ENCAPSULATION OF TRANSFORMED PROTEASE AND ITS APPLICATION IN DETERGENT INDUSTRY

Hasan Muneer*, Muhammad Hafeez, Saman Ali, Maham Shahid and Shafaq Aiyaz Hassan

[1]*Department of Biosciences, Mohammad Ali Jinnah University Karachi*, 22-E, Block-6, PECHS, Karachi-75400.*Pakistan*
[2]*Department of Biotechnology, University of Karachi, Karachi-75270, Pakistan.*
hasan.muneer@jinnah.edu; hafeezhanifmemon@gmail.com; m.hamza081023@gmail.com; saman.ali1911@gmail.com; maham.shah2015@gmail.com; shafaqah@uok.edu.pk
*****corresponding author: hasan.muneer@jinnah.edu

ABSTRACT

Protease enzyme playing important role in detergent industries because of their hydrolytic properties to breakdown proteins. Detergent industries use protease enzyme in detergents for the removal of protein based stain such as blood. Among all protease enzymes Serine protease are the one having unique properties and stability in alkaline nature. Serine protease is produced by multiple sources, with the increasing demand of protease in the market, and for cost effectiveness. In this research work *E.coli Bl21* is used to produce serine protease having recombinant gene for protease production. E.coli is most common, fast growing, easy to handle and least harming bacteria. Encapsulation of protease was done by using sodium alginate and check the activity against casein to identify the presence of protease. Presence of white zone near encapsulated protease beads indicates the presence of enzyme. The activity of protease against blood stain or protein based stain was checked by washing blood stained cloth with beads containing detergent and another stained cloth with detergent without having protease beads. The cloth washed with detergent having beads is pure clean while other one have some stains on it.

Keywords: Cloned Protease, Detergent, Protein Stain, Immobilization, Cell Free Filtrate, E.coli BL21.

INTRODUCTION

PROTEASE:

The enzyme protease plays a consequential use in making industrial products and processes. It is the procedure of breaking down proteins. Microbial proteases are generally extracellular (Kour *et al.,* 2019) The considerable essential bacterial origin for protease enzymes is the Bacillus. In this study the aim was stabilize the cloned serine protease in standard strain of E.coli from bacillus specie. Enzyme protease hydrolysis the peptide bond into the peptide chains (Verhamme *et al.,* 2019) protease has comprehensive research because of its enhanced applications such as formulation for detergent to remove blood strain from fabrics, dehairing of skin, food processing and production of bioactive peptides (Contesini *et al*., 2018) . The proteases of different Bacillus species have been extracted for commercial use. There are six types of this compound, and they are categorized according to the functional group of their active site. They are Aspartic, cysteine, glutamic, metallo, serine, and threonine protease (Marathe *et al.,* 2019). As well as detergent protease, alkaline serine protease occupies the largest share of the enzyme market, which allows the enzyme to be active with in a neutral range of pH, to alkaline pH ranges (Naveed *et al.,* 2021). Construction of recombinant protease from Bacillus subtilis some persuasive minerals for protease are magnesium sulphate, potassium dihydrogen phosphate, and manganese sulphate indicates the impact in the production of protease (Sharmin *et al*., 2017).

TYPES AND APPLICATIONS OF PROTEASE:

- → ALKALINE PROTEASE
- → ACIDIC PROTEASE
- → NEUTRAL PROTEASE

ALKALINE PROTEASE:

Commercially significant alkaline protease, which is active at alkaline pH values between 8.0 and 9.0, depends on the genus *Bacillus* (Contesini *et al*., 2018). The alkaline protease are found in soil, water, and extremely alkaline

conditions. Segregation of alkaline proteases has been observed from a variety of sources, including soil, dried fish, slaughterhouses, and detergent contamination. (Ashraf *et al.*, 2023). Alkaline proteases, which are serine proteases with an alkaline pH range, are most commonly used by the detergent industry (Anwar and Saleemuddin, 1998). When used for different applications in the food, pharmaceutical, and other relevant industries, alkaline proteases maintain a steady alkaline pH and exhibit distinctive action (Matkawala *et al*., 2021). Due to an increasing need for proteases and their affordability, alkaline proteases are manufactured from a variety of sources (Adetunji *et al*., 2023). Marine shipworms and a strain of Bacillus species, MPTK 712, which was isolated from dairy slush and contains alkaline protease, have a symbiotic connection (Babu *et al*., 2023).

