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Experimental Preliminary Study for Production of Recombinant Subtilisin Enzyme by pET28b Cloning Vector

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ABSTRACT:
Purpose: The purpose of this study is to enable the efficient production of the industrial enzyme subtilisin, a serine protease with extensive applications in various industries such as detergents, food processing, textiles, and pharmaceuticals. By leveraging recombinant DNA technology to produce subtilisin locally, the study aims to decrease reliance on foreign imports and enhance the sustainability of enzyme supply chains. This initiative seeks not only to meet the growing demand for subtilisin but also to promote economic independence, reduce costs, and foster innovation within the local biotechnology sector.
Material and Methods: Following bioinformatic calculations and processes for pET28 and subtilisin, the enzyme was produced.
Results: The results confirm successful cloning and expression of the subtilisin gene in the pET28b vector, creating the recombinant construct pET28b-subt. Agarose gel electrophoresis verified the transformation, showing distinct bands for the pET28b backbone and subtilisin insert. The recombinant subtilisin was purified from lysed *E. coli* using Ni-NTA affinity chromatography, with SDS-PAGE analysis revealing a molecular mass of 41,646.82 Da (Figure 5, Figure 6). These findings demonstrate successful production of subtilisin, though further optimization is needed for industrial applications.
Conclusion: Production has been carried out and the sds-page result supports this. After this, it was seen that it was necessary to focus on activity studies.
Keywords: *Recombinant Protein; Subtilisin; pET28b*

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INTRODUCTION

Enzymes are biological catalysts that catalyze chemical reactions in the cells of living organisms, which are in the form of proteins. As such, they work under the conditions that the metabolic requirements of a widely diversity of cell types. In general, these metabolic requirements can be described as ▪ Chemical reactions must happen under the conditions of the media of the organism, ▪ Specifity by each enzyme, ▪ High-level reaction rates (Damhus et al, 2008)

Enzyme-catalysed processes are replaced chemical processes in industry (Figure 1) for several areas. Enzymes have all the features of actual catalysts. In the presence of a suitable enzyme, a chemical reaction takes place at a much high level but the enzyme is not depleted by the reaction. Their ability to officiate quite specific biotransformations has made them gainly popular in industries where less likely specific chemical processes bring out undesired by products. Simplicity and predictability are confidential significance in food industry where by products may be harmful or affect flavour and due to their specifity, pharmaceutical manufacturer favour biotransformations in the improving of new therapeutic agents. The greatest challenge is a research and improving program for matching an enzyme with a process. Increasingly, new organisms

are found out that survive in unsual habitats and these prove a great source of varied enzymes (Saul, 2010).

Recombinant DNA (rDNA) technologies give chance the production of wide range of polipeptides from naturally nonproducing living being. Firstly, pharmaceutical products were but nowadays many proteins are obtained by this way for various applications especially industrial enzymes (Porro et. al, 2005).

The requirement for enzymes and microorganisms in the industrial field has improved "Genetic Engineering and rDNA technology". The technology enables genetic regulation of microorganisms to generate the needed enzyme. Protein engineering job is finding out the structure of enzymes and modifying them full benefit. Studies focus on improving and producing of functional enzyme. Therefore, biotechnology becomes more attractive than chemical processes. The main advantages provided by enzymes; they are produced from natural resources and they are not harmful for environment etc (Hasan et. al, 2010). For example, biotech origin cleaning materials harmful for environment. Besides having specific activity, they can show this activity at lower temperatures. Their wastes are with lower chemical oxygen demand and not corrosive for nature. Enzyme based cleaning materials are increasingly preferred in the food industry compared to caustic and acidic cleaning agents (D'Souza & Mawson, 2005).

