Investigating the Enzymological Properties of Casease from various organisms: Kinetics, Inhibition, Substrate Specificity and Metal ion Dependent Activity.

Abstract: Proteolytic enzymes like casease are essential biological catalysts, valued for their ability to break down complex proteins such as casein into smaller, more digestible molecules. Among these, casease a serine protease holds major industrial importance, particularly in the food, pharmaceutical, and environmental sectors, owing to its role in protein hydrolysis and nutrient release. In this study, we isolated, produced, and characterized casease from two bacterial species, *Bacillus subtilis* and *Serratia marcescens*, both known for efficient extracellular enzyme secretion. Enzyme activity was confirmed by the formation of clear zones on skim milk agar plates, signifying successful casein degradation. Comparative profiling revealed distinct production dynamics: *Bacillus subtilis* generated casease early, peaking at 24 hours, while *Serratia marcescens* showed delayed but more intense activity, reaching its maximum at 48 hours. Characterization studies indicated that *Serratia marcescens* had higher overall proteolytic activity. The response of enzymes to chemical inhibitors like hydrogen peroxide and hydrochloric acid suggested structural and functional differences between the two. Both enzymes also displayed broad substrate specificity, acting on proteins such as gelatin, glycinin, and zein.Furthermore, enzymatic efficiency was significantly impacted by the presence of metal ions including calcium, copper, iron, magnesium, and zinc which could enhance or inhibit activity. These findings illustrate that both *Bacillus subtilis* and *Serratia marcescens* are effective producers of extracellular casease with promising applications in food technology, pharmaceuticals, and environmental biotechnology, particularly for sustainable waste management and protein-rich waste biodegradation.

Keywords: Casease, *Bacillus subtilis* , *Serratia marcescens* , Proteolytic Enzymes, Casein Hydrolysis, Extracellular Enzyme Production

1. INTRODUCTION

Proteases, or proteolytic enzymes, are indispensable agents in both nature and various industries because of their ability to break down complex protein molecules. By cleaving peptide bonds, these enzymes are at the core of processes like protein recycling, metabolism, and nutrient transformation. Casease is a prime example of a serine-type protease, with a particular knack for attacking casein, the principal protein constituent in milk. Due to its tightly folded and hydrophobic character, casein is resistant to enzymatic cleavage under ordinary physiological conditions. Casease, however, overcomes this barrier, efficiently converting casein into peptides and free amino acids. This enzymatic activity underpins its significant contribution to dairy manufacturing, specialty food processing, pharmaceutical formulations, and solutions for environmental sustainability [1]. Historically, the practical benefits of casease were harnessed by cheesemakers long before its role was scientifically recognized. The spontaneous fermentation used in traditional cheese production depended on native bacteria within milk. These microorganisms naturally generated casease, leading to the formation of curds and the development of distinctive textures and flavors in cheese. With the advent of microbiology, scientists began to unravel the central role of proteases, especially casease, in controlling fermentation outcomes [2]. Such insights enabled the formulation of new dairy products with

improved digestibility, consistency, and targeted nutritional benefits. Modern applications now span infant nutrition and medical protein supplements, in which casease-driven hydrolysis enhances absorption for individuals with specific dietary requirements [3].

The widespread adoption of casease in industry is fueled by the productivity of microorganisms. *Bacillus subtilis* stands out for its robust secretion of extracellular enzymes, making it a staple choice for commercial enzyme manufacture. Other bacteria, like *Lactobacillus* and *Streptomyces*, along with various fungi and yeast, have been tapped as alternative sources, given their versatility and ability to grow under tailored conditions [4].Continuous progress in biotechnology and strain improvement have yielded microbial producers with superior enzyme output, stability, and resilience to demanding industrial environments. Selective breeding, molecular optimization, and adaptive evolution ensure these strains deliver reliable casease performance in large-scale fermentation. Casease's functionality is intimately linked to its molecular makeup [5]. At its core, a triad of serine, histidine, and aspartic acid orchestrates the hydrolytic attack on casein substrates. The enzyme shows a preference for slightly alkaline environments and moderate heat, though exceptional cases, isolated from extremophile microbes, can maintain catalytic activity under high temperatures or variable pH traits highly sought after for industrial applications [6].

