ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Comparative Study of Proteolytic Microbes from Various Places and the Alkaline Protease Optimization Produced by *Bacillus sp.* using Taguchi's Statistical Optimization Method.

Sonika Jha*, and Suneetha V.

School of Biosciences and technology, Vellore Institute of Technology, Vellore, Tamilnadu.

ABSTRACT

The screening of novel bacterial colonies from soil samples collected from various places was done followed by the plate screening using well diffusion method in casein skim milk agar plate. The desired bacterial colonies from each sample were subjected to bacterial growth curve study in order to observe the exponential phase of each colony. These bacterial colonies were then observed for highest protease activity by Folin Lowry's assay. The best bacterial colony was from the soil sample of milk packet dumping area followed by paper waste dumping area, cattle farm area and the least was observed in the soil sample from VIT lakeside. The best colony was chosen for further physical and chemical characterization which confirmed it to be a *Bacillus* strain. The optimization of pH showed the highest activity in alkaline conditions pH (at 9)which confirmed it to be alkaline protease. The statistical optimization for physical and nutritional parameters was done using Taguchi's orthogonal array method. Signal to noise ratio analysis from Taguchi's loss of function was done and the formula "larger is better" was used. From the main effects plot for SN ratio it was interpreted that the optimized pH for the bacillus is 9, optimized temperature is 45°C, optimized agitation is 100rpm, optimized NaCl is 0.6 g, and optimized peptone is 0.4g.

Keywords Well diffusion method, Folin Lowry's assay, Taguchi, MINITAB, alkaline protease

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Proteases are of great importance because of the wide range of commercial applications and physiological roles that they offer. Along with the degradative functions, synthetic functions are also performed by them [1-3]. Their occurrence is ubiquitous in wide diversity of sources because they are of great physiological importance for living organisms [1,4,5]. The sources are plants, animals and microorganisms. There are two types of protease based on their action away from termini or at the termini [5,7]. They are exopeptidases which act at the termini and endopeptidases which act away from the termini. Based on the nature of functional group on active sites, the classification of proteases can be done as serine, aspartic, metallo and cysteine proteases [6,8,9]. Proteases have critical role in pathophysiological and physiological processes [1,14]. There are four different types of catalytic mechanisms which are operative based on their classification [3,7]. In food and dairy industry, the use of protease is very prominent [6,13]. Alkaline proteases are widely used in leather and detergent industry and it is necessary also because of the need of developing environmentally friendly technologies. It is important in order to eliminate the currently used toxic chemicals for bating and dehairing of hides in leather industries [10,12]. The advancement of analytical techniques demonstrates that proteases are also responsible for selective and specific modification of protein for example the lysis of fibrin and blood clots, zymogenic form of enzyme's activation etc [16,17].Included among one of the three largest groups of industrial enzymes, the involvement of proteases in complex or normal physiological processes of the cell along with its involvement in pathophysiological conditions is very prominent [6,11,13,15]. Taghuchi's approach of optimization is the first novel methodology used in this research.

MATERIALS AND METHODS

Collection of soil samples

The collection of soil sample was done from various sites located in Vellore. The site of collection were VIT lakeside, paper waste dumping area, cattle farm and milk packet dumping area. (Figure 1).

Figure 1: (A) VIT lakeside. (B) Paper waste dumping area. (C) Cattle farm. (D) Milk packet dumping area. The collection of topsoil samples was done from 0-10 cm depth in each plot. During sampling it was made sure that the surfaces of soil in the plots were dry.

Screening of potential bacteria

Sample inoculation was done in test tube and it was kept for 10-20 minutes for proper suspension of microbes in the liquid. Then 1 ml was taken from this test tube and transferred to the next test tube having 9 ml of distilled water and so on till the 10^{th} dilution. After that 1 ml was taken from each test tube and using spread plate method is was spreaded on the nutrient agar plates using bent glass rod. After two days of incubation, bacterial colonies were obtained in 5^{th} and 6^{th} dilution. In order to obtain pure colonies, sub culturing was done. For each soil samples, colonies were obtained after the spread plate method followed by

incubation (figure 2). All the colonies were sub cultured using streak plate method and later screened for proteolytic bacteria.

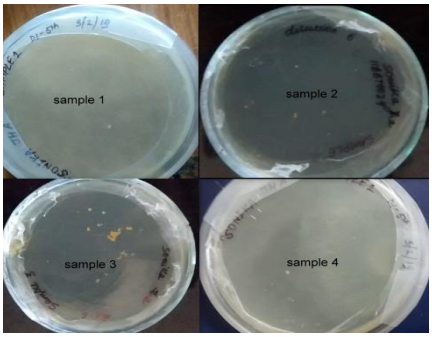


Figure 2: Isolated colonies from the soil samples of (1) VIT lakeside. (2) Paper waste dumping area. (3) Cattle farm. (4) Milk packet dumping area.