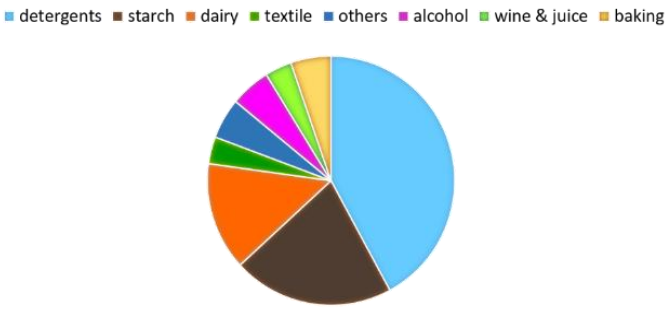

Figure.1. Distribution of industrial enzyme sales according to sectors (Falch, 1991)

Proteinases are one of the most substantial industrial enzymes, accounting for nearly 60% of total worldwide enzyme sales (Ward, 1985; Kalisz, 1988; Outtrup & Boyce, 1990). Since microorganisms can be fermented in a short time and in large quantities, they are the most preferred protease sources. Microbial based alkaline proteinases command the world market with the ratio of 2/3 of detergent industry (Gupta et. al, 2002; Mienda et. al, 2014).

Microbial peptides are long-lasting without loss activity as long as they are stored appropriate conditions. They are extracellular and can directly express in the medium of fermentation. Therefore, they have simplicity for producing that they are preferred than their counterparts in plants and animals. Microbial sources for commercial utilization are not toxic and not pathogenic that decided as safe (Gupta et. al, 2002).

Genus *Bacillus* is the most popular source for producing alkaline peptidases. Various species can live in different environments, which have ability for producing proteases. A large number of microorganisms produce alkaline peptidases of the serine type that they belong to bacteria, fungi (Kumar & Takagi, 1999).

Subtilisins (EC 3.4.21.62) are a family of subtilase super family, very important enzyme for industry that one of the serine proteases, which are, produced the largest amount by the *Bacillus spp*. They are used in laundry, dishwashing detergents and contact lens cleaning applications (Rao et. al, 1998; Bryan, 2000; Yang et. al, 2000; Saeki, 2007; Anonymous, 2020a).

Subtilisin has highly antimicrobial activity especially for gram-positive bacteria so that they have a broad usage such as food conversation, detergent industry, etc (Okeley et. al, 2003).

There is a significant demand for subtilisin in various industrial sectors, creating a substantial global market that attracts considerable interest. This study aims to produce an enzyme utilizing our own resources to meet this growing demand.

MATERIAL and METHODS

Bacterial Strains and Plasmid Construction

The gene sequence for subtilisin from Bacillus subtilisin (Accession number: HQ699519.1) was retrieved from the NCBI database following a comprehensive literature review and bioinformatics analysis. The pET28b vector (Figure 2) was selected as the cloning vector for this study. Additionally, codon optimization of the subtilisin gene was conducted using bioinformatics tools to enhance expression in the Escherichia coli K12 strain. In codon optimization studies, codons in the nucleotide sequence of the subtilisin gene and expressed at low frequency in *E. coli* K12 microorganism were replaced with codons expressed at higher frequency. For codon optimization, "JCat" codon optimization program was used (http://www.jcat.de/) and DNA alignment was performed using the "CLUSTAL Omega (1.2.1) Multiple Sequence Alignment" program.