For optimal production, precise fermentation parameters are critical. Microbial cells require balanced carbon and nitrogen sources, along with controlled aeration, pH, and temperature. Advanced fermentation setups now include measures to counteract foam build-up and ensure sufficient oxygenation especially vital for aerobic species like *Bacillus subtilis*. Innovations such as solid-state fermentation, which employs natural solid substrates like agricultural waste, offer environmentally friendly and cost-effective alternatives to traditional liquid fermenters, enhancing overall enzyme yield [7].Strain engineering has been transformed through molecular genetics and recombinant DNA technology. Targeted manipulation whether through classical mutagenesis, genome editing, or gene cloning has empowered producers to tweak regulatory pathways, optimize enzyme secretion, and improve casease's properties. By introducing casease-encoding genes into high-growth hosts like *E. coli* or *Pichia pastoris*, manufacturers can achieve scalable, consistent enzyme production suited to various industrial needs.Once harvested, the enzyme undergoes rigorous purification. Sequential steps, precipitation, various chromatographies, and membrane filtration remove impurities and concentrate casease for end-use [8]. Finishing processes like freeze-drying (lyophilization) allow stable, transportable enzyme products that retain activity for extended periods. Casease's relevance extends far beyond dairy foods. Its peptide hydrolysis capabilities contribute to drug development (through bioactive peptides), medical nutrition, and therapeutic interventions like enzyme replacement. Moreover, in the context of green technology, casease supports the breakdown and valorization of protein-rich waste, transforming environmental challenges into economic opportunities [9]. The enzyme's use in detergents enables effective removal of protein-based stains, while in leather processing, it provides a milder alternative to harsh chemical treatments.Ongoing research is centered on advancing casease's performance through protein design, computational modeling, and the discovery of unconventional enzyme sources. The integration of genomics, metagenomics, and synthetic biology offers promising routes to develop even more robust and specialized enzyme variants. These efforts underscore the growing significance of casease not only in established industries but also in shaping future biotechnological innovations and sustainable practices [10].

2. MATERIALS AND METHODS

2.1 To screen for casease-producing microorganisms, skim milk agar plates were prepared, sterilized, and inoculated with bacterial samples. After incubating at 37°C for 24 hours, clear zones around colonies indicating casein breakdown were measured to assess casease activity [11].

2.2 To characterize the production pattern of casease and its nature the Casein peptone broth was inoculated and incubated with the microorganism. At 24, 48, and 72 hours, samples were centrifuged to separate supernatant and cell extracts. Both were incubated with casein, and absorbance at 580 nm was measured. Decreases in absorbance indicated casein hydrolysis and casease activity at different time points in both fractions [12].

2.3 To quantify the casease production. The Casein was dissolved in a phosphate buffer (1% w/v) and mixed with enzyme extract, then incubated at 37°C for 30 minutes. The reaction was stopped using 10% trichloroacetic acid, followed by centrifugation to remove remaining proteins. The supernatant's absorbance was measured at 280 nm to quantify tyrosine released. A tyrosine standard curve was used, and casease activity was calculated as µg tyrosine released per minute per mg protein [13].

2.4 To perform a bioinformatics-based characterization of casease enzyme the Amino acid sequences for casease from Bacillus subtilis and Serratia marcescens were obtained from NCBI and UniProt. BLASTp was used to find homologous proteins and assess sequence similarity. Conserved domains were identified via InterProScan and Pfam, while ProtParam analyzed molecular weight, pI, instability index, and aliphatic index.

2.5 To predict the 3D structure of casease using homology modeling and analyze its active site, As the crystal structure of casease was not available in the PDB, homology modeling was completed using Swiss-Model, AlphaFold, and MODELLER, with templates chosen for sequence and structural similarity. Model validation included Ramachandran plots and Verify3D. Active site residues were identified using CASTp and MOE.