Primary screening of proteolytic bacteria by well diffusion method

The pure colonies were screened for their proteolytic activity using plate assay method. Agar plate was prepared using 1% Casein and 1% skimmed milk powder. After the solidification, holes were punched on the plates. Crude culture supernatant was taken and loaded into the wells. Plates were kept at 37°C for incubation for time period of 72 hours. The plates were showing zone of proteolysis for some cultures. Further experiments were done on these selected proteolytic bacterial samples. The screened proteolytic bacterial samples were sub cultured by streak plate method. Next experiments were conducted on total of 8 bacterial colonies. 2 proteolytic colonies from each soil sample (figure 3). These colonies were named as p1 and p2 (from VIT lakeside), q1 and q2(from paper waste dumping area), r1,and r2 (from cattle farm) , s1 and s2 (from milk packet dumping area)

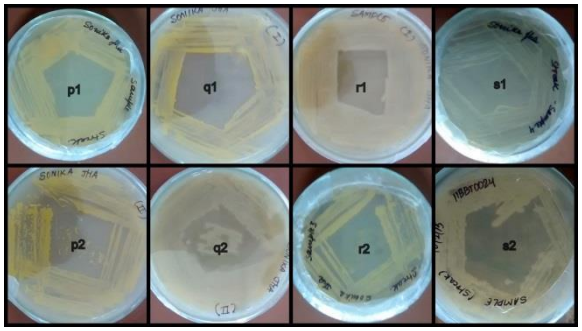


Figure 3: subculture plates of the test samples (p1,p2,q1,q2,r1,r2,s1,s2)

Bacterial growth curve

In order to determine the exponential phase, bacterial growth curve study was done for the samples p1, p2, q1, q2, r1, r2, s1, s2. After the growth curve study, the time period of exponential phase was noted down and the enzyme assay was performed in that time period because according to previous research works, the bacterial enzyme activity is highest in the exponential phase. 30 ml falcon tube was used for the study. Using of falcon tube for bacterial growth and so less time is required to achieve the result.

Folin Lowry's assay for the comparison of protease enzyme activity

Casein was used as substrate for the Folin lowry's assay [18]. The colonies p1,p2,q1,q2,r1,r2,s1,s2 were subjected to the assay. The liberation of tyrosine takes place after the digestion of casein by test protease. This free/liberated tyrosine when reacts with Folin's reagent, blue colored chromophore is observed and the absorbance (OD) value is taken on spectrophotometer at 660 nm. Better is the release of tyrosine, stronger is the chromophore which indicates strong activity of protease. Standard curve preparation is done

and this OD value is compared with it. In order to prepare the standard curve, known amount of tyrosine is made to react with FC reagent. When we notice the changes in the absorbance, it is correlated with the amount of tyrosine in micro molecules. In this procedure, usage of 50 ml broth per sample in the conical flask was done in order to get the comparative enzyme activity.

Physical characterization

Gram staining

A glass slide was taken and smear was prepared on it followed by addition of crystal violet. It was washed after 1 minute with distilled water. After the washing step, Gram's iodine was added to it and then it was kept for 1 minute incubation time. After 1 minute, again it was washed with distilled water. In order to make the dye disappear, the decolorizer was added drop by drop till the color fades. The addition of counterstain was done and was kept for 30 seconds incubation followed by washing with tap water. Blotting paper was used to dry the slides and then it was observed under microscope using different lenses.

Chemical characterization

Catalase test

Bacterial culture was placed on slide using the inoculation loop. Few drops of hydrogen peroxide were added to it and it was kept for 2 minutes in order to observe the result. If it is observed that some bubble formation is taking place after few minutes then it means that the result is positive indicating the presence of catalase enzyme.

Oxidase test

Oxidase discs were taken and placed on slide. Using the sterile loop, culture was spread on the disc. The color change (purple) indicates positive result which shows the presence of Cytochrome oxidase.

TSI agar test

TSI agar slant was prepared and the bacterial culture was stabbed and streaked on it aseptically. The incubation period was 24 hours at 37°C after which the slant was observed for color change.

Indole test

The inoculation of bacterial culture was done in LB broth using an inoculation loop. It was then incubated at 37°C for 48 hours followed by the addition of Kovac's reagent (10-12 drops). It was then observed for color change. Pink or red ring indicates positive result.

Methyl red test(MR)

Using sterile inoculation loop, the culture was inoculated in MR-VP broth. The inoculated broth was kept for incubation at 37°C for 48 hours. After the incubation time, addition of 6-8 drops of methyl red is done which leads to red color formation for positive result.

Voges Proskauer test (VP)

Sterile inoculation loop was used to inoculate pure culture in MR-VP broth followed by 48 hour incubation at 37°C. 1 ml of broth was taken from this and was tested for VP test by adding 15-20 drops of Barritt A and 6-10 drops of Barritt B. After shaking it well, the observation was done for color change. Pink or red color shows positive result. Negative reaction will show no color change.

ISSN: 0975-8585

Simmons citrate media test

A sterile test tube was taken and Simmons citrate agar was added to it. This was kept in slant position till it gets solidified and then the culture was streaked on it. After 24 hours incubation at 37°C, the result was observed for color change.

Optimization by using Taguchi's method (a new statistical approach)

Optimization of the nutritional factors and physical factors was done. Taguchi's orthogonal array method [19] was used to carry out the optimization. All the parameters in this method at each combination and different level will appear in equal number of times. Size of experiment is given in symbolic designation. A relative value is present which represents all the factors.

To carry out the optimization, 100ml sterile flask was used in which 50ml nutrient broth with 1% of culture inoculum was prepared.

5 factors with 2 different levels were taken in the optimization.

Factors	Level 1	Level 2
Agitation	100 rpm	150 rpm
pH	9	10
Temperature	38°C	45°C
Peptone	0.4g/100ml	0.6g/100ml
NaCl	0.4g/100ml	0.6g/100ml

After that the design of experiment was done using Taguchi's method in MINITAB16 for the above mentioned factors and levels.

