

Original Article

Production and Optimization of Protease (Caseinase) Enzyme by Bacterial Genus *Alcaligenes* Isolated from Dairy Effluents

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Article Info

Article history:

Received: September 15, 2025

Accepted: October 23, 2025

Published: November 8, 2025

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Keywords: Caseinase, DPE, *Alcaligenes faecalis*, Optimization studies

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Abstract

Proteases are the class of enzymes constituting 60% of the total enzyme market with its valuable contribution towards a wide variety of functions and biotechnological applications. Caseinase (a type of protease) has immense industrial applications in dairy industry, agro-based and pharmaceuticals, bioremediation, improving detergent efficiency, texturizing in baking and helpful in textile and leather processing. These diverse applications make them important to study their production and isolation especially from dairy effluents. In view of the importance of Caseinase, the current research was aimed to isolate and screen dairy effluents for Caseinase producers, to study the production and optimization of Protease (Caseinase) enzyme. The caseinase producer was later identified as *Alcaligenes* spp. based on the biochemical tests and 16SrRNA sequencing result. The study was aimed to evaluate the highest proteolytic activity from the isolates of *Alcaligenes* spp. designated as DPE-1, DPE-2, DPE-3, DPE-4 and DPE-5. Two higher caseinase producing isolates (isolates DPE-3 and DPE-5) were determined for their performance efficiency by screening them and studying the quantitative Caseinase enzyme assay by modified Tsuchida method. The results demonstrated the highest zone of proteolysis by DPE-3 and DPE-5, thereby, selecting them for optimization studies and Km, Vmax studies. These findings suggest that *Alcaligenes* spp. (DPE-3 and DPE-5) could be used to produce caseinase enzyme on a commercial scale to enhance the production of Protease (Caseinase) enzyme. However, a systematic and statistical production optimization studies are a way to go further.

1. Introduction:

Proteases are one of the most important enzymes with many functions [1]. Proteases are a group of enzymes which play an important role in bioremediation and are used in pharmaceutical and agricultural industries [2]. These are ubiquitous in biosystems and perform multiple roles in the biochemical, physiological and regulatory functions of cells and organisms. Proteases are used in detergent, food processing, leather and fabric improvement, as catalysts for organic synthesis and in therapeutics [5].

Proteolytic enzymes are degradative enzymes which effect the complete hydrolysis of proteins and which specifically attack the internal peptide bonds of proteins and peptides [3]. The increase in consumer demand for cheese is now leading to new products with different organoleptic properties, resulting in a great amount of research on alternative milk coagulants. The requirements for proteases used in cheese making are determined by the relationship between proteolytic activity and milk-clotting activity [6]. It is an exo-enzyme, excreted from the cells into the surrounding environment

that causes the breakdown of the milk protein called casein into smaller peptides and single amino acids that are then absorbed by the organism for energy or structural components. *Alcaligenes faecalis* is an important bacterium known for its ability to remove nitrogen from wastewater. Previous research has mostly focused on improving its denitrification performance [7]. However, its diverse metabolic profile also makes it a strong candidate for producing industrial enzymes. Although the research did not initially aim to isolate this bacterium from dairy waste, its unintended discovery in this protein-rich area offered a great chance to explore its biochemical abilities, especially the creation of extracellular proteases. Thus, the main goal of this study is to examine the production and optimization of the caseinase enzyme from this newly isolated *Alcaligenes* sp. It is expected that since this strain was found in a dairy-rich setting, it has a strong ability to produce caseinase. This production can be greatly improved by studying the effects of important environmental and nutritional factors. Therefore, systematic optimization studies were carried out to assess pH, temperature, different carbon, nitrogen sources, and specific inducers and their affect on enzyme yield for possible industrial use.

2. Materials and Methods:

2.1 Collection of sample

Dairy effluent samples were collected in sterile containers from selected industrial waste sites and immediately conveyed to the laboratory. The samples were stored at 4°C to maintain sample integrity prior to bacterial enrichment and isolation procedures.

2.2 Isolation and identification of caseinase producing bacteria

Proteolytic bacterial strains were isolated by screening individual colonies of the dairy effluent samples on Skimmed Milk Agar (SMA) medium. The inoculated plates were incubated at 30°C for 48 hours and monitored for distinct zones of clearance surrounding the bacterial growth indicating active caseinolytic expression. This localised hydrolysis reaction converts the opaque casein protein into soluble, transparent end products such as small chains of amino acids, dipeptides and polypeptides [4]. Those strains producing a distinct clear zone were considered as good caseinase producers and selected for subsequent quantitative enzyme analysis.