2.6 To perform sequence analysis and functional annotation of casease enzymes to classify them within the serine protease/metalloprotease families, Casease sequences were analyzed for conserved motifs and functional sites using InterProScan and Pfam. Classification as serine protease or metalloprotease was confirmed by identifying catalytic triads and metal-binding residues. Functional annotation with GO terms and KEGG pathways outlined casease's roles in protein degradation.

2.7 To conduct a phylogenetic analysis of casease enzymes , Casease homologs from various bacteria were aligned using MUSCLE, and a phylogenetic tree was built in MEGA-X with the Neighbor-Joining method and 1000 bootstrap replications. Evolutionary distances were calculated to classify casease within the serine protease family.

2.8 Casease production was optimized by varying pH (4.0–10.0), temperature, substrate (casein) concentration, and enzyme concentration. Enzyme activity was measured at each condition using the Kunitz method (absorbance at 280 nm) to identify optimal pH, temperature, substrate, and enzyme levels for maximum casein hydrolysis [13].

2.9 To Determine the kinetic parameters of casease-catalyzed casein hydrolysis, including Km and Vmax, to assess enzyme efficiency and substrate interaction varying concentrations of casein (0.5%–10% w/v) were incubated with a fixed volume of enzyme extract at 37°C for 30 minutes. After stopping the reaction with 10% TCA and centrifugation, absorbance at 280 nm was measured using the Kunitz method. Initial reaction velocities were plotted using the Michaelis-Menten and Lineweaver-Burk methods to calculate Km and Vmax, assessing enzyme affinity and efficiency [14]

2.10 Various chemical inhibitors dissolved in phosphate buffers were tested for their effects on casease activity. Enzyme and casein were incubated with each inhibitor at 37°C for 30 minutes, and reactions were stopped with TCA. After centrifugation, supernatant absorbance at 280 nm was measured. Reduced absorbance compared to controls indicated inhibition of casease activity. [15].

2.11 To assess inhibitor type, enzyme activity with and without inhibitors was measured using the Kunitz method. After incubation at optimal temperature and pH, reactions were terminated with TCA and centrifuged. Absorbance at 280 nm was compared to controls. A partial reduction in activity indicated reversible inhibition, while complete or permanent loss signified irreversible inhibition. Results were evaluated by calculating the percentage of activity retained.

2.12 To evaluate the influence of different metal ions on casease activity, Casease activity was assessed in the presence of various metal ions using the Kunitz method. Casein solution and metal ions were mixed with enzyme extract, incubated at 37°C, and the reaction was stopped with trichloroacetic acid. After centrifugation, supernatant absorbance at 280 nm was measured. Activity changes for each metal ion were calculated relative to the control to evaluate their effects on casease [16].

2.13 To assess casease substrate specificity, non-casein proteins (1% in pH 7.5 buffer) were incubated with casease extract(1 mg/mL) at 37°C for one hour. After stopping the reaction and centrifugation, absorbance at 280 nm was measured using the Kunitz method. Comparing results with controls indicated the enzyme's ability to degrade non-casein proteins for industrial waste management [17].

3. RESULTS AND DISCUSSION

3.1 To identify microorganisms with potential for casease production and evaluate their casease production capabilities.

Organism	Zone of clearance (mm)	Casease Production
Bacillus subtilis	27	Positive
Pseudomonas aeruginosa	22	Positive
Staphylococcus aureus	0	Negative
Escherichia coli	0	Negative
Serratia marcescens	25	Positive
Proteus mirabilis	0	Negative
Klebsiella pneumoniae	0	Negative
Saccharomyces cerevisiae	0	Negative
Streptococcus pyogenes	24	Positive
Salmonella typhi	12	Negative (weak)