SN RATIO

Signal to noise ratio was analysed from Taguchi's loss function and then it was calculated using larger is better formula.

Optimization of Ph

pH optimization was carried out to check if the protease is alkaline or acidic. 100 ml flasks with 50 ml broth having 1% bacterial inoculum in each were taken and kept for different pH 5,6,7.5,9,10. After this, Folin lowry's assay was performed.

RESULTS AND DISCUSSIONS

Screening of proteolytic bacteria

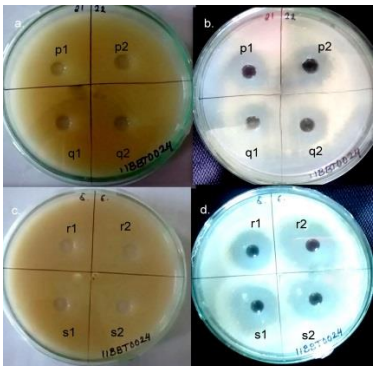


Figure 4: Showing the screening of proteolytic bacteria by well diffusion method in (1%) casein+skimmed milk agar plates. (A) and (C) Showing pictures before incubation. (B) and (D) showing pictures after incubation(72 hours)

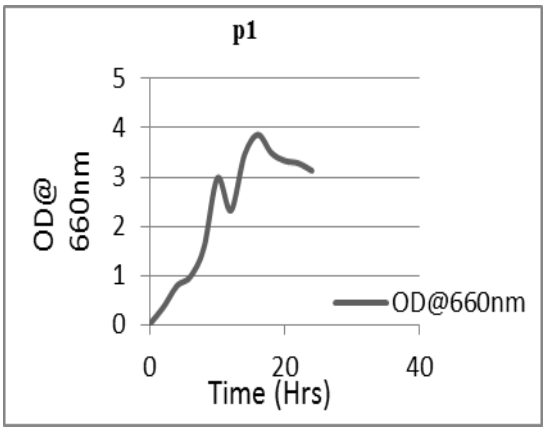
ISSN: 0975-8585

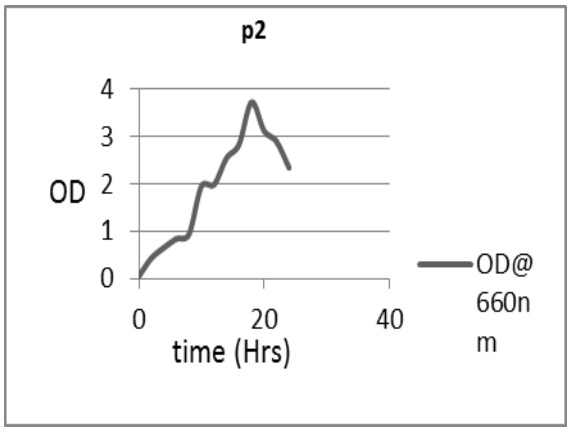
Zone of proteolysis was shown in the plates for some cultures after performing the well diffusion method (figure 4), indicating the presence of protease hence these bacterial samples are screened for proteolytic activities.

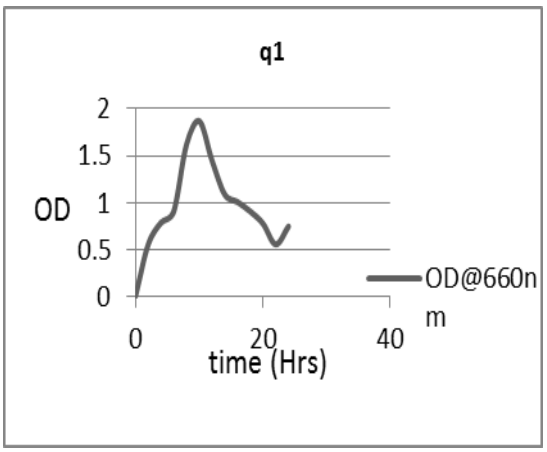
Bacterial growth curve study

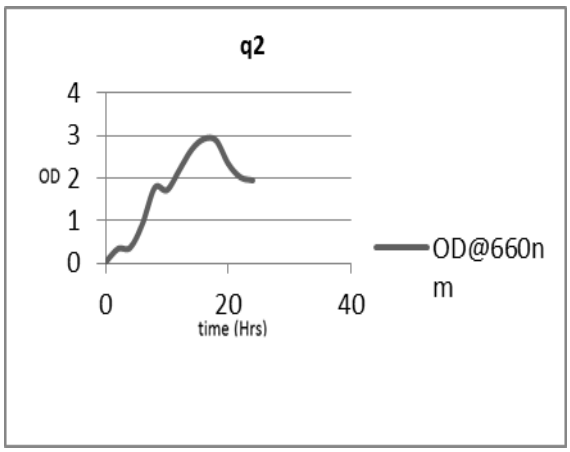
Bacterial growth curve study for colonies p1,p2,q1,q2,r1,r2,s1 and s2.

