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PROTEASE ENZYME PRODUCTION BY *BACILLUS SUBTILIS* AND *STAPHYLOCOCCUS AUREUS* ISOLATED FROM SOIL UTILIZING AGRO-RESIDUES AS FERMENTATION SUBSTRATE – A REVIEW

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ABSTRACT:
The substrates utilized for the production of proteases from microorganisms are often expensive, which significantly increases the overall cost of enzyme production. Hence, identifying suitable low-cost alternatives and optimizing the production process are critical to making large-scale production economically viable. Proteases constitute one of largest groups of enzymes used in industrial application. Studies have demonstrated the effectiveness of various substrates, including rice bran and wheat bran, in maximizing protease yield. Among the evaluated carbon sources, wheat bran and soybean meal have shown exceptional potential when tested at different concentrations. Additionally, other cost-effective substrates such as hulled wheat grains, soybean meal, wheat flour, and rice bran have proven to be promising candidates. These organic substrates possess advantageous properties and are considered highly suitable for efficient enzyme production. Proteases find applications in diverse industries, including stain removal, digestion of natural proteins, and food processing, where they aid in breaking down complex proteins.

Keywords: - Protease enzyme, B. subtilis, S. aureus, Isolation, Low-cost substrates, Enzyme assay.

INTRODUCTION :

Proteases are one of the most significant groups of industrial enzymes due to their diverse applications. These enzymes are produced by various organisms and are naturally present in plant and animal tissues, with bacteria and fungi being the primary sources of industrial proteases (Elif Demirkan et al., 2020). Global demand for proteases continues to rise, accounting for approximately 59% of the total industrial enzyme market (Hanan S. Alnahdi, 2012). To meet this growing demand, alternative production strategies leveraging microorganisms have become crucial. The selection of an appropriate microbial strain plays a key role in maximizing enzyme yield. Fungi such as Rhizopus, Penicillium and Aspergillus are also capable of synthesizing proteases. While fungal proteases exhibit greater diversity, bacterial proteases are generally more reactive and thermally stable (Semih Yılmaz, 2023).

Proteases are extracellular enzymes primarily produced by Bacillus species. From an industrial perspective, the cost and availability of substrates play a pivotal role in enzyme production. The selection of a suitable substrate depends on several factors, with cost-effectiveness and accessibility being the most significant (C.E. Kotlar et al., 2012). A variety of industrial and agricultural wastes can serve as substrates for protease production. However, the efficiency of protease synthesis is strongly influenced by the carbon-to-nitrogen ratio and the overall composition of the medium (U.O. George-Okafor et al., 2012). Agricultural residues, including dried powders of wheat bran, rice bran, sugarcane bagasse, sugarcane molasses, rice mill waste, and fish waste, have been successfully utilized as low-cost substrates for protease production (Aishwarya Ramkumar, 2016). Using these substrates not only reduces production costs but also helps manage industrial and agricultural waste, thereby

mitigating environmental pollution (Temam Abrar Hamza, 2017). Proteases are utilized across various industries. For example, they are a key component in laundry detergents, aiding in the removal of protein-based stains. Additionally, they are employed in textile industries to impart unique finishes to wool and other fibers. Proteases also serve as effective cleaning agents for contact lenses (Mohsen M.S. Asker, 2013).

LITERATURE REVIEW :

Nihan Sevinc and Elif Demirkan (2011) conducted a study titled "Production of Protease by Bacillus sp. N-40 Isolated from Soil and Its Enzymatic Properties" their research highlighted that factors such as carbon and nitrogen sources, as well as metal ions, significantly impact protease production. Among the tested carbon sources, fructose demonstrated the highest potential for protease synthesis. Skim milk was identified as the most effective organic nitrogen source, while a combination of Ca^{2+} and Mg^{2+} ions enhanced production. The enzyme exhibited optimal activity at pH 7.0 and 55°C, with stability in the alkaline pH range of 6.0–9.0. The isolated Bacillus strain was found to be a promising producer of protease, indicating potential for industrial applications. However, further studies are necessary to optimize production for commercial scalability.

