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High-Yield Protease Production by a Gram-Negative Soil Bacterium Under Optimized Conditions

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ABSTRACT

Proteases are vital enzymes that catalyze the hydrolysis of peptide bonds and are extensively used in diverse industrial sectors including food, pharmaceuticals, leather, and detergents. This study aimed to isolate and characterize potent protease-producing bacteria from soil samples collected from Eco Park, Bhopal. A total of 11 bacterial isolates were screened for protease activity using skim milk agar, of which isolate 4 showed the highest zone of clearance (+++), indicating strong protease production. Morphological analysis revealed isolate 4 as a greyish, circular, raised colony with an entire margin. Gram staining indicated it was a Gram-negative rod-shaped bacterium. Biochemical characterization showed the isolate to be catalase, nitrate reduction, ONPG, indole, and glucose positive, aligning with selective metabolic traits favourable for protease synthesis.

Optimization studies revealed that isolate 4 exhibited maximum protease activity at pH 7 and 30°C, suggesting neutral and moderate thermal conditions support optimal enzyme production. Among the carbon sources tested, glucose significantly enhanced protease activity, followed by sucrose and starch. Similarly, among nitrogen sources, peptone supported the highest enzyme activity, with yeast extract and tryptone also showing favorable results. These findings underscore the potential of isolate 4 as a robust microbial candidate for industrial-scale protease production due to its high enzymatic yield under optimized culture conditions. Further molecular identification is recommended to determine its species and explore genetic pathways involved in enzyme regulation.

INTRODUCTION

Enzymes are biological catalysts (also known as biocatalysts) that speed up biochemical reactions in living organisms (Lehninger et al., 2008). Proteases are enzymes with highly specialized proteolytic functions. They are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation (Rao et al., 1998). Protease has been categorized based on several standards; they are classified according to the position of the peptide bond cleaved into two major groups: exopeptidases and endopeptidases (Barrett & Rawlings, 2001). They can also be classified according to their optimal pH range as acidic (pH 2.0 to 6.0), neutral (pH 6.0 to 8.0), or alkaline proteases (pH 8.0 to 13.0) (Gupta et al., 2002).

Proteases can be produced from plants, animals, and microorganisms. The production of proteases by microorganisms has grown in recent years due to the wide variety of enzymes they can produce. They represent about two-thirds of the world's production of proteases (Rao et al., 1998). The enzyme that carries out proteolysis, the protease, represents almost 60% of the total enzyme market (Gupta et al., 2002). Based on their structure and properties of the catalytic site, proteases are classified into several types such as carboxyl-, metallo-, serine-, neutral-, acidic-, and alkaline proteases (Barrett & Rawlings, 2001).

Proteases regulate the fate, localization, and activity of many proteins, modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information, and generate, transduce, and amplify molecular signals (Turk, 2006). Microbial proteases are generally non-pathogenic and can be easily cultivated in culture media, which contributes to their wide industrial applications (Joo et al., 2002). Microbial proteases are in greater demand for industrial applications compared to enzymes isolated from plants and animals due to their rapid proliferation and reduced space requirements (Gupta et al., 2002).

Proteases are very selective during hydrolysis and are widely used in various industries, including food, laundry detergent, leather, and pharmaceuticals (Rao et al., 1998). For industrial production of proteases, microorganisms are preferred to achieve large-scale enzyme production (Ellaiah et al., 2002). Bacillus, Aspergillus, Pseudomonas, etc., are common producers of protease. Bacteria, especially the genus Bacillus, are the most important alkaline protease producers because of their capacity to produce high yields of proteases with significant activity and stability at high pH and temperature (Gupta et al., 2002; Joo et al., 2002).

Under physiological conditions, proteases catalyze the hydrolysis of peptide bonds. However, under non-physiological conditions, such as an organic environment, a biphasic system (solid/liquid), or precipitation of reaction products, they can catalyze the formation of peptide bonds (Wang et al., 2008). Microbial proteases have diverse applications in baking, brewing, detergents, leather processing, pharmaceuticals, meat tenderizing, cosmetics, and medical diagnostics (Rao et al., 1998; Gupta et al., 2002).