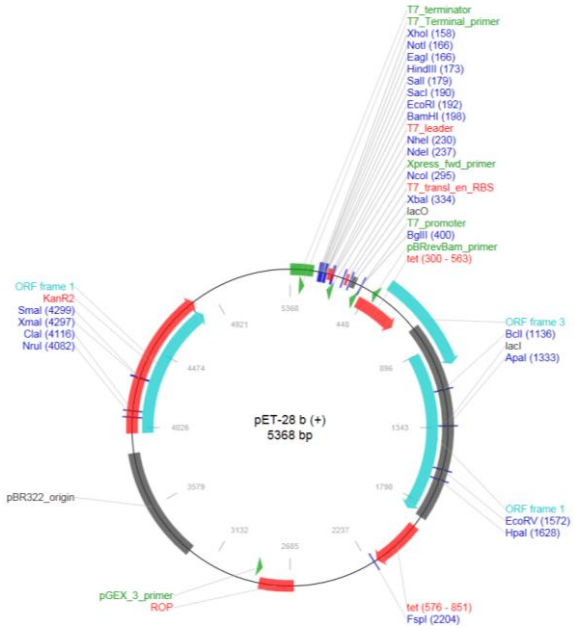

Figure 2. pET28b plasmid map (Anonymous, 2020b)

For the double cut 100 % cutting enzyme pairs (*NcoI* & *XhoI*) were created in the same buffer which was checked at the "NEB cutter" site for the recognition of these enzymes in the gene. These enzymes were placed at the beginning and end of the gene. Convenience in the purification was provided by adding six His-tag (Figure 3). At the end, the sequence was synthesized by "BIOMATIK Company".

Protein expression

BL21 (DE3) pLysE (*Novagen*), which is a strain of *E. coli* was used as host cell for expressing subtilisin protein. The strain was transformed by recombinant pET28b-subt plasmid (Hanahan, 1985). Transformed cells were spread on LB (Luria Bertani) agar plates which containing 100 $\mu M.mL^{-1}$ kanamycin. Then all cells were grown at 37°C for a night. A colony of recombinant cell was taken and transferred in to 3 mL LB medium for growing at 37°C and shaking (250 rpm) for 16 hours. Then overnight culture was inoculated into 600 mL of LB [containing kanamycin (1:10) & chloramphenicol (1:1)] and incubated until

the OD_{600}= 0.6 and was induced by isopropyl ß-D-1-thiogalactopyranoside (IPTG). Then the culture was incubated for 3 hours. The culture was collected by centrifugation and the cell pellets were resuspended in buffer A (100mM Tris – HCl -*pH= 7-*, containing 1 $g.L^{-1}$ lysozyme, 1 mM PMSF and 1 mM benzamidine). The cells were lysed with a vibra cell processor (sonics VCX130) and centrifuged (Vision VS-30000i) at 30000 rpm for one hour at 4°C. After centrifugation soluble and insoluble fractions were seperated. All samples were analyzed by 12% SDS-PAGE and dyed by commassie brillant blue (Laemmli, 1970).

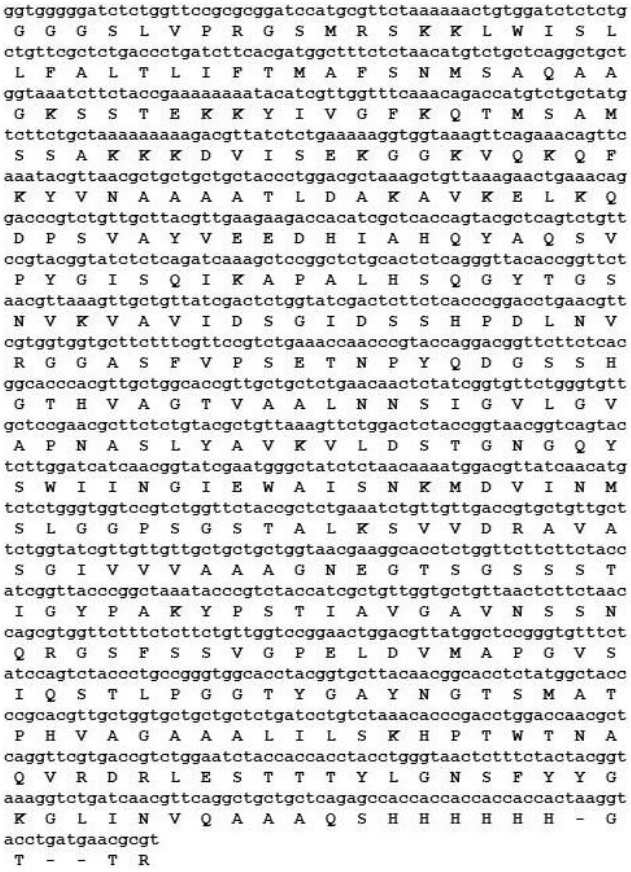

Figure 3. Gene sequence of recombinant subtilisin which synthesized after bioinformatics calculations