P. Vijayaraghavan and S.G. Prakash Vincent (2014) explored the use of agro-residues as substrates for fibrinolytic enzyme production in their study "Agro-residues were used as substrates for the production of fibrinolytic enzyme in solid-state fermentation" their findings revealed that various agro-residues could serve as effective fermentation media for protease production. Nutritional factors such as casein and sodium dihydrogen phosphate were found to significantly enhance fibrinolytic enzyme synthesis. Optimization of the medium resulted in greater enzyme activity compared to unoptimized conditions.

Kashif Younas Butt, Sumaira Kousar Butt, Ghori, M. I., Khan, M. A., and Younas, A. (2019) conducted a study titled "Production of a Serine Alkaline Proteinase from *Bacillus subtilis* by Using Low-Cost Substrate and Its Purification." their research demonstrated that Bacillus subtilis could efficiently produce proteases using low-cost substrates, followed by a purification process suitable for eco-friendly applications. The enzyme was purified using a ammonium sulfate precipitation, dialysis, ion-exchange chromatography, and gel filtration chromatography. The study concluded that B. subtilis possesses significant potential for protease production. Moreover, optimal production parameters were identified, with the enzyme showing maximum activity at a pH of 7.5 and a temperature of 60 °C. The purified serine protease holds strong potential for applications in industries such as detergents and leather processing.

Jermen Mamo and Fassil Assefa (2018) explored "The Role of Microbial Aspartic Protease Enzyme in Food and Beverage Industries." their findings highlighted the versatile applications of proteases across multiple industries. While aspartic proteases are most commonly utilized in cheese manufacturing, their potential extends to other sectors, such as the food and beverage, bakery, and brewery industries. Enzymes derived from bacterial and fungal sources have proven effective in producing curd for cheese production, removing haze in wine and beer, and improving bread quality in baking. Despite these uses, evidence supporting broader industrial applications of aspartic proteases remains limited.

Amal Hammami, Ahmed Bayoudh, O. Abdelhedi, and M. Nasri (2018) investigated "Low-Cost culture medium for the production of proteases by *Bacillus mojavensis* SA and their potential use for the preparation of antioxidant protein hydrolysates from meat sausage by-products."

their study aimed to enhance protease production using *Bacillus mojavensis* SA and explore its application in producing bioactive protein hydrolysates from meat by-products. The research revealed that poultry feathers were not effective substrates for protease production. Instead, wheat bran and soybean meal were identified as optimal carbon sources. Additional substrates such as hulled wheat grains and rice bran also demonstrated effectiveness. This research underscores the significance of selecting appropriate substrates for maximizing enzyme production.

Abd- ElKhalek, A. M., Seoudi, D. M., Ibrahim, O. A., Abd-Rabou, N. S., and Abd El Azeem, E. (2020) conducted a study titled "Extraction, Partial Purification, Characteristics, and Antimicrobial Activity of Plant Protease from Moringa oleifera Leaves." the primary objective of this research was to evaluate the enzyme's antimicrobial effect against specific pathogenic bacteria. Their findings revealed that purification using ammonium sulfate yielded the highest specific activity, while gel filtration using Sephadex G-100 produced the best specific activity and the highest purification fold, with a yield ranging from 34% to 43%. The optimal pH and temperature for protease activity were determined to be 7 and 50 °C, respectively. Notably, the enzyme's efficacy was enhanced when combined with antibiotics against certain bacterial strains. The study concluded that protease derived from Moringa oleifera leaves is a promising, cost-effective, and safe enzyme source, making it suitable for various industrial applications.

M.E. Uddin, T. Ahmad, M.M. Ajam, M. Moniruzzaman, D. Mandol, S.K. Ray, A. Sufian, M.A. Rahman, E. Hossain, and T. Ahammed (2017) investigated "Thermotolerant Extracellular Proteases Produced by *Bacillus subtilis* Isolated from Local Soil Representing Industrial Applications." their research highlighted that enzyme production through biological processes is more efficient and sustainable compared to chemical methods, with significant potential for application in industries such as leather processing. A novel protease-producing strain of Bacillus subtilis was isolated from local soil samples. The enzyme demonstrated optimal activity at 60 °C and pH 8.5. Purity testing using SDS-PAGE revealed a molecular weight of approximately 30 kDa. Due to its stain-removal properties, this protease enzyme also shows potential for use in detergent, poultry, and leather industries.