ACIDIC PROTEASE:

Acidic proteases are frequently used in soy sauce, protein hydrolysate, digestive aids, and the creation of flavoring material because they are stable and active between pH 3.8 to 5.6 (Razzaq *et al*., 2019). Acidic proteases with a molecular weight of 30–45 kDa have an ideal pH of 3–4, and their isoelectric point range is between 3 and 4.5 (Vishwanatha *et al*., 2010). Additionally, acidic proteases are used to soften fibril muscle, improve wheat paste texture, and clarify fruit juice and beer (Arogundade *et al*., 2023). Compared to alkaline proteases, fungal species such *Aspergillus niger*, *Aspergillus oryzae*, and *Aspergillus saitoi* are the primary producers of these extracellular acidic proteases (Shivakumar, 2012). *Aspergilla opepsins* are the most common type of fungus extracellular acidic proteases (Ashraf *et al*., 2023). The active sites for catalytic activity of aspartic proteases are made up of 380–420 long chains of amino acid residues (Pinheiro *et al*., 2015).

NEUTRAL PROTEASE:

Neutral proteases are characterized by their ability to function at pH values that are neutral, slightly acidic, or slightly alkaline (Sharma *et al*., 2019). The genus Bacillus contains mostly neutral proteases that have a restricted thermotolerance between pH 5 and pH 8. In addition to their medium rate of reactivity, they produce less bitterness when food proteins hydrolyze, making them more valuable in the food sector (Tavano *et al*., 2018). The brewing industry uses neutrase because it is resistant to plant proteinase inhibitors (Malik *et al*., 2017). Neutral proteases are identified and described based on their strong affinity for hydrophobic amino acids (Singh *et al*., 2018).Due to their limited thermotolerance, neutral proteases' reactivity can be somewhat controlled throughout the food hydrolysate synthesis process (Somavarapu *et al*., 2021). Neutral proteases of the metalloprotease type are ineffective without a divalent metal ion (Achmad *et al*., 2016). Metalloproteases have the greatest diversity among protease types (Gurumallesh *et al*., 2019).

SERINE PROTEASE:

Serine protease belongs to one of the important families of hydrolase enzymes. They involve in proteolytic reactions and hydrolysis of peptide bonds between amino acids of protein (Yang *et al.,* 2015). It contain serine amino acid at active site and are the most common bacterial protease (Banerjee *et al.,* 2017). Among other species *Bacillus caseinilyticus* is a novel specie for the production of alkaline serine protease. *Bacillus caseinilyticus* is characterized by its usage in detergent industries (Mothe *et al.,* 2016). Some other species for the production of serine protease include *Bacillus pumilus, Thermus aquaticus, Bacillus licheniformis, Penaeus indicus, Aspergillus fumigatus and Aspergillus terreus* (Da Silva *et al.,* 2017). The conditions required for the production and activation of alkaline serine protease include pH range from neutral to alkaline (7 to 10) while temperature range varies from (30 to 65°C) organism to organism which is used for its production and it requires nutrients as inducers in the medium (Matkawala *et al.,* 2021). Serine is essential catalytic amino acid residue in serine protease containing histidine and aspartic acid (Vojcic *et al.,* 2015)

ENCAPSULATION:

Encapsulation is a concept that originated approximately 65 years ago based on extensive research that was carried out to develop this new technology based on the results of this research to develop it more practically (Timilsena *et al.,* 2015). As a result of the enzyme protease being encapsulated, it provides solidity against harsh conditions, such as alkaline environments or high temperatures, which can lead to the enzyme protease being damaged (Wang *et al.,* 2024). The enzymes encapsulated in a protective layer of the detergent are slowly dispersed during the washing process so that the surface is thoroughly cleaned and the force is sustained during the process (Mamusa *et al.,* 2021). This research was aimed to monitored both the ability of the beads to load proteins as well as their proteolytic activity as part of the evaluation of the effectiveness of the technique (Matuła *et al.,* 2020).

