation time on protease production

Sr.No.	Incubation Time (hr)	Protease production (U/ml)
1	24	15
2	48	18
3	72	7
4	96	5



2. Effect of Temperature: -

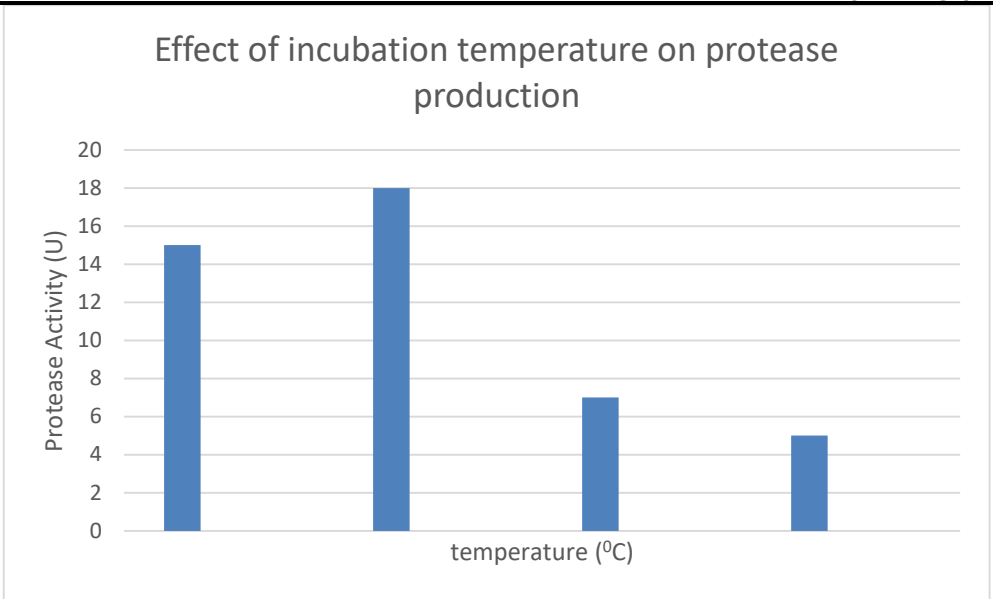
The effect of temperature on protease production by the isolate was studied. It was found that concentration of protease increases with increase in temperature. The maximum protease production was found that at a 37°C which was 18Unit /ml, but when the temperature was further increased, concentration of protease was reduced. This indicate that the optimum temperature for protease production by isolate was 37°C.

M. Akhtaruzzamanetal. (2012) concluded that protease enzyme showed peak at 37°C and found that leguminous seed can be source of proteases for industrial purposes.

Amlesh Samanta, Pinaki Pal et al. (2012) found that the isolated strain of bacteria from Municipal solid waste showed that highest biomass production was observed at 30°C whereas the highest enzyme activity was found at 37°C.

Table no.3-Effect of temperature on protease production

Sr.No.	Incubation Temperature (^{0}C)	Protease production (U/ml)
1	30	12
2	37	18
3	52	11
4	60	09