Muthu Padmapriya and B. Christudhas Williams (2012) examined "Purification and Characterization of Neutral Protease Enzyme from *Bacillus subtilis*." they concluded that the enzyme exhibited maximum activity at a neutral pH of 7, with an optimal temperature of 37 °C and stability within the range of 30–60 °C. Starch and whey protein were identified as the best carbon and nitrogen sources for protease production. An agitation speed of 180 rpm proved optimal for protease production. Proteolytic activity was further confirmed through casein zymography. The study emphasized cost reduction in enzyme production while increasing yield, thereby enhancing profitability and industrial applicability.

RESULT :

Bacterial strains are most significant in protease production compared to fungal strains (H. Kahraman and C.C. Karaderi (2020). A variety of microorganisms are capable of producing proteases. In this study, the selected isolates were identified as proteolytic Bacillus species through gram staining, cellular morphology, and biochemical tests, as described by Muthu Padmapriya et al. (2012). Proteolytic strain of Bacillus was observed to form clear zone on skim milk agar plates (Nihan Sevinc et al., 2011). Agro-industrial byproducts, including banana peels, sugarcane molasses, rice bran, wheat bran, and

coconut cake, serve as inexpensive and readily available substrates. Utilizing these substrates significantly lowers production costs (Temam Abrar Hamza, 2017). Enzyme purification was performed using DEAE-cellulose ion-exchange chromatography (Hiba T. Rasheed, 2022).

Maximum protease production occurred at 60 °C in an alkaline pH environment, with a fermentation duration of 48 hours under continuous shaking at 220 rpm (Kashif Younas Butt et al., 2019). To understand the factors influencing protease production, a series of experiments examined the effects of simple and complex carbon and nitrogen sources (Anissa Hadar, 2010). The study identified fructose as the optimal carbon source, skim milk as the best nitrogen source, and calcium (Ca^{2+}) and magnesium (Mg^{2+}) as the most effective metal ions for protease production by Bacillus sp. N-40. Combining these compounds created a new medium that resulted in maximal enzyme production (Nihan Sevinc and Elif Denmark, 2011). Proteases exhibit unique therapeutic properties, making them valuable for developing medications to treat microbial and inflammatory infections, as well as other applications. These properties underscore their potential for use in biotechnological and industrial sectors (Kashif Younas Butt et al., 2019).

DISCUSSION :

The potential of using cost-effective substrates such as wheat bran, rice bran, and sugarcane bagasse for protease production, significantly reducing overall production costs. The high protease activity observed suggests that these microorganisms are suitable candidates for large-scale enzyme production. This indicates that affordable substrates like rice bran, wheat bran, and soybean meal can serve as effective alternatives to expensive refined substrates, which typically increase production costs. Consequently, this research focused on identifying low-cost substrates and optimizing cultural conditions to enhance protease production by Bacillus species. These findings provide a foundation for large-scale protease production at a reduced cost, offering advantages for industrial applications. Proteases produced in this manner have applications in diverse industries, including textiles, food processing, and medical fields. This literature review also concludes that proteases can be used as additive agents in detergents for breaking down protein-based stains on fabrics. However, further studies are necessary to explore ways to enhance enzyme yields for commercial-scale processes.

CONCLUSION:

This study demonstrated the potential of utilizing cost-effective substrates for protease production by Bacillus subtilis and Staphylococcus aureus. The findings pave the way for developing an economical process for protease production with significant industrial applications. Green biotechnology emphasizes the use of agro-industrial materials, such as agricultural waste, for producing valuable industrial products like enzymes in a more efficient and cost-effective manner. This research area is gaining momentum across various industries due to the availability and affordability of agricultural byproducts. From the literature, it is evident that proteases can be produced using organic, low-cost substrates instead of relying solely on synthetic chemicals. These organic substrates offer excellent properties, making them highly effective for protease production.

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