Table 1 Screening of Microorganisms for Casease Production

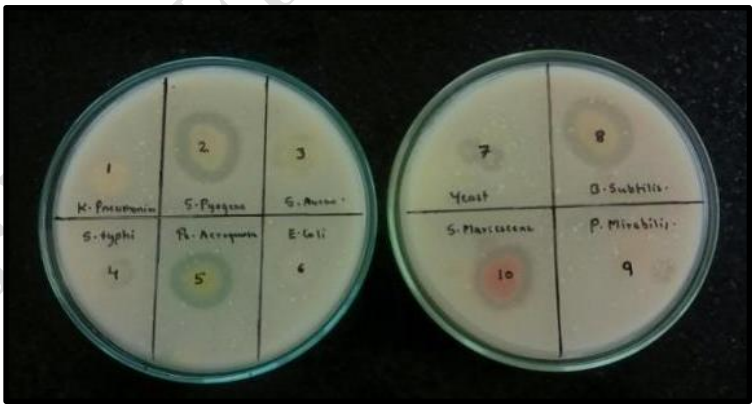

Figure 1 Screening of Microorganisms for Casease Production

A qualitative screening on skim milk agar revealed casease activity in *Bacillus subtilis* , *Serratia marcescens* , *Pseudomonas aeruginosa* , *and Streptococcus pyogenes* , indicated by clear zones around colonies. *Bacillus subtilis* showed the largest zone (27 mm), followed by *S. marcescens (25 mm), S. pyogenes (24 mm), and P. aeruginosa (22 mm). Salmonella typhi showed weak activity (*12 mm*), while Staphylococcus aureus , Escherichia coli , Proteus mirabilis , Klebsiella pneumoniae ,* and *Saccharomyces cerevisiae* showed no casease production.

3.2 To characterize the production pattern of casease and determine its nature .

Time (Hours)	Test Od (580nm)	Control Od (580nm)	Δ Od (Test-Control)
24	0.43	0.08	0.35
48	0.15	0.01	0.14
72	0.9	0.02	0.07

Table 2 Casease activity in the supernatant of *B.subtilis* at different time intervals.

Time (Hours)	Test Od (580nm)	Control Od (580nm)	Δ Od (Test-Control)
24	0.04	0.01	0.03
48	0.06	0.02	0.04
72	0.02	0.01	0.01

Table 3 Casease activity in the cell extract of *B.subtilis* at different time intervals.

Time (Hours)	Test Od (580nm)	Control Od (580nm)	Δ Od (Test-Control)
24	0.17	0.0	0.17
48	0.30	0.11	0.19
72	0.07	0.02	0.05

Table 4 Casease activity in the supernatant of *S.marcescens* at different time intervals.

Time (Hours)	Test Od (580nm)	Control Od (580nm)	Δ Od (Test-Control)
24	0.02	0.01	0.01
48	0.04	0.01	0.01
72	0.03	0.01	0.01

Table 5 Casease activity in the cell extract of *S.marcescens* at different time intervals.

Bacillus subtilis showed peak extracellular casease activity at 24 hours, followed by a decline at 48 and 72 hours. Intracellular activity remained minimal, suggesting degradation or regulatory inhibition. *Serratia marcescens* showed delayed enzyme production, with partial intracellular

retention or delayed release. The postponed biphasic trend suggests regulation influenced by quorum-sensing or stress-associated pathways.

3.3 Quantification of casease production.

Tyrosine Conc. (mcg/ml)	Od (at 280 nm)
0	0
50	0.25
100	0.58
150	0.74
200	0.94
250	1.24
300	1.62
350	1.84
400	2.05

Table 6 Standard Tyrosine Curve Plot for Casease Activity Determination

Organism	Test OD (280 nm)	Control OD (280nm)	Δ OD
Bacillus subtilis	3.21	2.33	0.86
Serratia marcescens	3.33	3.02	0.31