DETERGENT:

Detergency is a term which describes the process of cleaning by a surface active agents. Detergents play important role in life (Landeck *et al.,* 2020). Surfactants, builders, corrosion inhibitors, brighteners, bleaching agents and enzymes are ingredients of detergents. (Gürkök *et al.,* 2019) Enzymes are the key components in formulation of detergents such as lipase, and protease (Niyonzima *et al.,* 2015). The essential ingredient in modern detergents is protease enzyme (Vojcic *et al.,* 2015). Detergents containing enzymes are effective in removing strains at high temperature and reduce water consumptions. (Gürkök *et al.,* 2019)

BACTERIA:

Bacterial species are a broad variety of earth ecology (Persat *et al.,* 2015). *Escherichia coli* is the most used microorganism for the production of biomolecules in biotechnology and for the production of recombinant proteins and enzymes. The strain BL21 is an expression strain. The molecules rich media such as Luria was used for the rapid division of bacteria and support enzymatic pathways (Li *et al.,* 2015). BL21 strain of E.coli containing recombinant of protease enzyme secretes extracellular protease to hydrolyze complex proteins or proteinaceous substances into small peptides and amino acids (Zhang *et al.,* 2015). Bacterial proteases are important because they are eco-friendly and microbial proteases have ability to tolerate harsh conditions, avoid autoproteolytic activity due to substrate specificity and pH stability (Razzaq *et al.,* 2019).

METHODOLOGY:

MATERIALS:

All the nutrients used in this research was purchased from Oxoid, U.K and the chemicals were purchased from Daejung Korea.

Borosilicate glassware:

Borosilicate glassware is highly valued in laboratories as a result of its excellent thermal resistance and chemical stability. There is a particular reason why this material is commonly used for items such as beakers, flasks, and test tubes, since it is capable of withstanding high temperatures and drastic temperature changes without breaking. Moreover, this type of glassware is also resistant to chemical corrosion, making it ideal for a variety of scientific experiments and procedures that require the use of glassware.

PREPARATION OF MEDIA:

As a medium for bacterial growth, Luria broth is used due to the fact that it contains a great deal of nutritional value as well as enabling the cultivation of a wide variety of bacteria, especially *E. coli*.

Table 1. Recipe of Luria broth for the growth of transforming.

S.No	INGREDIENTS	QUANTITY
1	NaCl	1g
2	Tryptophan	1g
3	Yeast extract	0.5g
4	Water	100mL

Then autoclave it at 15 psi for 15 minutes.

INOCULATION:

To avoid contamination, the area was cleaned with 70% ethanol and work was performed under sterile conditions. Final concentration of 1mm kanamycin antibiotic in the test tube containing broth, Addition of antibiotic only allow the growth of bacteria containing recombinant plasmid as they have antibiotic resistant gene. Take bacterial culture and pour it into antibiotic containing broth and then place it in incubator for 24hr to provide the optimum bacterial growth temperature which was 37°C.

ACTIVITY AGAINST CASEIN AGAR:

Solution was mixed until all the ingredients dissolved completely and after addition of agar placed in microwave till it boils. Then poured casein agar solution in petri plates and left them for 10 to 15 min on room temperature to solidify. Well was prepared in casein agar plate and CFF of transformed culture was added in the well. Immobilized enzyme was added in another plate of casein agar.

Table 2. Recipe of Casein Agar for the activity of Protease.

S.No	INGREDIENTS	QUANTITY
1	Tris	0.06g
2	Casein	0.5g
3	water	50mL
4	Agar	1g
5	pH	8.0

ENCAPSULATION THROUGH SODIUM ALGINATE:

For the preparation of the immobilized enzyme beads, 50ml of sodium alginate and 50ml of CFF in a flask was added. A large beaker was placed on magnetic stirrer the chilled calcium chloride was placed on stirrer along with ice around it to prevent the beads from being broken. The mixture of sodium alginate and CFF was added drop by drop during the stirring process, make sure to slow the speed of the stirrer in order to stabilized the beads.