The bacterial isolates from dairy industry effluents were identified as DPE-1, *Alcaligenes* sp. AMT-03 gene

sequence for 16S rRNA partial sequence, DPE-2, *Alcaligenes* sp. AMT-03 gene sequence for 16S rRNA partial sequence, DPE-3, *Alcaligenes faecalis* AMT-03 gene sequence for 16S rRNA partial sequence and DPE-5, *Alcaligenes faecalis* strain CD234 gene sequence for 16S rRNA full length sequence.

2.3 Culture preservation

The productive bacterial cultures were preserved in refrigerator at 4°C on nutrient agar slants. These cultures were then utilised for enzyme assays and optimisation studies.

2.4 Screening of caseinase enzyme producing isolates

The screening of the caseinase enzyme producing efficient bacterial isolates was carried out by using a modified method of Tsuchida et al., 1986 [8] with casein as a substrate.

2.5 Caseinase enzyme assay

The enzyme was assayed using the cell free supernatant as the source of crude extracellular enzyme. Protease activity was assayed by a modified method of Tsuchida *et al.* by using casein as substrate. 100 µl of enzyme solution was added to 900 µl of substrate solution (2 mg/ml (w/v) casein in 10 mM Tris–HCl buffer, pH 8.0). The mixture was incubated at 45°C for 30 minutes. Reaction was terminated by the addition of an equal volume of 10% (w/v) chilled trichloro acetic acid. Then, the reaction mixture was allowed to stand in ice for 15 minutes to precipitate the insoluble proteins.

The supernatant was separated by centrifugation at 10,000 rpm for 10 minutes at 4°C; the acid soluble product in the supernatant was neutralized with 5 ml of 0.5M Na₂CO₃ solution. The colour developed after adding 0.5 ml of 3-fold diluted Folin–Ciocalteu reagent was measured at 600nm. All assays were done in triplicate. (Figure 1)

2.6 Optimization studies of different parameters for enzyme activity

The efficient bacterial cultures were grown in sterile nutrient broth with 2 mg/ml casein adjusted at various pH conditions i.e pH-4, pH-7 and pH-9 and incubated at temperature 30°C to optimize the pH condition for caseinase enzyme production. Since the enzyme activity was found more at the pH-4, to optimize the temperature condition, the efficient bacterial cultures were incubated at various temperature conditions such as, 30°C, 37°C and 42°C. Since the enzyme production was found more under these optimized conditions, further variations were evaluated. To check the effect

of various carbon sources, the efficient bacterial cultures were separately inoculated in the production medium with 1% glucose, sucrose and starch respectively; to check the effect of various nitrogen sources, the efficient bacterial cultures were separately inoculated in the production medium with 1% peptone, beef extract and ammonium sulfate, respectively; and to check the effect of inducers for enhancing the enzyme production, the efficient bacterial cultures were separately inoculated in the production medium with 3% skimmed milk and 1% casein as inducers respectively.

In all experiments, the production of enzyme was determined by quantitative assay of caseinase enzyme by Tsuchida et al. method [8] using casein as a substrate.

3. Results:

3.1 Quantitative assay of caseinase enzyme

The visual presentation of the protease assay setup and its corresponding quantitative analysis is shown below. (Figure 1) shows the typical colour development in the assay tubes after the Folin–Ciocalteu reaction. The associated standard graph and the enzyme activity profile was recorded at 600 nm. (Figure 2)