MATERIALS AND METHODS

Isolation of Bacterial Colonies

Soil samples were collected from a depth of approximately 15 cm at Eco Park, Rameshwaram Bagmugaliya, Bhopal using a sterile spatula and stored in sealed plastic bags to maintain sterility. For bacterial isolation, 1 gram of the collected soil was suspended in 10 mL of sterile distilled water to prepare a stock solution, followed by serial 10-fold dilutions up to 10^{-10} using sterile water, as described by Cappuccino and Sherman (2014). Nutrient agar medium was prepared by dissolving 13 g of nutrient broth and 20 g of agar in 1000 mL of distilled water, and the mixture was sterilized using an autoclave at 121°C for 15 minutes under 15 psi pressure (Pelczar et al., 2001). The sterile medium was poured into Petri plates under aseptic conditions inside a laminar airflow cabinet to avoid contamination. Subsequently, one drop from the 10^{-3} dilution was spread uniformly on the agar surface using a sterile spreader, and the plates were incubated at 37°C for 24 hours to facilitate the growth of distinct bacterial colonies (Tortora et al., 2019).

Purification of Bacteria:

Individual bacterial colonies were sub-cultured onto fresh Nutrient Agar Medium (NAM) slants to ensure purity and were preserved at 4°C for short-term storage, as described by Aneja (2003).

Identification of bacteria

The isolated bacterial colonies were initially examined for their morphological characteristics, such as colony shape, color, elevation, and margin, to distinguish among different isolates (Tille, 2015). Screening for protease production was conducted on skim milk agar plates by incubating the bacterial isolates at 37°C for 24 hours. After incubation, the plates were flooded with crystal violet; clear zones around the colonies indicated casein hydrolysis and protease activity (Jadhav et al., 2013).

Gram's staining was performed to determine the Gram reaction and cellular morphology of the isolates under light microscopy, distinguishing Gram-positive from Gram-negative bacteria based on cell wall structure (Beveridge, 2001). Further biochemical identification was carried out using the HiBacillus™ Biochemical Test Kit (HiMedia, Mumbai), following the manufacturer's protocol. Additionally, several standard biochemical tests were conducted. In the catalase test, 3% hydrogen peroxide (H_2O_2) was added to a bacterial colony on a glass slide; bubble formation indicated a positive result, confirming the presence of catalase enzyme (MacFaddin, 2000). The oxidase test involved applying oxidase reagent directly to the colony; a blue or purple coloration within 30 seconds was considered positive, indicating the presence of cytochrome c oxidase (Cheesbrough, 2006).

For starch hydrolysis, isolates were streaked on starch agar plates, incubated, and then flooded with iodine solution. The formation of clear zones around colonies indicated extracellular amylase production (Prescott et al., 2005). Similarly, in the casein hydrolysis test, isolates were streaked on skim milk agar plates and incubated; the presence of transparent halos around colonies confirmed the ability to degrade casein, indicating protease production (Rao et al., 1998).

Optimization of Culture Conditions

To determine optimal pH for protease production, nutrient broth was adjusted across a range of pH 5 to 9 using sterile 1N H_2SO_4 and NaOH solutions. After inoculation, the flasks were incubated at 37°C for 24 hours, and optical density (OD) was measured at 600 nm to assess bacterial growth (Singh et al., 2012). For temperature optimization, inoculated flasks were incubated at temperatures ranging from 20°C to 60°C for 24 hours, followed by centrifugation at 4,000 rpm for 18 minutes. The OD of the supernatant was measured at 600 nm to determine the effect of temperature on protease activity (Amoozegar et al., 2007). To study the influence of carbon sources, various sugars (glucose, lactose, xylose, sucrose, starch) were individually incorporated into the media. Cultures were incubated under similar conditions and OD values recorded post-centrifugation (Sathya & Ushadevi, 2014). For nitrogen source optimization, media containing different organic nitrogen compounds (yeast extract, peptone, beef extract, casein, and tryptone) were used. After 24 hours of incubation at 37°C, centrifuged samples were analyzed for OD to evaluate the best nitrogen source for growth and protease production (El-Hadi et al., 2016).