DESTAINING AGAINST BLOOD:

Took two pieces of white color cloth and stain it with blood drops and allowed it to dry. Both the jars fill with water and add detergent (enzyme-free) in both jars. encapsulated beads of protease enzyme was added in one jar. Place piece of blood stained fabric in both jars and place both jars on shaking incubator for shaking for 15 minutes and then results was observed.

RESULT

The culture was grown at 37°C. overnight culture was grown for 16 hours. With final concentration of 1mM of antibiotic kanamycin. The culture contains recombinant protease gene.

GROWTH OF BACTERIA:

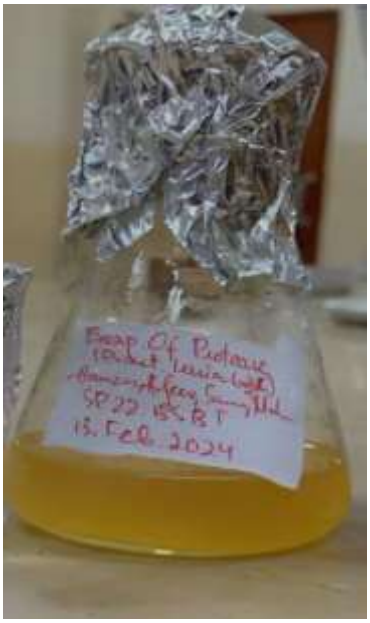

Fig. 1. Growth of E. coli Bl21.

Fig. 2. Immobilized protease with sodium alginate.

ENCAPSULATION THROUGH SODIUM ALGINATE:

The sodium alginate beads were formed by mixing cell free filtrate and 1% sodium alginate in the ratio of 1:1. The protease was covered in a protection of sodium alginate beads. Which will stabilize the enzyme.

ACTIVITY AGAINST AGAR:

The casein was used as a substrate against immobilized protease at 37°C for overnight. It was observed that immobilized protease formed 20 mm zone of degradation against the casein.

Fig. 3. Immobilized protease enzyme against casein.

DESTAINING AGAINST BLOOD:

The activity of immobilized protease was checked against the blood stain. It was observed that the test sample means detergent containing immobilized protease shows more efficiency than the detergent with out enzyme. Test sample cleared all blood patches. While control (without enzyme) have some patches of blood.

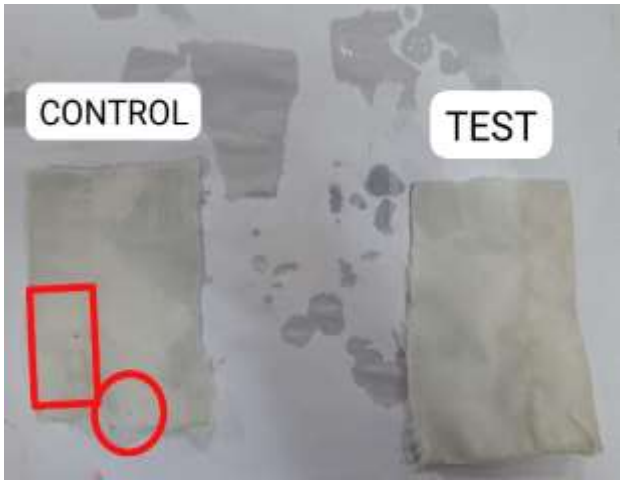

Fig. 4. The test having Protease Enzyme shows more activity against the Protein Stain.

CONCLUSION

As a result of the immobilization of the enzyme in this experiment, enzyme became more stable than it was. if it is used in a detergent which functions at pH 8.0. Next, the enzyme was added to a normal detergent to see. The better results were observed when the enzyme was added to the detergent, as compare the detergent without enzyme, Protease enzymes should be incorporated into detergent formulations to improve their stability and performance, to reduce adverse environmental effects, to make formulations more compatible, to make enzymes last longer, to deliver enzymes more efficiently, and to reduce cost. Encapsulated protease is becoming increasingly valuable as a component of environment friendly and advance detergent products because of these advantages, making it an essential ingredient in the development of such products. The field of encapsulation technology offers a lot of opportunity for more research and development in order to continue to make progress in this field and develop detergent formulations that are even more effective and sustainable in the future. These results in detergent formulations that are even better and more sustainable than they are today.