RESULTS

In this study, the XhoI-NcoI fragment containing the subtilisin gene was successfully cloned into the pET28b plasmid, resulting in the recombinant construct pET28b-subt Figure 4.. The process involved molecular techniques and bioinformatics analyses to ensure the accuracy of the inserted gene. Assessments on the subtilisin sequence included analysis of its structure and potential post-translational modifications, along with comparisons to entries in databases like NCBI and UniProt. The selection of restriction sites was crucial for effective ligation, and after digesting the vector and insert, competent E. coli cells were transformed with the recombinant plasmid. Verification of successful insertion was achieved through colony PCR and restriction digestion analysis, followed by IPTG induction for protein expression. Characterization of the protein was performed using SDS-PAGE to confirm the expected molecular weight, and tools like ProtScale and the SOLpro server were utilized to predict expression levels and solubility, aiding in the optimization of purification conditions. Overall, this work demonstrates the feasibility of producing subtilisin in a bacterial system and lays the groundwork for future functional studies.

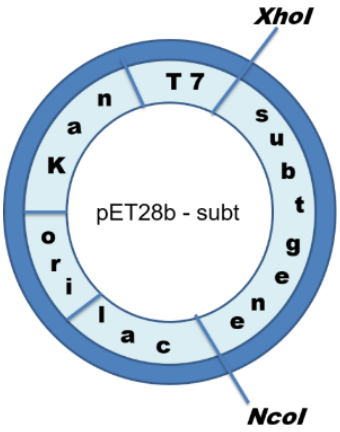

Figure 4. Map of pET28b-subt which subtilisin gene transferred to pET28b (+) vector

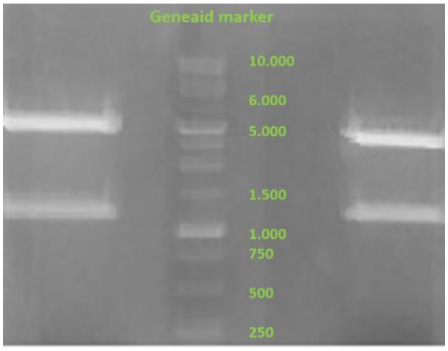

Figure 5. Verification of recombinant pet28b-subt by restriction enzyme digestion

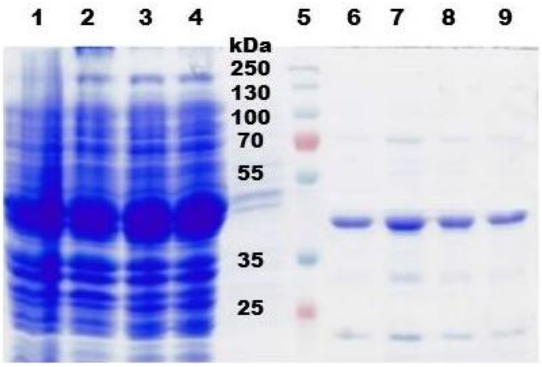

Figure 6. *1, 2, 3* ve *4*: *E. coli* lysate induced with IPTG. *5:* PageRuler Plus Prestained Protein Ladder. *6, 7, 8*, ve *9:* The eluates were collected from Ni-NTA agarose affinity column after washing with imidazole

Production has been carried out effectively, and the results from agarose electrophoresis (Fig. 5) and SDS-PAGE (Fig. 6) provide clear evidence for this outcome. Agarose electrophoresis enabled the visualization of nucleic acid fragments, confirming the presence and integrity of the cloned subtilisin gene. Meanwhile, the SDS-PAGE analysis confirmed the expression of the recombinant subtilisin protein, showing distinct bands that align with the expected molecular weight. Together, these analytical techniques illustrate that the recombinant protein was expressed as intended and substantiate the overall production process.