Table 7 Casease Activity (Quantitative) in *B.subtilis* and *S.marcescens*

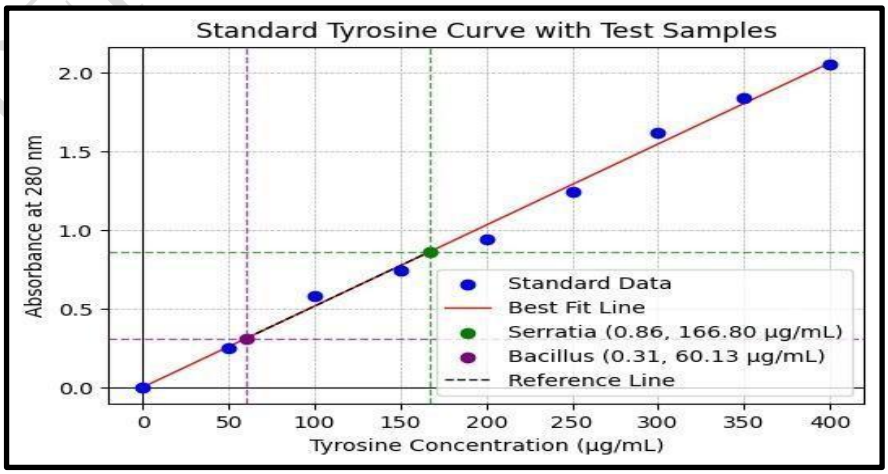

Figure 2 Quantification of the casease production.

A standard tyrosine curve was generated by plotting absorbance at 280 nm against tyrosine concentration. The best-fit line shows a linear relationship between absorbance and concentration. Two casease enzyme samples were tested. *Serratia marcescens*: 0.86 absorbance, corresponding to 166.80 µg/mL of tyrosine release. *Bacillus subtilis* : 0.31 absorbance, corresponding to 60.13 µg/mL of tyrosine release. The higher absorbance of *Serratia marcescens* indicates greater enzymatic activity.

3.4 To perform a bioinformatics-based characterization of casease enzyme

Property	*Bacillus subtilis*	*Serratia marcescens*
Sequence Length (aa)	381	487
Molecular Weight (Da)	38,866.38	52,234.06
Isoelectric Point (pI)	8.72	4.64
Instability Index	12.83 (Stable)	24.13 (Less stable)
Aliphatic Index	76.92	75.99

Table 8 Structural and functional properties of casease enzyme from two Organism

Comparative bioinformatics analysis shows that S. marcescens casease is larger and more acidic (487 aa, pI 4.64) than B. subtilis casease (381 aa, pI 8.72). B. subtilis casease is more structurally stable (instability index 12.83 vs. 24.13), while both have similar thermostability. These differences reflect distinct biochemical characteristics and application potentials.

BLASTp analysis of *Bacillus subtilis*

Subject ID	Organism	Seq. Length	E-value	Identity (%)	Align Length
WP_003233171.1	*Bacillus subtilis*	381	0	100.0	381
WP_326223890.1	*Bacillus subtilis*	381	0	99.74	381
WP_014479360.1	*Bacillus subtilis*	381	0	99.74	381
WP_029726299.1	*Bacillus subtilis*	381	0	99.74	381

KDE25138.1	*Bacillus subtilis*	381	0	99.74	381

Table 9 BLASTp Analysis of *Bacillus subtilis*

BLASTp Analysis *Serratia marcescens*

Subject ID	Organism	Seq. Length	E-value	Identity (%)	Align Length
WP_033644999.1	*Serratia sp.*	487	0	100.0	487
WP_216474504.1	*S. ureilytica*	487	0	99.79	487
WP_176589442.1	*S. ureilytica*	487	0	99.79	487
WP_308349048.1	*S.marcescens*	487	0	99.79	487
WP_197764609.1	*S. ureilytica*	487	0	99.79	487