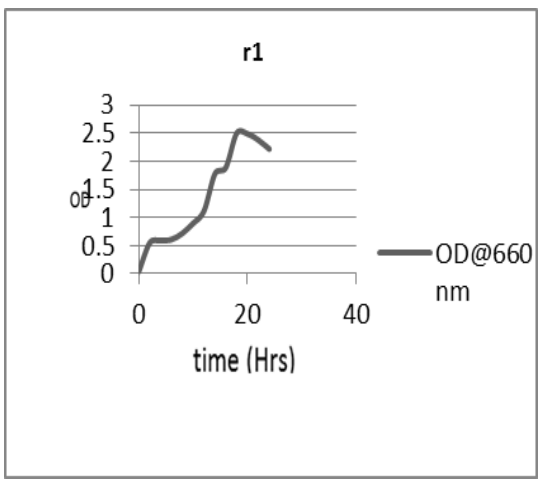
The enzyme assay is done using the exponential phase time for each colony because of the higher enzyme production rate at this phase **(**Fig 5**.** p1,p2,q1,q2,r1,r2,s1,s2**):**

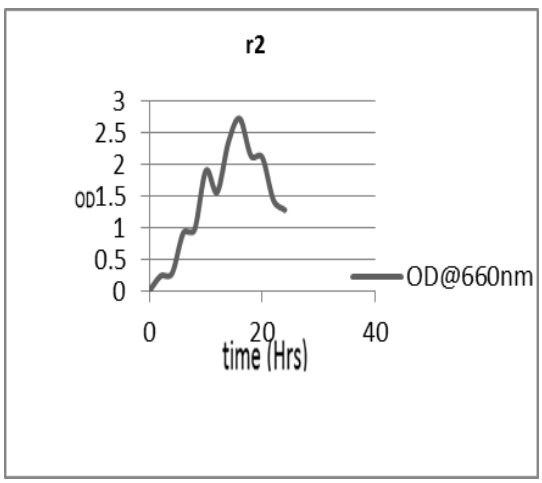












ISSN: 0975-8585

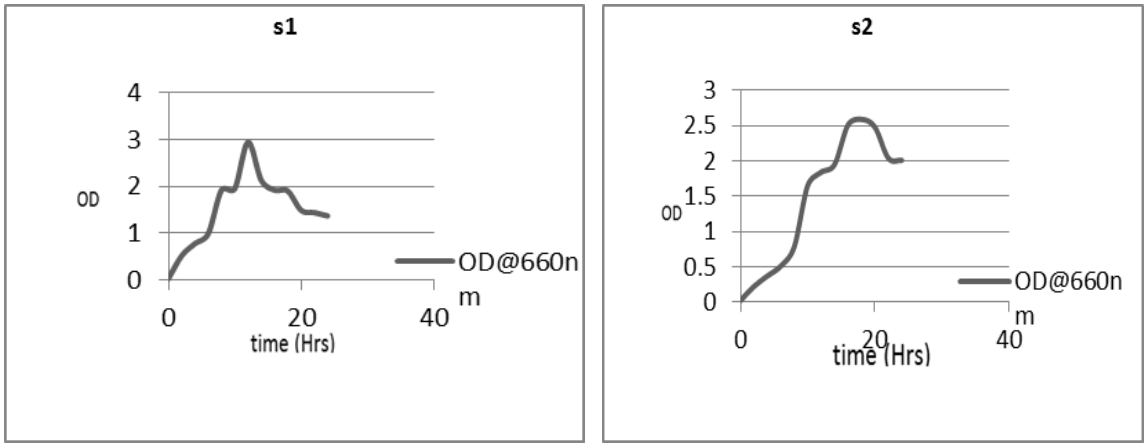
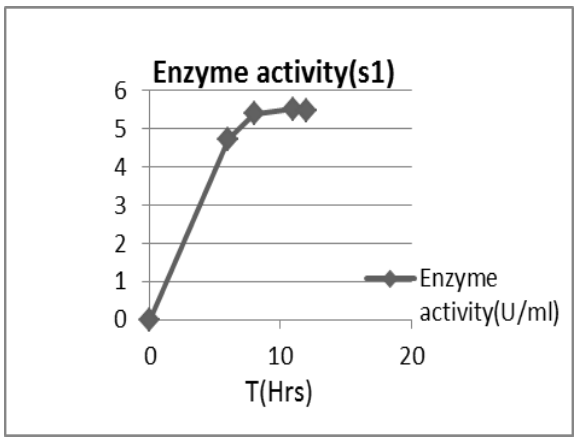
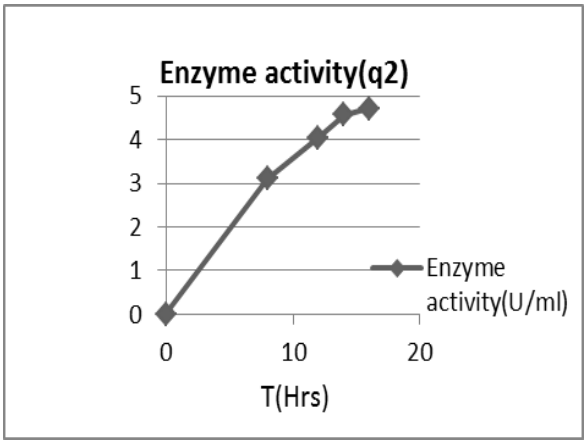
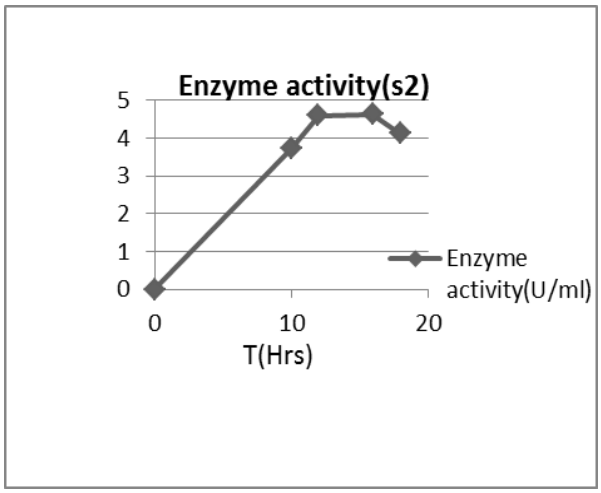
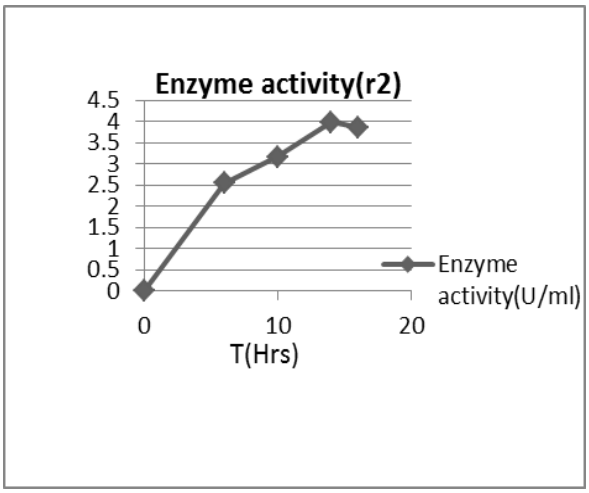