DISCUSSION

The analysis of the restriction enzymes NcoI and XhoI was conducted on agarose gel containing the "Geneaid" marker for the purified plasmid DNA (pET28b-subt) after the transformation process (Figure 5). he observed size of the pET28b vector, which falls within the 5000-6000 bp range, alongside the 1146 bp fragment corresponding to subtilisin, positioned in the 1000-1500 bp range, provides clear evidence of proper transformation and accurate insertion of the target gene. This ligation confirms that the plasmid retains the functional elements required for expression in *E. coli*. Amplification of the subtilisin gene from *Bacillus subtilis* was conducted using standard polymerase chain reaction (PCR) techniques to ensure the fidelity of the gene fragment. After amplification, the fragment was cloned into the pET28 vector with NcoI and XhoI restriction endonucleases. This new recombinant system, named "pET28b-subt," serves as a key platform for further studies on protein expression.

Following cloning, the recombinant subtilisin protein was purified using Ni-NTA affinity chromatography, taking advantage of the 6xHis tag that allows for selective binding to the resin. This purification method effectively isolates His-tagged proteins, achieving the necessary purity for subsequent applications. Analysis of the eluted samples via Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) revealed that the purified subtilisin migrated within the expected molecular weight range of 35,000-55,000 Da (Figure 6). The molecular mass calculated at 41,646.82 Da, determined using ExPASy ProtParam tools, closely aligns with the experimental results, further supporting the methods used for expression and purification.

Although the cloning and purification of subtilisin are important outcomes, further optimization is essential to improve enzymatic efficiency and activity. Existing literature highlights that achieving high enzymatic performance often requires meticulous preliminary studies (Billman-Jacobe et al., 1995; Ghasemi et al., 2012; Joshi & Satyanarayana, 2013). Variables such as expression conditions, including temperature and IPTG concentration, as well as purification strategies, can significantly affect the enzyme's yield and functionality.

Future research should focus on optimizing these parameters, potentially exploring different growth media, induction times, and post-harvest processing methods. Such optimizations are vital for maximizing the yield and stability of recombinant subtilisin, facilitating its application in biocatalysis and other industrial processes. Moreover, the work involving the cloning, expression, and purification of subtilisin lays a solid groundwork for further research. The methodologies developed in this investigation offer valuable insights into producing recombinant proteins in E. coli, highlighting the potential of subtilisin in various biotechnological applications. Continued optimization will be critical to fully harness the capabilities of this enzyme in practical scenarios.

CONCLUSION

This preliminary study on the production of recombinant subtilisin using the pET28b cloning vector has yielded promising results. Confirmation of the successful insertion of the subtilisin gene from *Bacillus subtilis* into the pET28b vector was achieved through molecular techniques, including agarose gel electrophoresis and SDS-PAGE analysis. The recombinant subtilisin protein migrated within the expected molecular weight range, with a calculated mass of approximately 41,646.82 Da, demonstrating the effectiveness of the expression system.

While the production of subtilisin has been successfully established, further optimization is essential to enhance its enzymatic efficiency and activity for industrial applications. Focus should be directed toward refining expression conditions and purification strategies to maximize yield and functionality. These efforts will not only improve the enzyme's performance but also expand its applicability across various sectors, including detergents, food processing, and pharmaceuticals.

The methodologies and findings from this study provide a solid foundation for ongoing research in enzyme production. Future investigations can explore a broader range of optimization parameters, ultimately facilitating sustainable and efficient production of subtilisin and potentially other

industrial enzymes. This initiative represents a significant step toward reducing dependency on external sources and fostering innovation within the local biotechnology landscape.

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