Table 10 BLASTp Analysis *Serratia marcescens*

3.5 To predict the 3D structure of a casease using homology modeling and analyze its active site.

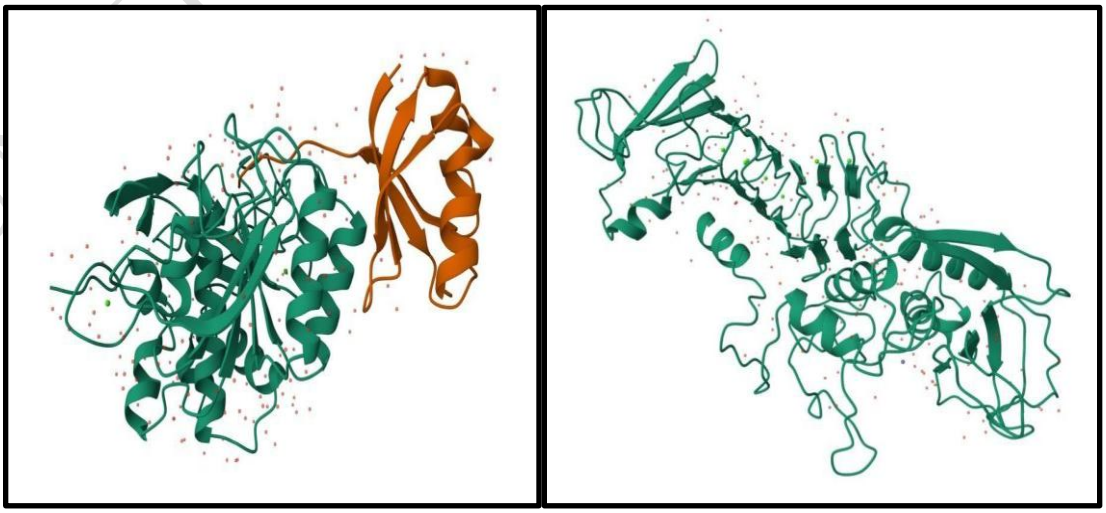

Figure 3a (*Bacillus subtilis*) Figure 3b (*Serratia marcescens*)

Figure (3a&3b) 3d Structure of casease enzyme from *B.subtilis* and *S.marcescens*

3.6 To perform sequence analysis and functional annotation of casease enzymes to classify them within the serine protease/metalloprotease families

Feature	*Bacillus subtilis*	*Serratia marcescens*
Type	Serine protease	Metalloprotease
Catalytic Mechanism	Ser-His-Asp catalytic triad	Zinc-dependent hydrolysis
PDB ID (3D Structure)	1SCJ, 3WHI, 6044, 6PAK	1SRP
Active Site Residues	Asp32, His64. Ser221	His176, His180. His186.
Cofactors	None	Zinc (Zn2+)
Optimal pH	Alkaline (pH ~8-9)	Neutral to alkaline (pH ~7-9)
Function	Protein degradation	Protein degradation
Industrial Use	Detergents, food processing	Bioremediation
Structural Stability	Highly stable	Less stable
Sequence Length	381 amino acids	487 amino acids
Sequence Identity	<30% identity	<30% identity

Table 11 Functional annotation of casease enzymes

Subtilisin E (serine protease, 381 residues) uses a Ser-His-Asp catalytic triad and functions in soil adaptation; it is widely used in industry for its stability. Serralysin (metalloprotease, 477 residues) relies on a Zn^{2+}-binding site, is involved in pathogenicity, and is useful in bioremediation and biofilm disruption.