Figure 5: (p1,p2,q1,q2,r1,r2,s1,s2): Bacterial growth curve study for colonies p1,p2,q1,q2,r1,r2,s1 and s2.
Comparison of protease enzyme activity by Folin Lowry's method









ISSN: 0975-8585

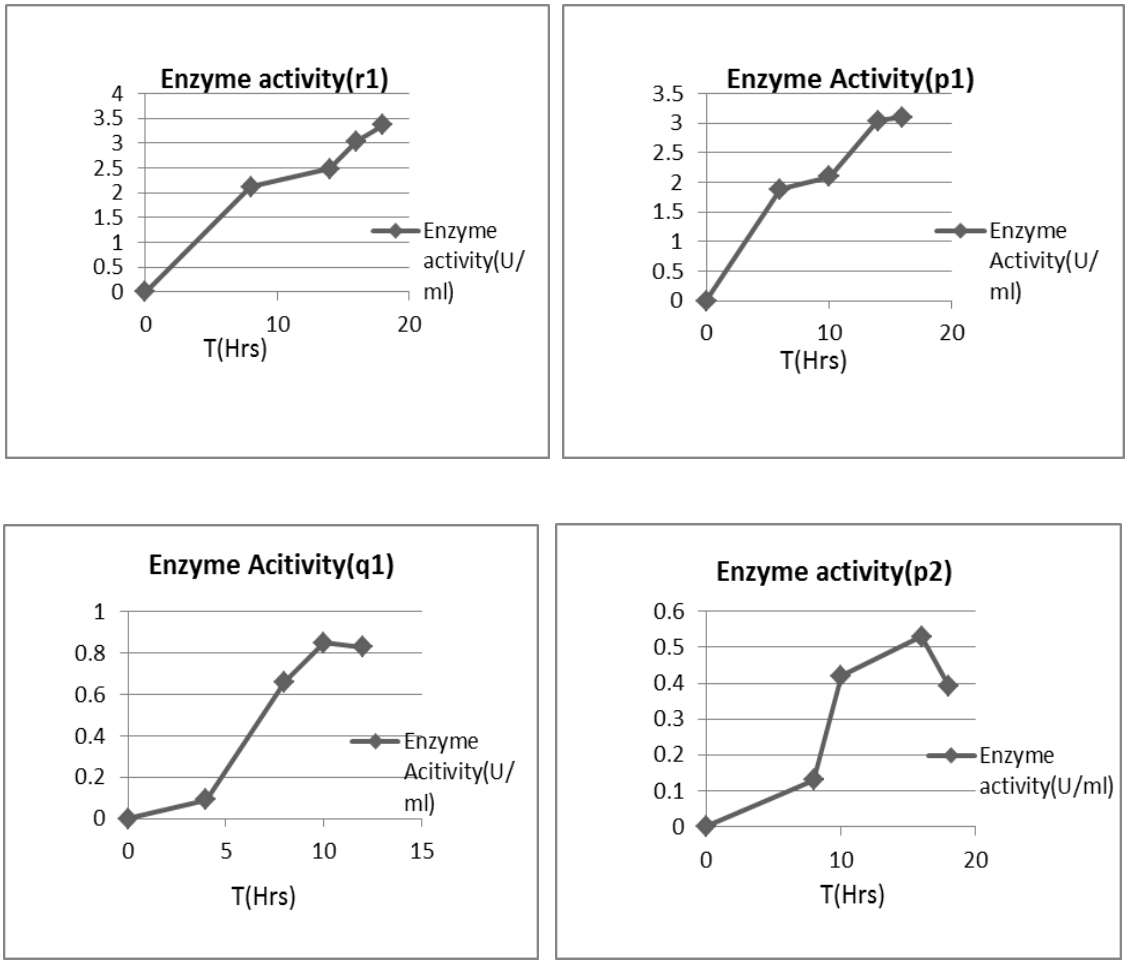


Figure 6: (s1,q2,s2,r2,r1,p1,q1,p2) : Showing the protease activities of s1,q2,s2,r2,r1,p1,q1,p2