REFERENCES

Achmad, A. A., M. S. Rohman, and I. D. Prijambada (2016). Biochemical properties of crude extracellular proteases from Chromohalobacter salexigens BKL5 and Micrococcus luteus 11A. *Indonesian Journal of Biotechnology*, 21: 56-62.

Adetunji, A. I., M. O. Olaitan, M. Erasmus and A. O. Olaniran (2023). Microbial proteases: A next generation green catalyst for industrial, environmental and biomedical sustainability. *Food Materials Research*, 3: article 12, 2023.

Anwar, A., and M. Saleemuddin (1998). Alkaline proteases: a review. *Bioresource technology*, 64: 175-183.

Arogundade, Femi Qudus and Ridwan Abiodun Lawal (2023). Screening and isolation of protease-producing bacteria from wastewater samples in Obafemi Awolowo University (OAU) Campus, Ile-Ife, Nigeria. BioRxiv: 2023-07.

Ashraf, M., N. Hussain, Z. Baqar and M. Bilal (2023). Bioprospecting microbial proteases in various industries/sectors. In: *Microbial Biomolecules*. Academic Press, pp. 301-324.

Babu, Deepika S., Mithra S. Mohan, and A. Jayakumaran Nair (2023). Sreelekshmy VS 20 Characterization of proteases from bacterial and fungal species and evaluation of their ability in meat. *Microbial Oxidative Enzymes: Biotechnological Applications*, 2023: 409.

Banerjee, G. and A. K. Ray (2017). Impact of microbial proteases on biotechnological industries. *Biotechnology and Genetic Engineering Reviews*, 33(2): 119-143.

Contesini, Fabiano Jares, Ricardo Rodrigues de Melo and Hélia Harumi Sato (2018). An overview of *Bacillus* proteases: from production to application. *Critical reviews in biotechnology*, 38 (3): 321-334.

Da Silva, R. R. (2017). Bacterial and fungal proteolytic enzymes: production, catalysis and potential applications. *Applied biochemistry and biotechnology*, 183: 1-19

Gürkök, S. (2019). Microbial enzymes in detergents: a review. *Int. J. Sci. Eng. Res*., 10(9): 75-81

Gurumallesh, P., K. Alaqu, B. Ramakrishnan and S. Muthusamy (2019) A systematic reconsideration on proteases. *International Journal of Biological Macromolecules.* 128: 254-267.

Kour, D., K. L. Rana, S. Thakur, S. Sharma, N. Yadav, A. A. Rastegari, ... and A.K. Saxena (2019). Disruption of protease genes in microbes for production of heterologous proteins. In: *New and future developments in microbial biotechnology and bioengineering*, pp. 35-75. Elsevier.

Landeck, L., L. A. Baden and S. M. John (2020). Detergents. *Kanerva's occupational dermatology*, pp.1131-1143.

Li, Z., M. Nimtz and U. Rinas (2014). The metabolic potential of Escherichia coli BL21 in defined and rich medium. *Microbial cell factories*, 13(1): 1-17.

Malik, Kamla, S. Ahlawata and Shikha Mehta (2017). Microbial proteases: current perspectives and potential applications. *Ann Biol*., 33: 207-210.

Mamusa, M., C. Resta, C. Sofroniou and P. Baglioni (2021). Encapsulation of volatile compounds in liquid media: Fragrances, flavors, and essential oils in commercial formulations. *Advances in Colloid and Interface Science*, 298: 102544

Marathe, K. R., R. H. Patil, K. S. Vishwakarma, A. B. Chaudhari and V. L. Maheshwari (2019). Protease inhibitors and their applications: An overview. *Studies in natural products chemistry*, 62: 211-242.

Matkawala, F., S. Nighojkar, A. Kumar and A. Nighojkar (2021). Microbial alkaline serine proteases: Production, properties and applications. *World Journal of Microbiology and Biotechnology*, 37: 1-12.

Matkawala, F., S. Nighojkar, A. Kumar and A. Nighojkar (2021). Microbial alkaline serine proteases: Production, properties and applications. *World Journal of Microbiology and Biotechnology*, 37: 1-12.