3. Effect of pH:-

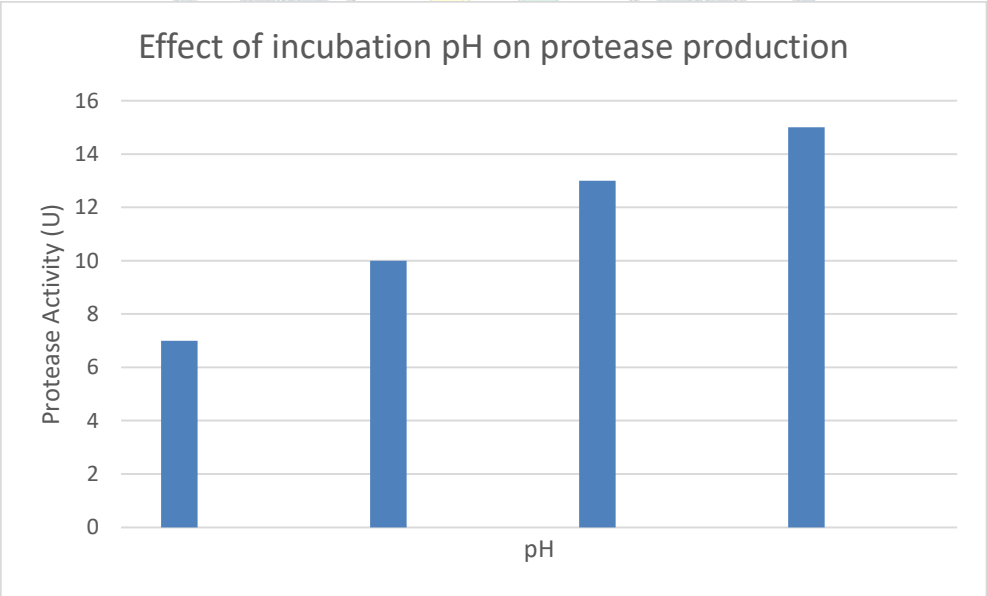
The effect of pH (from 5 to 12) on the protease production by the isolate was studied. It was found that the concentration of protease production increases with increase in pH. Maximum protease production observed at pH 12 after 72 hrs which was 15 Unit/ml. But when the phi was further increased concentration of protease was reduced. This indicates that the optima pH for protease production by the isolate was 12. The influence of pH on enzyme production was found to be an important parameter.

Ohudumila Omolara Rachearl et al. (2015) showed that protease enzyme was relatively stable at pH 9-12 then add pH 3-8.

Abdelnasser S. S Ibrahim, Ali A. Al-Salamah et al. (2015) concluded that Bacillus sp, NPST AKIS could grow and produce alkaline protease over a wide pH range from 7-12.

Table no.3-Effect of pH on protease production

Sr.No.	pH	Protease production (U/ml)
1	5	7
2	7	10
3	11	13
4	12	15



4. Effect of different carbon source:-

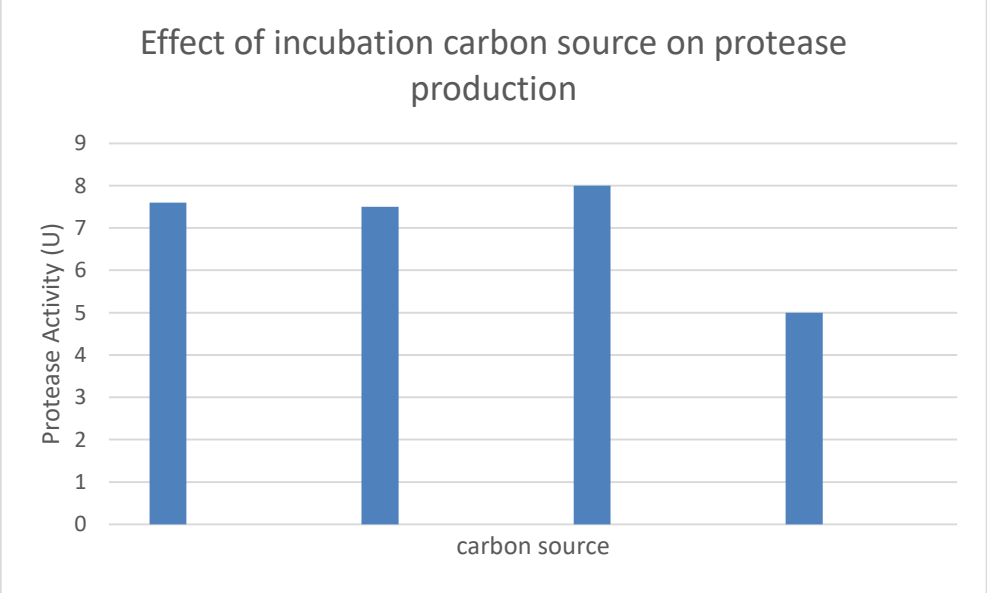
Protease production with different carbon sources was studied. The maximum protease production was obtained when sucrose used as carbon source which was Unit/ml followed by glucose was 7.6 Units/ml. The lowest protease production by isolate was obtained by fructose used as a carbon source.