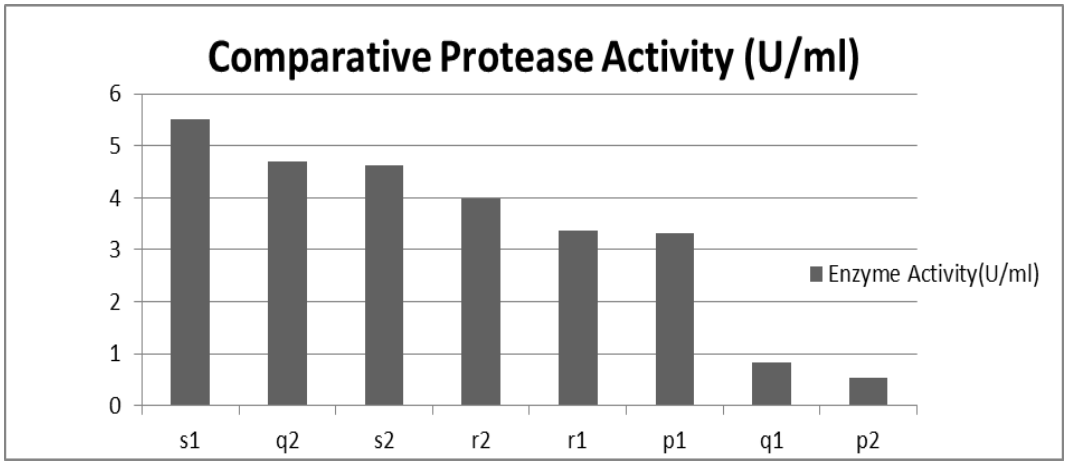


Figure 7: Showing comparative protease activities of s1,q2,s2,r2,r1,p1,q1,p2

Physical and chemical characterization

Physical and chemical characterization was done for the bacterial sample showing highest protease activity. S1 isolated from milk packet dumping area was showing highest enzyme activity so its identification was done using Gram's staining (figure 8), IMViC tests(figure 9) , TSI agar test, Catalase test and oxidase test (figure 10).

ISSN: 0975-8585

Gram staining

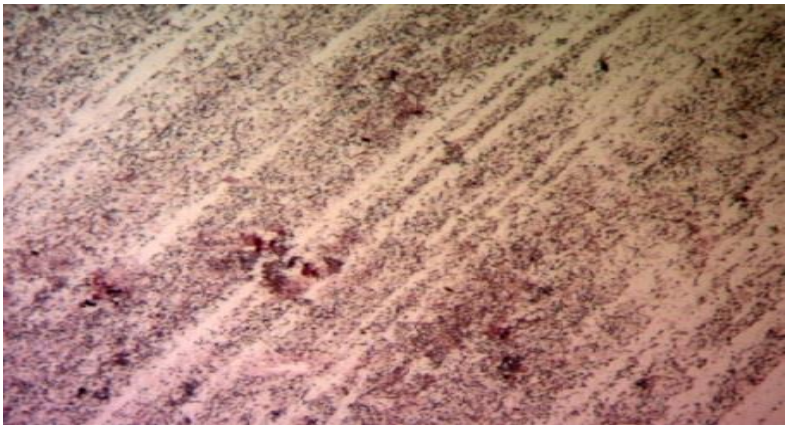


Figure 8: showing the gram negative bacillus at 10X magnification.

Chemical characterization

Test	Result
Indole test	-ve
Methyl red test	+ve
VP test	+ve
Citrate test	+ve
Catalase test	-ve
Oxidase test	+ve
TSI agar test	Glucose(+), lactose(-), Sucrose(-), H2S(-)

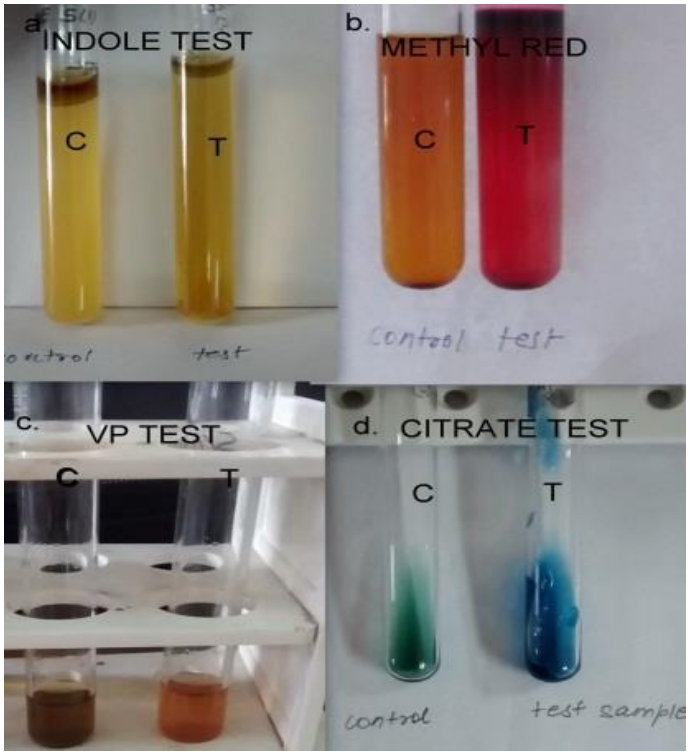


Figure 9: showing the results for IMViC tests (A) Negative for indole test. (B) Positive for Methyl red test shown by red color formation. (C) Positive result for VP test shown by pink color formation. (D) Positive result for Simmon Citrate test shown by blue color formation