RESULTS

4.1 SCREENING OF PROTEASE ACTIVITY:

ISOLATES	ZONE OF INHIBITION	
	POSITIVE	NEGATIVE
1	+	-
2	-	+
3	+	-
4	+++	-
5	-	+
6	-	+
7	+	-
8	-	+
9	-	+
10	-	+

11	++	-

Table 1 – Screening of protease activity

The screening of protease activity among 11 microbial isolates was assessed by observing the zone of inhibition (clear halo) on casein agar plates, indicating protease production. Out of the 11 isolates tested, six isolates (1, 3, 4, 7, and 11) showed positive protease activity, with isolate 4 exhibiting the strongest activity (+++), followed by isolate 11 with moderate activity (++), and others showing mild activity (+). In contrast, five isolates (2, 5, 6, 8, 9, and 10) were negative for protease production, as indicated by the absence of any clear zone around the colony. These results suggest that isolate 4 is a particularly potent protease producer and could be selected for further studies and optimization of enzyme production.

4.2 MORPHOLOGY

Isolate	Colour	Form	Elevation	Margin
1	Yellow	Circular	Convex	Entire
2	Off White	Circular	Flat	Entire
3	Marigold	Circular	Raised	Entire
4	**Greyish**	**Circular**	**Raised**	**Entire**
5	White	Circular	Raised	Entire
6	Pale Yellow	Circular	Convex	Entire
7	White	Irregular	Crateriform	Entire
8	White	Irregular	Flat	Entire
9	Off White	Circular	Raised	Entire
10	Off White	Circular	Flat	Entire
11	White	Irregular	Flat	Entire

Table 2 - Morphological Characteristics of Isolated bacteria's

The morphological analysis of the 11 bacterial isolates revealed variations in colony colour, form, elevation, and margin. Most isolates exhibited a circular form, with the exception of isolates 7, 8, and 11, which were irregular. The colony elevation varied from flat (isolates 2, 8, 10, and 11), to convex (isolates 1 and 6), and raised (isolates 3, 4, 5, and 9). Notably, isolate 7 displayed a unique crateriform elevation, distinguishing it from the others. In terms of colour, a range was observed: yellow (1), off white (2, 9, 10), marigold (3), greyish (4), white (5, 7, 8, 11), and pale yellow (6). Despite differences in form and elevation, all isolates shared a common entire margin, indicating smooth, unbroken colony edges. These morphological features are essential for preliminary differentiation and identification of bacterial species and may correlate with their functional characteristics, such as enzyme production.

4.3 Gram staining of selected isolates

Isolate	Shape	Color	Result
4	Rod	pink	Negative

Table 3 – Result of Gram staining

The gram staining result for Isolate 4 indicates that it is a Gram-negative, rod-shaped bacterium with a pink coloration under the microscope. This pink staining is characteristic of Gram-negative bacteria due to their thinner peptidoglycan layer and the presence of an outer membrane, which does not retain the crystal violet stain and instead takes up the counterstain (safranin or fuchsine). The rod shape (bacillus form) suggests that it may belong to genera such as *Pseudomonas*, *Escherichia*, or *Serratia*. This morphological and staining characteristic, combined with its strong protease activity observed earlier, makes Isolate 4 a promising candidate for further biochemical and molecular characterization

.BIOCHEMICAL TEST

S.NO.	Characterization	E. coli
1	Catalase	+
2	Coagulase	-
3	Citrate utilization	-
4	Nitrate reduction	+
5	ONPG	+
6	Vogus Proskaur	+
7	Indole	+
8	Glucose	+
9	Sucrose	-
10	Lactose	+

11	Mannitol	-
12	Arabinose	-

Table 4 - Effect of Biochemical test on protease production.

The biochemical profiling of the isolate, when compared to *E. coli*, provides insight into its metabolic capabilities, which may be linked to its protease-producing potential. The isolate tested positive for several key reactions including catalase, nitrate reduction, ONPG (ß-galactosidase activity), Voges-Proskauer, indole, and glucose fermentation, indicating its ability to survive oxidative stress, reduce nitrate, and metabolize certain sugars and amino acids. Notably, it showed negative results for coagulase, citrate utilization, and fermentation of sucrose, mannitol, and arabinose, suggesting limited usage of some carbon sources and a lack of extracellular clotting enzymes. The positive indole and ONPG reactions indicate active tryptophan metabolism and lactose hydrolysis, respectively, though lactose fermentation was positive while sucrose and mannitol were not utilized. These biochemical traits, when taken together with its Gram-negative rod morphology and high protease activity, suggest that the isolate could belong to an enteric or environmental genus with selective enzymatic capabilities that support protease production.