Gitishree Das and M.P. Prasad found that when protease producing strains of bacteria were provided with different carbon source such as sucrose, lactose, dextrose and maltose and dextrose was considered the suitable carbon source as compared to glucose.

Mary Suja R. et al. (2017) studied that the effect of carbon sources of protease production after 48hr incubation revealed maximum protease production in maltose supplemented medium and minimum protease production in sucrose added medium.

Table no.3-Effect of carbon source on protease production

Sr.No.	carbon source	Protease production (U/ml)
1	Glucose	7.6
2	Galactose	7.5
3	Sucrose	8
4	Fructose	5



5. Effect of different Nitrogen source:-

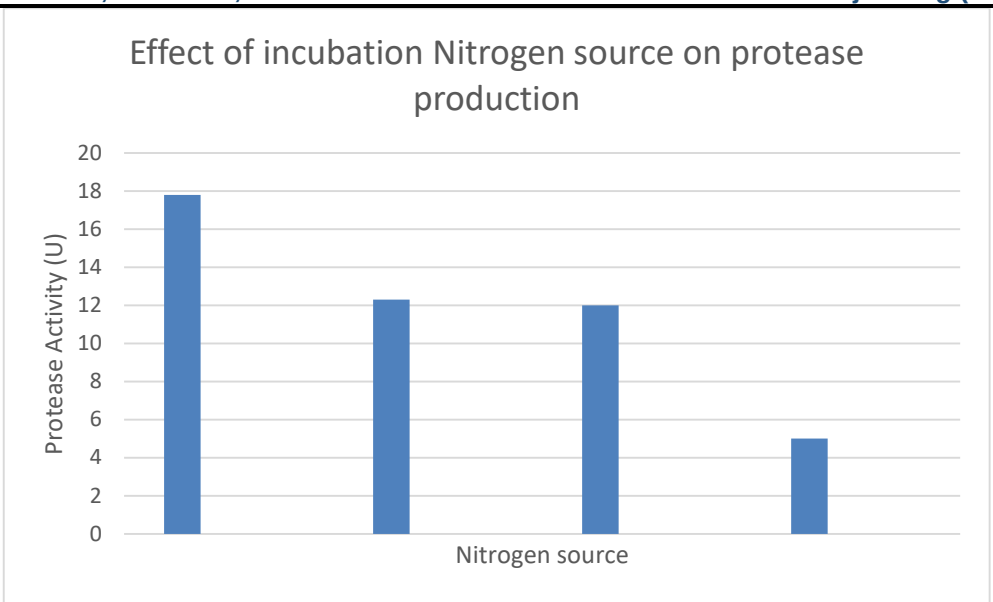
The effect of different Nitrogen sources on sources on production was studied. The maximum protease production was obtained when ammonium sulphate used as nitrogen source which was 17.8 Unit/ml followed by ammonium chloride was 12.3 U/ml. The lowest protease production by isolate was obtained when tryptophan used as nitrogen source.

Udandi Boominedhan et. al. (2009) reported that beef extract though was found to be best organic nitrogen source for augmenting the protease enzyme production for bacillus spp. Among the different inorganic nitrogen study ammonium carbonate was found to be the best for the *Bacillus* isolate.

Titilayo Olumunke Femi-Ola et al. (2014) found that the best nitrogen source for protease production was casein and also yeast extract produce a large amount of protease al compared to ammonium chloride, ammonium nitrate, sodium nitrate, skimmed milk, and potassium nitrate used as nitrogen source.

Table no.3-Effect of Nitrogen source on protease production

Sr.No.	Nitrogen source	Protease production (U/ml)
1	Ammonium sulphate	17.8
2	Ammonium chloride	12.3
3	Ammonium nitrate	12
4	Tryptophan	5



Destaining efficiency of enzyme:-

Enzyme which exhibit its activity in the alkaline range is known to be used as potential detergent additive and it is used for removing stains.

To test the application of alkaline protease as a detergent additive, the experiment was performed which used the soaking the dirty white cloth pieces in detergent for 10min. It was found that the mixture of alkaline protease showed better action by giving faintness of the blood spot on the cloth. The isolated enzyme showed efficient removal of spot darkness from cloth.