ISSN: 0975-8585

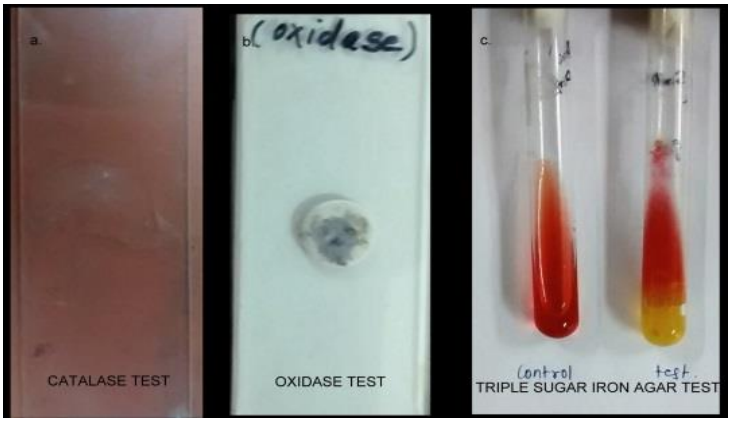


Figure 10: (A) Catalase –ve because of absence of bubble formation. (B) Oxidase +ve due to the purple color formation. (C) Glucose fermentation is there as the butt of slant turned yellow.

Optimization of pH

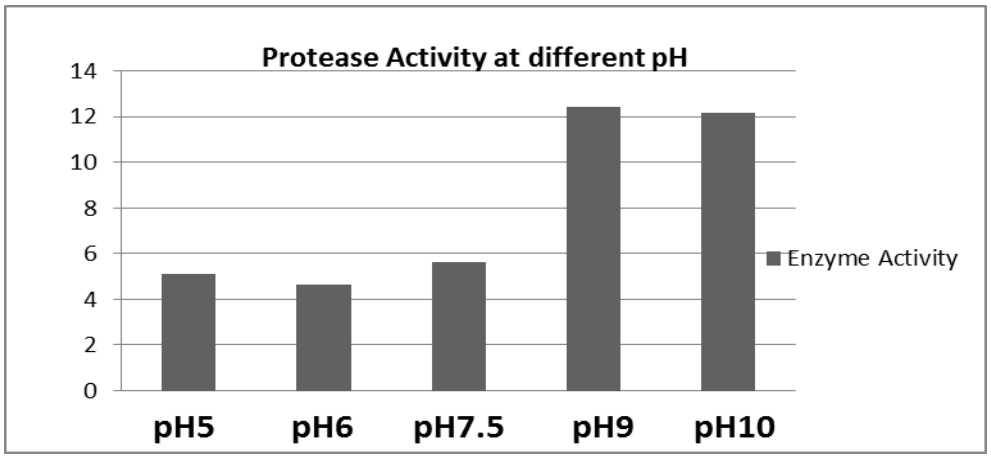


Figure 11: Showing the protease activity of strain s1 at 660nm carried out in various pH.

The optimization of pH was done which showed highest activity at pH9. It shows the alkaline nature of protease produced by bacterial strain s1 (figure 11).

Statistical Optimization of pH, temperature, agitation, Peptone and NaCl using Taguchi's method