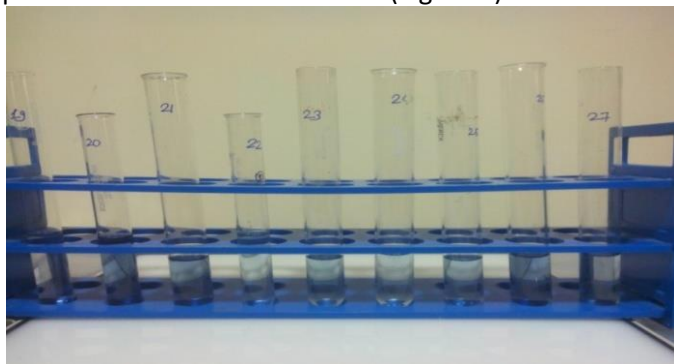


Figure 1: Quantitative assay of caseinase enzyme

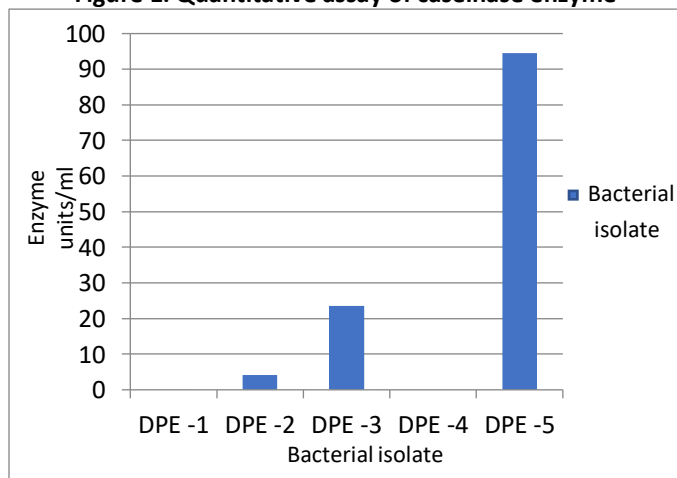


Figure 2: Graph of quantitative assay of caseinase enzyme

3.2 Isolation and screening of proteolytic bacteria

A total of 53 bacterial isolates were isolated from the dairy effluents. When tested for their proteolytic activity, 5 bacterial isolate (DPE-1, DPE-2, DPE-3, DPE-4 and DPE-5) demonstrated clear zones around the colony on skimmed milk agar. Among these isolates, only 2 isolates, DPE-3 (*Alcaligenes faecalis* strain AMT03) and DPE-5 (*Alcaligenes faecalis* strain CD2334) showed highest zone of proteolysis 12 mm (DPE-3) and 17 mm (DPE-5). Therefore, these points were selected for quantitative caseinase enzyme assay, optimization studies (pH, temperature, carbon sources, nitrogen sources and inducers) and Km, Vmax Studies.

3.3 Effect of pH on caseinase enzyme activity

The obtained results showed that the maximum enzyme production **38.9 U/ml** by bacterial isolate DPE-5 (*Alcaligenes faecalis* strain CD2334) was noted at acidic pH 4.0, whereas no enzyme production was detected by isolate DPE-3 (*Alcaligenes faecalis* strain AMT03) (Figure 3).

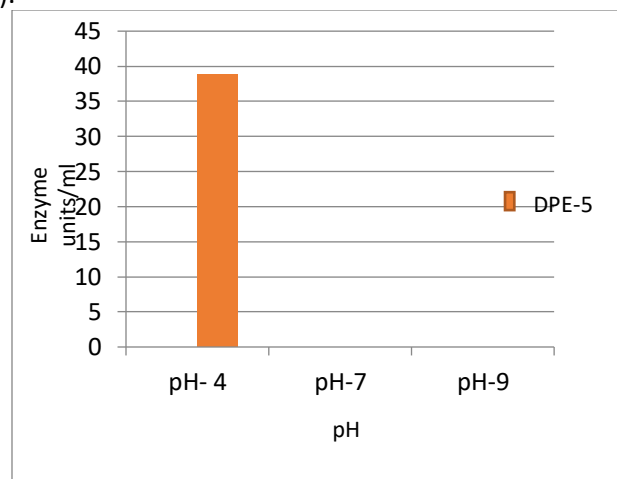


Figure 3: Effect of pH on enzyme activity

3.4 Effect of temperature on caseinase enzyme activity

The optimum temperature of DPE-5 (*Alcaligenes faecalis* strain CD2334) for protease production was found to be **96.8 U/ml** at 30°C, whereas no enzyme production was detected by isolate DPE-3 (*Alcaligenes faecalis* strain AMT03) (Figure 4).