pH activity

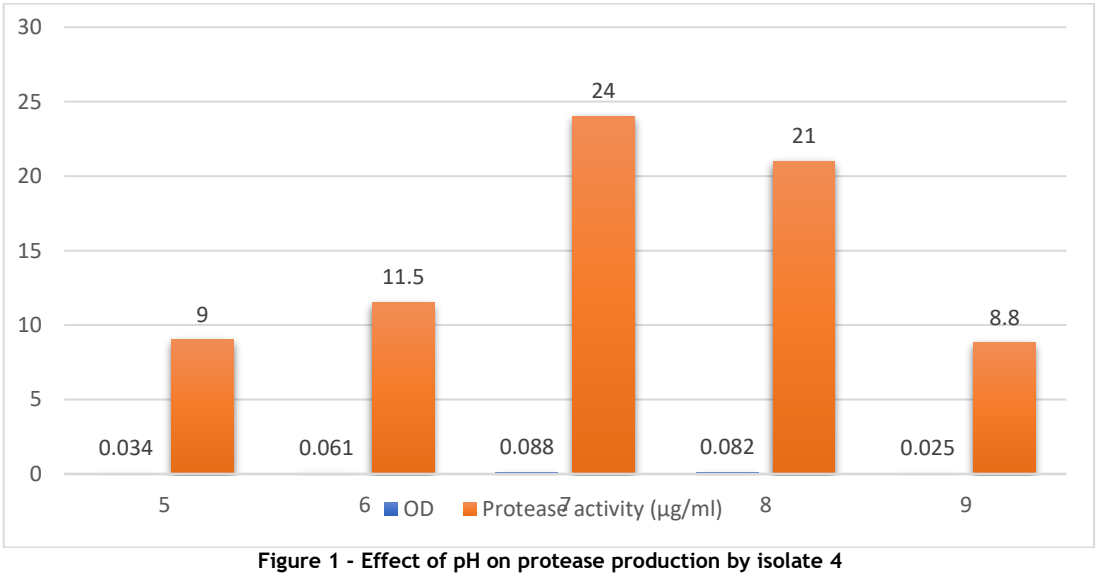

Figure 1 - Effect of pH on protease production by isolate 4

The bar graph illustrates the effect of pH on protease activity, demonstrating how enzyme production varies across a pH range of 5 to 9. The results show that protease activity is highly dependent on the pH of the medium, with a clear optimum at pH 7. At pH 5, the activity is relatively low (9 µg/mL), indicating that acidic conditions are not favourable for the enzyme's activity or the growth of the protease-producing microorganism. As the pH increases to 6, there is a slight rise in activity (11.5 µg/mL), suggesting a gradual adaptation of the enzyme system. The highest protease activity (24 µg/mL) is observed at pH 7, indicating that neutral pH provides the most suitable environment for enzyme production and functionality. At pH 8, the activity slightly decreases to 21 µg/mL, which shows that the enzyme remains active under mildly alkaline conditions but is less efficient than at neutral pH. A further increase to pH 9 results in a sharp decline in activity (8.8 µg/mL), suggesting that more alkaline conditions may negatively affect enzyme structure or microbial growth. Overall, the data suggest that the protease under investigation is most efficiently produced and functions optimally at a neutral pH, with reduced activity in both more acidic and more alkaline environments.