Matuła, K., F. Rivello and W. T. Huck (2020). Single-cell analysis using droplet microfluidics. *Advanced Biosystems*, 4(1): 1900188

Mothe, T. and V. R. Sultanpuram (2016). Production, purification and characterization of a thermotolerant alkaline serine protease from a novel species *Bacillus caseinilyticus*. *3 Biotech*., 6: 1-10

Naveed, M., F. Nadeem, T. Mehmood, M. Bilal, Z. Anwar and F. Amjad (2021). Protease—a versatile and ecofriendly biocatalyst with multi-industrial applications: an updated review. *Catalysis Letters*, 151: 307-323.

Niyonzima, F. N. and S. More (2015). Detergent-compatible proteases: microbial production, properties, and stain removal analysis. *Preparative Biochemistry and Biotechnology*, 45(3): 233-258.

Persat, A., C. D. Nadell, M. K. Kim, F. Ingremeau, A. Siryaporn, K. Drescher, ….. and H. A. Stone (2015). The mechanical world of bacteria. *Cell*, 161(5): 988-997.

Razzaq, A., S. Shamsi, A. Ali, Q. Ali, M. Sajjad, A. Malik and M. Ashraf (2019). Microbial proteases applications. *Frontiers in Bioengineering and Biotechnology*, 7: 110.

Sharma, M., Y. Gat, A, Shalini and V. Kumar (2019). A review on microbial alkaline protease: an essential tool for various industrial approaches. *Industrial Biotechnology*, 15: 69-78.

Sharmin, S. (2017). *Application of lactobacillus species in the development of probiotic poultry feed as an alternative to antibiotic growth promoters.* (Doctoral dissertation, BRAC University).

Shivakumar, S. (2012). Production and characterization of an acid protease from a local *Aspergillus* sp. by Solid substrate fermentation. *Archives of Applied Science Research*, 4: 188-199.

Singh, S., P. Gupta and B. K. Bajaj (2018). Characterization of a robust serine protease from *Bacillus subtilis* K-1. *Journal of Basic microbiology*, 58: 88-98.

Somavarapu, S., B. Ramesh, G.V.S. Reddy and K. Srinivasan (2021). Enzymes from Microorganisms. *Innovations in Biotechnology for a Sustainable Future*. Pp. 45-58.

Tavano, O. L., A. Berenquer-Murcia, F. Secundo and R. Fernandez-Lafuente (2018). Biotechnological applications of proteases in food technology. *Comprehensive reviews in food science and food safety*, 17: 412-436.

Timilsena, Y. P., M. A. Haque and B. Adhikari (2020). Encapsulation in the food industry: A brief historical overview to recent developments. *Food and Nutrition Sciences*, 11(6): 481-508

Verhamme, I. M., S. E. Leonard and R. C. Perkins (2019). Proteases: pivot points in functional proteomics. *Functional Proteomics: Methods and Protocols*, pp.313-392.

Vishwanatha, K. S., A. G. Appu Rao and S. Annapurna Singh (2010). Acid protease production by solidstate fermentation using Aspergillus oryzae MTCC 5341: optimization of process parameters. *Journal of Industrial Microbiology and Biotechnology*, 37: 129-138.

Vojcic, L., C. Pitzler, G. Karter, F. Jakob, R. Martinez, K. H. Maurer and U. Schwaneberg (2015). Advances in protease engineering for laundry detergents. *New Biotechnology*, 32(6): 629-634.

Wang, P., F.Cai, Y. Li, X. Yang, R. Feng, H. Lu, …..... and J. Han (2024). Emerging trends in the application of hydrogel-based biomaterials for enhanced wound healing: A literature review. *International Journal of Biological Macromolecules*, 129300

Yang, Y., Y. Jun Wen, Y. N. Cai, I. Vallée, P. Boireau, M. Y. Liu and S. P. Cheng (2015). Serine proteases of parasitic helminths. *The Korean Journal of Parasitology*, 53(1): 1

Zhang, X. Y., X. X. Han, X. L. Chen, H. Y. Dang, B. B. Xie, Q. L. Qin …..... and Y. Z. Zhang (2015). Diversity of cultivable protease-producing bacteria in sediments of Jiaozhou Bay, China. *Frontiers in Microbiology*, 6: 1021.

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