IV. CONCLUSION:-

The isolated species from dairy waste soil is a good producer of protease and can be utilized for production of proteolytic enzymes. The observed optimum conditions for the production of protease by isolate I were as follows:-

1. Time period -48 hrs. (Enzyme units 18 U/ml)
2. pH- pH 12 (enzyme units 15 U/ml)
3. Temperature-37^0 C (enzyme units 18 U/mL)
4. Carbon source-Sucrose (enzyme units 8 U/ml)
5. Nitrogen source- Ammonium sulfate (enzyme units 17.8 U/mL)

V. ACKNOWLEDGMENT

Mr.Girish Kukreja, Head of Microbiology Department, New Arts commerce and science college, Ahmednagar for their valuable support in the research and also authors are thankful to Miss.Vidya Mehetre for guiding throughout the process of research project and publishing research paper.

REFERENCES

1. A. Petriwiningrum, F D Anggraini, N. A. Fitrianto, R Rochijan (2017) Isolation and identification of bacterial protease enzyme from leather waste Journal of the Indonesian Tropical Animal Agriculture, vol -42. Page no 33-41.

2. Abedelnasser S.S. Ibrahim, Ali A. Al-Salmah, Yaliya B. Elbadawi, Mohamed A. EL-Tayb and Sheble Salah Sheblelbrahim (2015) Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus spp.* NPST-AK15 isolated from hyper saline soda lakes, Electronic Journal of Biotechnology.

3. A. Vidyalakshami and S. Esakiselvi (2013) Protease Activity of Floral Extract of Jusminum grandiflorum L. a Wound Healing Herb. Volume 1, Page no.11-15.

4. Amalesh Samanta, Pinaki Pal, Anurup Mandal, Chandrima Sinha, Asif Lalee, Mousumi Das, Sudipta Kaity and Debmalya Mitra (2012) Estimation of Biosurfactant Activity of and Alkaline Protease Producing Bacteria Isolated from Municipal Solid Waste Central European Journal of Experimental Biology. Page no. 26-35.

5. Amruta Kothare, VinalPardhi, Prajkta Chivte, Sukharam Muley, Tejashree Shanbhag (2017) Purification, partial characterization and activity profiling of Bromelain protesse. International Journal of Pharmacy and Biological Sciences" 7. Page no 1-9

6. Anupama V, Mari Muthu M. Una Sundaram, Gunamoorthi P. (2013) Optimization Isolation and Characterization of proteases from under-utilized and common food legumes International Research Journal of Pharmacy page no 99-106.

7.Ashwini A Jadhav, Khatib Sayeed lsumail, Mahesh A Harale, Shashikant Gade and stanita T. (2014) Study of protease enzyme from *Bacillus species* and its application contact lens cleanser. Department of Microbiology, Topiwala, National college and B.Y.L Niar Charitable Hospital Mumbai. Page no. 291-302.

8. B. Asha and M. Palaniswany (2018) Optimization of Alkaline Protease Production by *Bacillus cereus* FT I isolated from soil.Vol.8 (02).

9. Dalal Rupali (2015) Screening and Isolation of Protease Producing Bacteria from Soil Collected from Different Areas of Burhanpur Region (MP) India. International Journal of Current Microbiology and Applied Sciences. Vol 4. Page no 597-606.

10. Ekhlas Uddin, Hossain MD Faruque, Tanvir Ahmed, Mohammad Khairul Alum Sarkar Muslima Khatun, and Mohommad Shah Alam (2015) Partial Characterization and Purification of protease enzyme from locally isolated soil bacterium Basic Research Journal of Microbiology, Vol. 2 (3) Page no 45-54.

11. Gitishree das and M.P.Prasad (2010) Isolation, Purification and Mass Production of Protease enzyme from *Bacillus subtilis.* International Research Journals of microbiology Vol 1 (2) Page no, 026-031.

12. Honagxia Cui. Liping Wang, Yang Yu (2015) Production and Characterization of Alkaline Protease from a High Yielding and Moderately Halophilic Strain of SDII Marine Bacteria. Hindawi Publishing Corporation Journal of Chemistry.Page no.1-8.