4.4.2 Effect of temperature

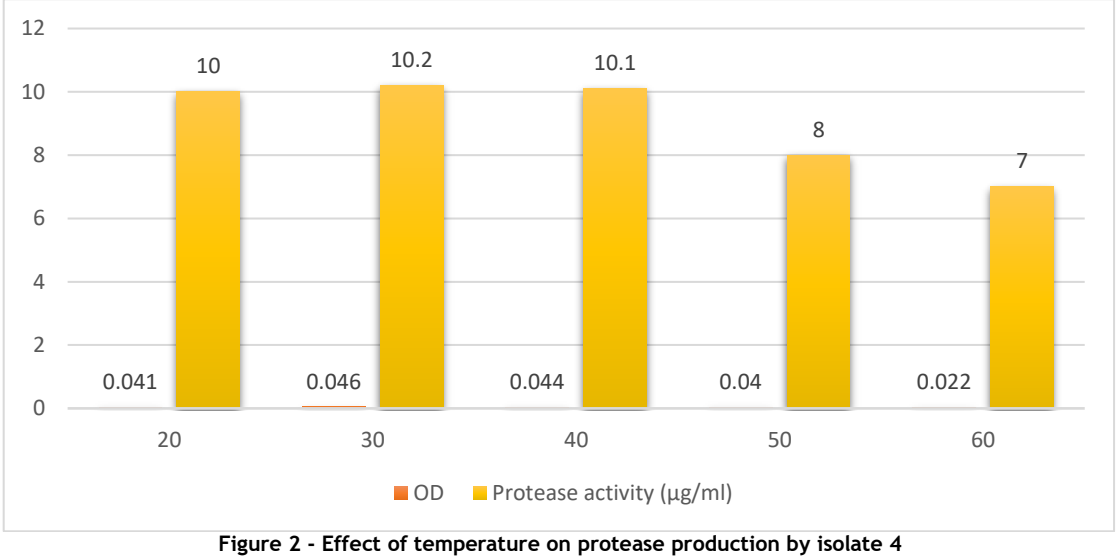

Figure 2 - Effect of temperature on protease production by isolate 4

The bar graph represents the influence of different incubation temperatures on protease activity. The data indicate that protease production is relatively stable between 20°C and 40°C, with the highest activity recorded at 30°C (10.2 µg/mL), closely followed by 40°C (10.1 µg/mL) and 20°C (10 µg/mL). This suggests that the enzyme-producing microorganism maintains optimal enzymatic output in a moderate temperature range, especially around 30°C, which appears to be the optimal temperature for protease production. At higher temperatures, the enzyme activity declines, with values dropping to 8 µg/mL at 50°C and further to 7 µg/mL at 60°C. This decrease in activity may be due to thermal denaturation of the protease enzyme or reduced microbial viability at elevated temperatures. Overall, the results suggest that moderate temperatures (especially around 30°C) are most favourable for protease activity, while higher temperatures adversely affect enzyme stability and function.