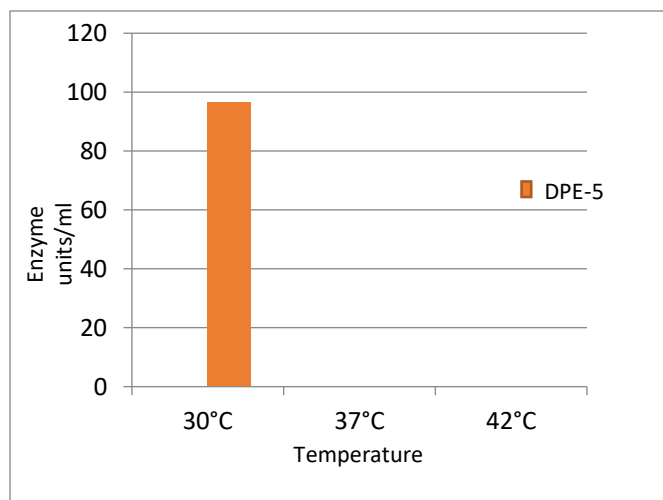


Figure 4: Effect of temperature on enzyme activity

3.5 Effect of carbon on caseinase enzyme activity

DPE-5 (*Alcaligenes faecalis* strain CD234) enhances the production of protease using glucose as carbon source and it was found to be **8.7 U/ml**, in presence of starch, the caseinase production was found to be **14.3 U/ml**. Protease production was found to be enhanced by isolate DPE-3 (*Alcaligenes faecalis* strain AMT03) using glucose as a carbon source was found to be **2.3 U/ml** in the presence of starch was **3.6 U/ml** and no enzyme production in presence of sucrose (Figure 5).

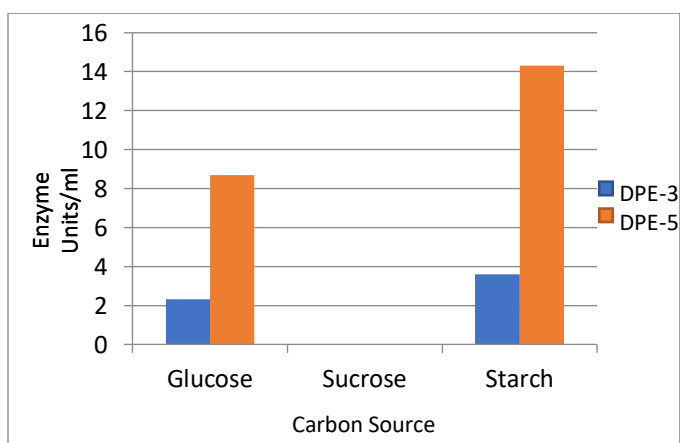


Figure 5: Effect of carbon source on enzyme activity

3.6 Effect of nitrogen on caseinase enzyme activity

Various nitrogen sources like peptone, beef extract and ammonium sulfate for the production of protease was investigated. DPE -5 (*Alcaligenes faecalis* strain CD234) enhance the production of protease using peptone as nitrogen source for protease production was found to be **63.8 U/ml** and in presence of beef extract the caseinase production was found to be **106.9 U/ml** and no enzyme production was found in presence of ammonium sulfate, whereas the enzyme production

was detected by isolate DPE-3 (*Alcaligenes faecalis* strain AMT03) using peptone as nitrogen source was found to be **77.1 U/ml**, in presence of beef extract the caseinase production was found to be **61.9 U/ml** and in presence of ammonium sulfate was **1.0 U/ml** (Figure 6).

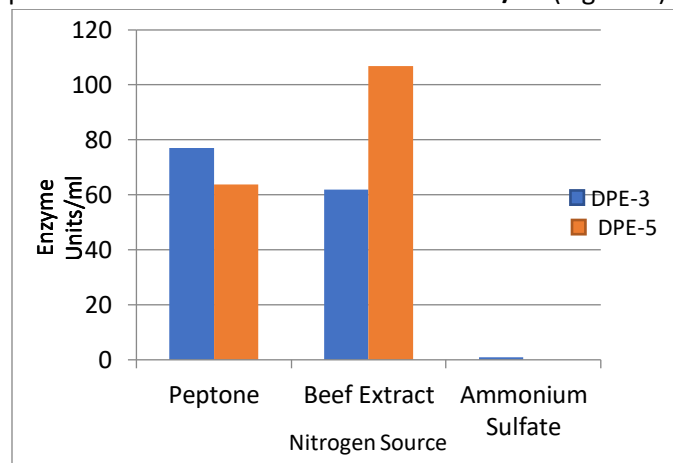


Figure 6: Effect of nitrogen source on enzyme activity

3.7 Effect of inducers on caseinase enzyme activity

DPE-5 (*Alcaligenes faecalis* strain CD234) enhanced the production of protease using skimmed milk as an inducer for protease production and was found to be **8.4 U/ml**, in presence of casein the caseinase production was found to be **88.1 U/ml**, whereas the enzyme production using skimmed milk detected by isolate DPE-3 (*Alcaligenes faecalis* strain AMT03) was **22.3 U/ml** and using casein showed no enzyme activity. (Figure 7)