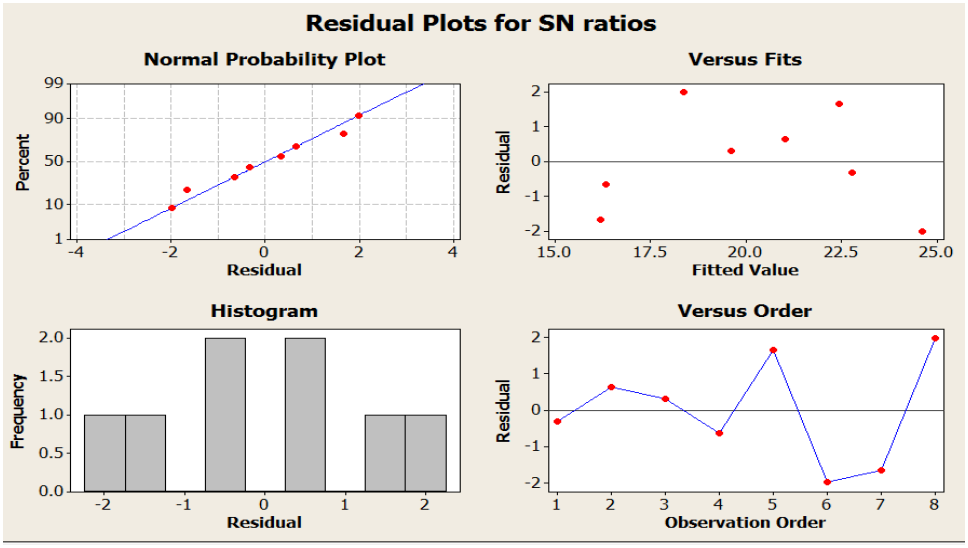


Figure 12: Showing residual plots for SN ratios

ISSN: 0975-8585

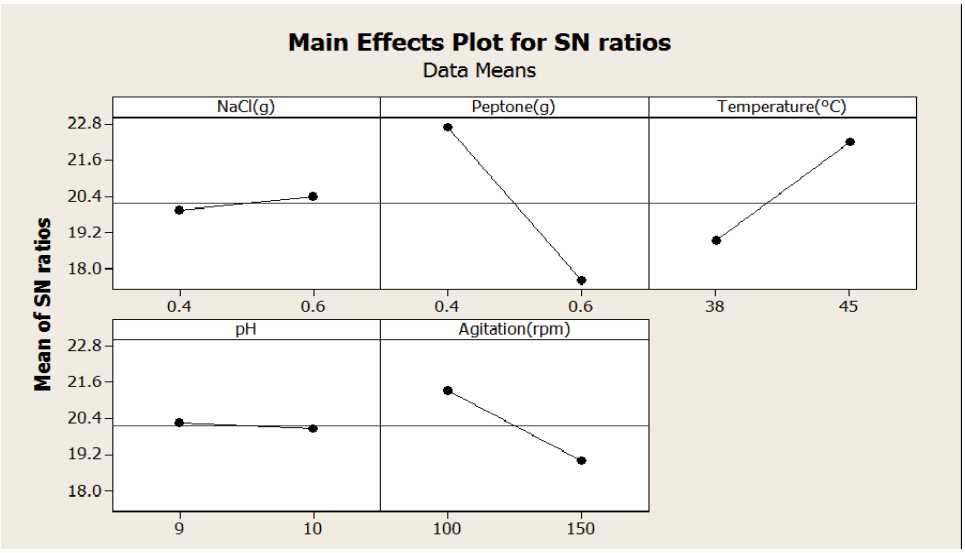


Figure 13: Showing mean effects plot for SN ratios

CONCLUSIONS

For the colonies which were isolated from the soil samples were first screened for protease activity by casein milk agar plate. After getting the desired proteolytic bacterial colonies they were subjected to Folin lowry's assay from which the selection of best bacterial colony was done. Here the best bacterial colony was s1 followed by q2,s2,r2,r1,p1,q1 and p2 based on their protease activity. From this a conclusion was made that the protease activity is highest in the proteolytic bacteria isolated from soil samples of milk packet dumping areas second best is from paper waste dumping area, third one is from cattle house area and the least proteolytic activity was shown by the bacteria from VIT lakeside soil sample. Bacterial colony showing the highest proteolytic activity was selected. Physical and chemical characterization of the colony was done proving it to be a *bacillus* strain. The pH optimization test showed that the colony is producing alkaline protease. MINITAB16 statistical software was used to do the optimization of the physical factors and nutritional factors. Taguchi's orthogonal array method was used to carry out the optimization. 5 factors agitation, pH, temperature (Physical) and peptone, NaCl (nutritional) were taken and optimized. The analysis of Signal to noise ratio from Taguchi's loss function was done followed by calculation using larger is better formula (figure 12 and 13). It was interpreted from the main effects plot for SN ratio that the optimized pH for the bacillus is 9, optimized temperature is 45°C, optimized agitation is 100rpm, optimized NaCl is 0.6 g, and optimized peptone is 0.4g.

ACKNOWLEDGEMENTS

We would like to thank our honorable and beloved Chancellor, Dr. G. Viswanathan for his guidance and encouragement towards research works.

REFERENCES

[1] Sumantha A, Sandhya C, Szakacs G, Soccol CR and Pandey A. Food Technol Biotechnol 2005;43: 313-319.
[2] Shafee N, Aris SN, Rahman RZA, Basri M and Salleh AB. J App Sci Res 2005;1:1-8.
[3] Rao MB, Tanksale AM, Ghatge MS and Deshpande VV. Microbiol Mol Biol Rev 1998;62:597-635
[4] Deng AH, Wu J, Zhang Y, Zhang,GQ and Wen TY. Biores Technol 2010;101:7100-7106.
[5] Ward OP. Proteolytic enzymes. In: Moo-Young, M. Ed., Comprehensive biotechnology, the practice of biotechnology current commodity products, Pergamon Press, Oxford, 1985;3:789-818.
[6] Adinarayana K and Ellaiah P. J Pharm Sci 2002;5:272- 278.
[7] Horikoshi K, Akiba T. Alkalophilic Microorganisms: A New Microbial World. Tokyo, Japan: Japan Scientific Societies Press and Berlin, Germany: Springer-Verlag, 1982.
[8] Durham DR. J Appl Bacteriol 1987;63:381–86.

[9] Gee JM, Lund BM, Metcalf G, Peel JL. J Gen Microbiol 1980;117:9–17
[10] Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K. Appl Environ Microbiol 1993;59:2311–16.
[11] Yan TR, Lin TT. Biotechnol Techniques 1995;9:215–20
[12] Jones BE, Grant WD, Collins ND, Mwatha WE. Alkaliphiles: diversity and identification. In: Priest FG, Ramos-Cormenzana A, Tindall B, editors. Bacterial Diversity and Systematics, New York: Plenum Press, 1994. pp. 195–230
[13] Grant WD, Mills AA, Schofield AK. J Gen Microbiol 1979;110:137–42.
[14] Mao W, Pan R, Freedman D. J Ind Microbiol 1992;11:1–6
[15] Nasuno S, Ohara T. Agric Biol Chem 1971;35:829–35.
[16] Sen S, Satyanarayana T. Ind J Microbiol 1993;33:43–47.
[17] Fujiwara N, Yamamoto K. J Ferment Technol 1987;65:345–48.
[18] Oliver H Lowry, Nira J Rosebrough, A Lewis Farr, and Rose J Randall. J Biol Chem 1951;193: 265-275.
[19] R Gopinath and Suneetha V. J Pure App Microbiol 2014;8(2):1749-1754