4.4.3 Effect of carbon source

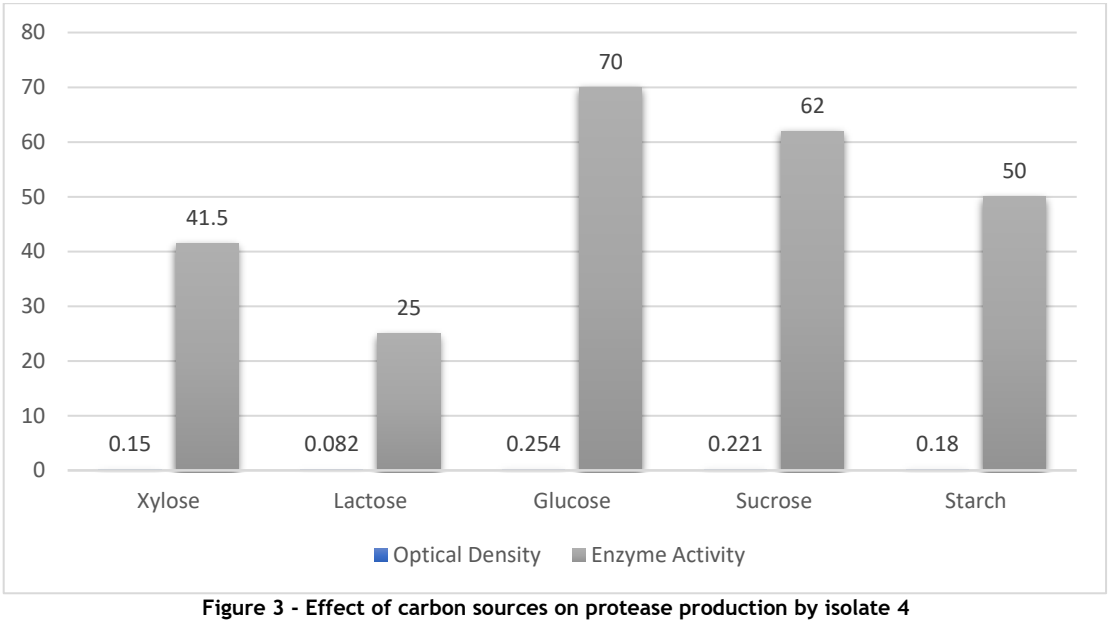

Figure 3 - Effect of carbon sources on protease production by isolate 4

The bar graph illustrates the effect of various carbon sources—Xylose, Lactose, Glucose, Sucrose, and Starch—on the optical density and enzyme (protease) activity of isolate 4. Among the tested carbon sources, Glucose showed the highest optical density (0.254) and the maximum enzyme activity (70 U), indicating that it is the most effective carbon source for promoting both cell growth and protease production. Sucrose followed closely with an optical density of 0.221 and enzyme activity of 62 U, also supporting significant enzyme production. Starch resulted in moderate growth and enzyme activity (OD: 0.180, Activity: 50 U), suggesting it can be used as a carbon source but with less efficiency. In contrast, Xylose and Lactose exhibited the lowest values, with Lactose showing the least optical density (0.082) and lowest enzyme activity (25 U), making them less favourable for protease production in this isolate. These results suggest that readily metabolizable sugars like glucose enhance both biomass and enzyme synthesis, likely due to their efficient uptake and utilization in metabolic pathways.

4.4.4 Effect of nitrogen source

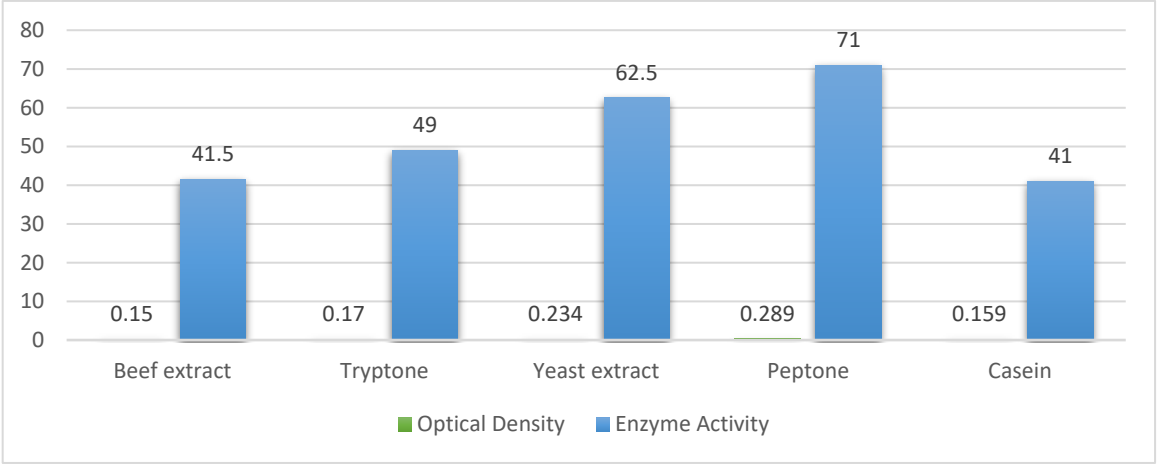

Figure 4 - Effect of nitrogen sources on protease production by isolate 4

The study evaluated five nitrogen sources for their effect on microbial growth and enzyme production. Peptone showed the highest optical density (0.289) and enzyme activity (71 U/mL), making it the most effective for promoting both cell growth and enzyme synthesis. Yeast extract also performed well (OD =

0.234, enzyme activity = 62.5 U/mL) due to its rich nutrient content. Tryptone showed moderate results, while beef extract and casein resulted in lower growth and enzyme activity, likely due to lower nutrient availability or slower utilization. Overall, peptone proved to be the best nitrogen source in this study.

CONCLUSION

The study successfully isolated and characterised a protease-producing bacteria from soil, known as *E. coli*. Under optimal circumstances, this isolate exhibited high protease activity. The study found that the optimal pH for protease synthesis is neutral (pH 7).
The ideal temperature for protease synthesis is about 30 °C. Glucose is probably the most efficient carbon source, followed by sucrose and starch. Peptone is the most active nitrogen source, followed by yeast extract. These optimisations can be utilised to boost protease production in industrial settings. Further research might look at the genetic and molecular mechanisms underlying protease synthesis in *E. coli* as well as possible uses in sectors such as detergent, food, and medicines.

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