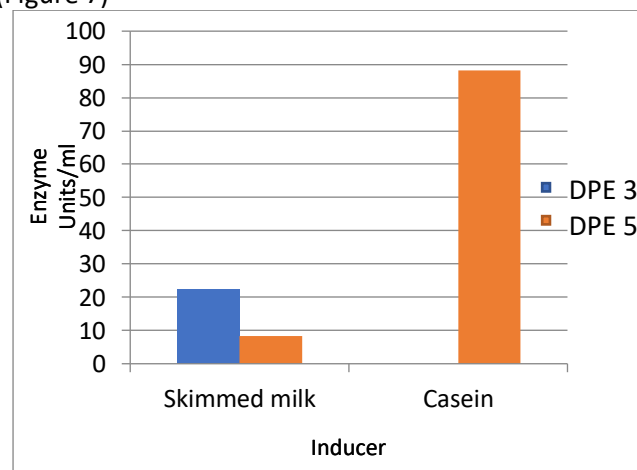


Figure 7: Effect of inducer on enzyme activity

3.8 Km and Vmax study (Michaelis Menton constant & maximum velocity)

The kinetic properties and substrate affinity of the improved caseinase enzyme was determined by measuring initial reaction velocities at different concentrations of casein. The basic kinetic parameters,

K_m and V_{max}, were then plotted using the Michaelis Menten plot (Figure 8). The results showed no activity at concentrations upto 1.5mg/ml with initial activity detected at 2.0 mg/ml (0.08). The reaction velocity increased with substrate concentration, showing maximum absorbance (V_{max}) of 0.425 at 4.0 mg/ml, while calculated Michaelis Menton constant (K_m) was determined to be 3.25 mg/ml (K_m) of casein.

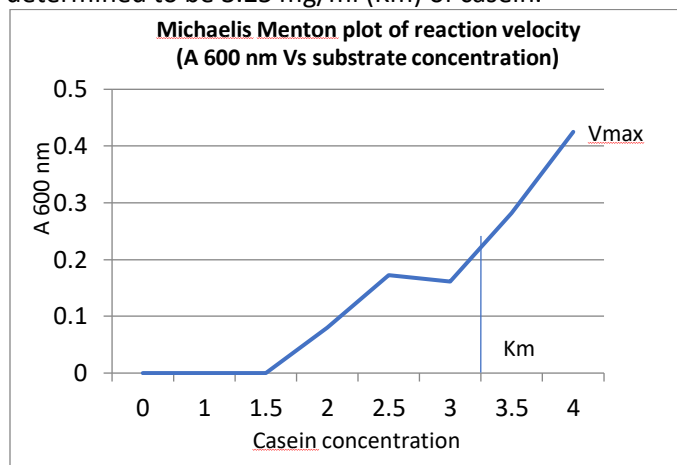


Figure 8: Michaelis Menton plot of reaction velocity

4. Discussion:

The obtained results showed that protease production was detected over different pH range, the maximum enzyme production was shown by bacterial isolate DPE-5 (*Alcaligenes faecalis* strain CD234) was noted at acidic pH 4.0, whereas no enzyme production was detected by isolate DPE-3 (*Alcaligenes faecalis* strain AMT03) (figure 4). This acid optimum for strain DPE-5 differs sharply with the published literature [9,10] which usually describes *Alcaligenes faecalis* as an alkaline protease producer. For instance, Senthilvelan et al. (2011) [9] observed that *Alcaligenes faecalis* AU01 produced caseinase enzyme activity optimally at pH 8.0 and 9.0, respectively. Similarly, Thumar and Singh (2007) [10] discovered an alkalophilic *Alcaligenes faecalis* with maximum protease activity at pH 9.0. The ability of DPE-5 to produce large enzyme yields at pH 4.0 suggests a novel acidophilic adaption, and its caseinase is distinct from the alkaline-preferring strains reported in prior research.