13. Josephinelenitta X. and J. Joel Gnanadoss (2014) Screening, Molecular characterization and phylogenetic analysis of Penicillin spp. Producing high levels of Protease. Microbial and environmental biotechnology research Centre, department of microbiology, page no. 10-17.

14. Kiran Anand (2016) Fungal Protease Production try Aspergillus niger und Aspergillus flavus using Rrce Bran as the Substrate Academic Journal of Agricultural Research Paper. Page no. 333-338.

15. Leelamma M. Panicker, Rajamma Usha, Samir Rey and Chhabina Mandal 2009 Purification of characterization of serine protease (CESP) from mature coconut endosperm National institute of health USA, Jadavpur university Page no 1-10

16. M. Akhtaruzzaman, N.H.M. Rubel Mozumder, Ripa lamal, Atikur Rahman, and Tanjina Rahman (2012) Isolation and characterization of protease enzyme from leguminous seeds Agricultural science research journal. Vol 2(8) page no 434-440

17. Mary Suja R. Arumugam P, Palavesam A and Christudhas William B (2017) Isolation and determination of protease enzyme synthesized by Pseudomonas spp from the gut of Estuarine fish Etroplussuratensis. William Research centre, Nagercoil, India Page no. 1-6

18. Mohsen Fathi Najafi, Dileep Deobagkar, Deepti Deobagkar (2005) Potential application of protease isolated from Pseudomonas aureoginosa. Electronic journal of biotechnology, vol 8 Page no.197-203.

19. Namrata Sharma (2012) Alkaline protease production by solid state fermentation of mustard oil cake. Department of biochemistry, Mata Gujri Mahila Mahavidyala Jabalpur (MP), India.vol.2. Page no 35-40.

20. Oludumila Omolara Racheal, Abu Temitope Folagbade Ahmed, Enujugha Victor Ndigwe, Sami David Morakinyo (2015) Extraction purification and characterization of protease from Aspergillus niger isolated from yam peels, International journal of nutrition and food science vol4(2)page no.125-131.

21. Pwars Wilson, and Zvauya Remiglo (2012) Production and characterization of the protease produced by a novel moderate thermophilic bacterian (EP1001) from an alkaline hot spring. Zimabawe. Department of biochemistry, university of Zimabawe, Mountpleasant, Harare Vol.6(27), page no 5542-5551.

22. Ponnaswamy Vijaya Raghavan, Samuel Gnana Prakash Vincent (2013) A simple for the detection of protease activity on agar plate using bromocresol green dye, page no 628-630.

23.R.C. Patil and B.L Jadhav (2017) Isolation and characterization of protease producing Bacillus species from soil of dairy Industry. International journal of current microbiology and applied sciences. Vol.6, page no 853-860.

24. Sharmila, 1. Jayanthi Rebecca, Merina Paul Das and Md Saduzzaman (2012) Isolation and partial purification of protease from plant leaves. Journal of Chemical and Pharmaceutical Research. Vol 4, page no3808-3812.

25. Sumardi. Rochmah Agustrina, Christina Nugroho Ekowati and Yovita Selvie Pasaribu (2018) Characterization of protease from Bacillus spp. on medium containing $FeCl_3$ exposed to magnetic field 0.2 mt Department of biology, faculty mathematic and natural science University of Lampung, page no 1-11.

26. Teman Abrar Hamza (2017) Isolation of Protease producing bacteria from soil for Polyester and Silver recovery from waste X-ray film. American Journal of Bioscience, vol. 5, page no74-79.

27.Titilayo Olufunke Femi-Ola, Obafeme Peters Akinsanmi and Olufemi Samuel Bamidele (2014) Production and Characterization of Protease from Serratia marcescens Department of Microbiology, Ekiti state University Vol 18 page no 1-10.

28. Udandi Boominadhan, Rajendran Rajakumar, Palanivel Karpaga Vinayaga Sivakumar and Manoharan Meln Joe (2009) Department of Microbiology, Faculty of Science, Annamalai University. Vol 2 page no 83-87.

29.V.N. Jeyadharshn (2013) Production and partial purification of Protease by Actinomyces species. Department of Bio-science, Vavuniya Camous of the University of Jaffna Sri Lanka. Vol 3-page no 1-3.