The characteristics of a cold-active alkaline protease produced by another strain of *Alcaligenes faecalis* documented in standard literature, [11] which also showed an ideal production temperature of 30°C, closely match this ideal temperature [11]. Our results,

however, differ from those of other strains of the species that have been reported; for example, [12] *Alcaligenes faecalis* AU01, which was isolated from industrial effluents, demonstrated peak enzyme synthesis and growth at a higher temperature of 37°C [12]. This variation strongly suggests that the metabolic and thermal thresholds for optimal enzyme secretion are critically determined by the environmental origin, such as the dairy effluent from which our strain was isolated.

The preference for carbohydrates like starch over simple sugars like glucose is common in protease production. For example, studies on *Alcaligenes* species have often found that glucose helps cells grow fast. It can also cause catabolite repression (a regulatory mechanism in bacteria and microorganisms that prioritizes the use of the most energetically favorable carbon source over others). This repression reduces protease expression [13]. We found that starch gives the yield. This matches what other studies on *Alcaligenes faecalis* have found [14]. They showed that carbohydrates like starch or maltose work better than simple sugars like glucose. These complex carbohydrates release carbon slowly. This slow release prevents enzyme repression. When we used sucrose there was no production. This suggests that these specific strains cannot efficiently use sucrose. They lack the tools, such as invertase to turn sucrose into a usable form, for making extracellular enzymes.

Alcaligenes species prefer complex organic nitrogen sources over inorganic salts. The basic components of the organic substrates, beef extract and peptone, supply essential amino acids, vitamins and nucleic acid precursors which directly stimulate the metabolic pathways involved in enzyme synthesis. These findings are consistent with previous studies on *Alcaligenes faecalis*, [15] which showed that complex organic nitrogen sources led to higher protease activity than inorganic nitrogen sources. In addition, the considerable inhibition by ammonium sulfate is consistent with the literature, [16] which states that the ammonium ions can cause severe nitrogen catabolite repression of protease genes to inhibit enzyme synthesis in the presence of bacterial growth.

Marathe et al. [17] utilized skim milk media to cultivate marine *Alcaligenes faecalis* isolates and observed prominent zones of casein hydrolysis, demonstrating the capacity of *A. faecalis* to synthesize

extracellular proteases in response to milk proteins. Comparative data indicate that different strains within the same species can exhibit distinct regulatory mechanisms, although screening studies often report that complex substrates such as skim milk act as positive inducers for the species [17]. These results indicate that not all strains of *A. faecalis* produce caseinase identically, and that strains CD234 (DPE-5) and AMT03 (DPE-3) may possess different substrate affinities, transport systems, or repressor mechanisms.

5. Conclusion:

Optimization studies revealed that *Alcaligenes faecalis* strain **DPE-5** produced higher caseinase than strain **DPE-3** under tested conditions. Starch was the preferred carbon source for both isolates.

Author contributions: SS: Developed the idea, wrote and verified the manuscript.

Competing interest: The author declare that no conflict of interest exists.

Ethical Statement: This work does not violate ethical guidelines.

Grant Support Details: The authors did not get funding from any agency to complete this work.

Acknowledgement: The authors are thankful to Dr. Bharat Shinde and Dr. Lalasaheb Kashid, Vice Principal, Vidya Pratishthan's Arts, Science and Commerce College, Baramati, Maharashtra, India. They are thankful to Nilima Pendharkar (HOD), Mrs. A. Neelima Devi, Dr. Amarja Bhosale, Ms Komal Kokare, Ms. Pallavi Rajguru, and Ms. Ruchita Honarao for their support in publishing this work.

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Cite this article as:

Suryawanshi SN. Production and Optimization of Protease (Caseinase) Enzyme by Bacterial Genus *Alcaligenes* Isolated from Dairy Effluents. *Int. J. Micro. Sci.* 2025; 6(2):59-65.

Abbreviations:

DPE: Dairy Processing Effluent (DPE-1 to DPE-5)

K_m: Michaelis constant

SMA: Skimmed Milk Agar

AM: Ammonium transporter

u/ml: Units per millilitre

nm: Nanometer

µg/ml: Micrograms per millilitre

V_{max}: Maximum reaction velocity

RNA: Ribonucleic acid

HCl: Hydrochloric acid

Mm: Millimolar

Na₂CO₃: Sodium carbonate

rpm: Revolutions per minute

w/v: Weight per volume