3.7 Phylogenetic analysis of casease enzymes

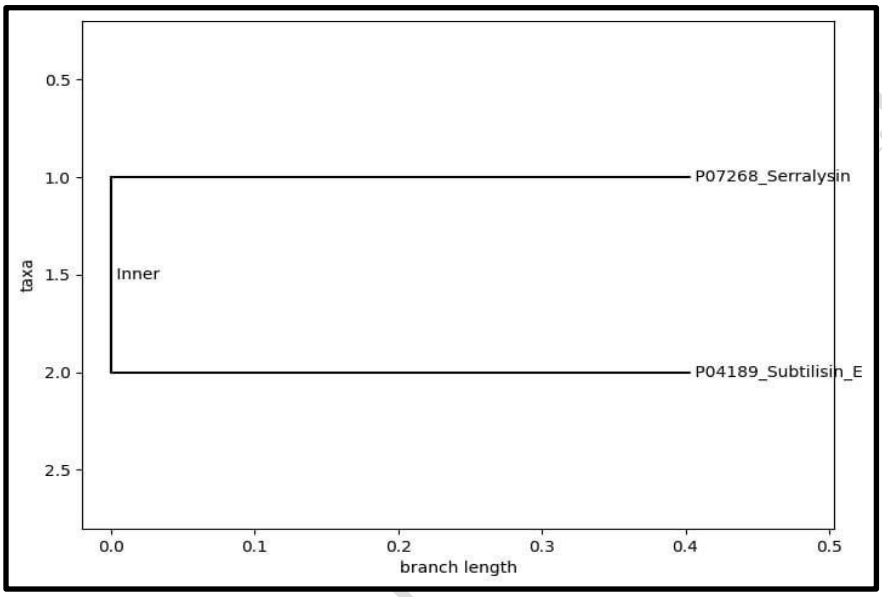

Figure 4 Phylogenetic analysis of casease enzymes

Subtilisin E and Serralysin form separate branches, indicating different evolutionary origins. Branch Length (~0.45 for both) Moderate divergence despite common function. Both enzymes play a role in protein degradation but with different catalytic mechanisms. Sequence Alignment (~35% Identity), Moderate similarity supports classification into distinct protease families.

3.8 Optimal conditions for casease production by evaluating the effects of pH, temperature, and inducer concentration on enzyme activity. pH Dependent Activity

Effects of pH

pH	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
4	0.05	0.05
5	0.08	0.12
6	0.12	0.24
7	0.32	0.54

8	0.30	0.71
9	0.25	0.46
10	0.19	0.35

Table 12 Effect of pH on Casease Activity

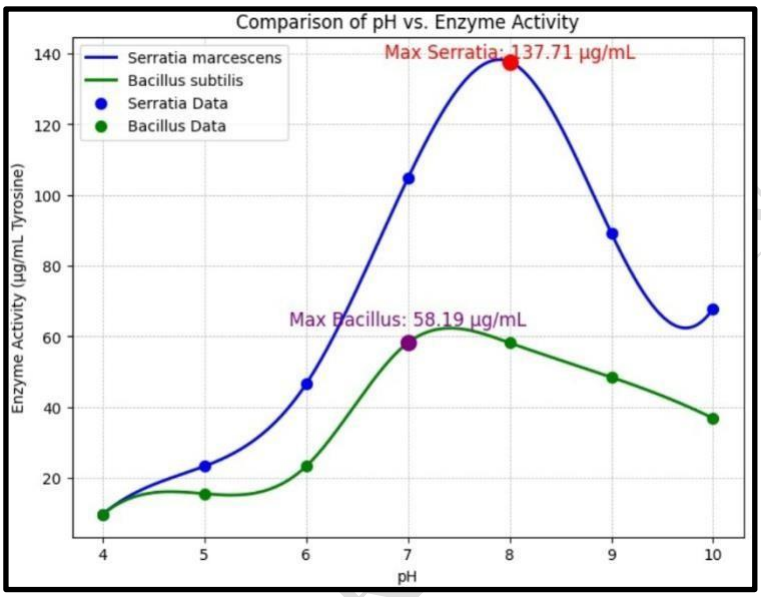

Figure 5 Comparison of enzyme activity of casease *B.subtilis* and *S.marcescens*

The pH-dependent activity profiles of *Bacillus subtilis* and *Serratia marcescens* caseases exhibit distinct optimal conditions and catalytic efficiencies. *B. subtilis* casease shows maximum activity at pH 7 with 58.19 µg/mL tyrosine release, whereas *S. marcescens* casease peaks at pH 8 with 137.71 µg/mL, indicating a broader alkaline preference. Both enzymes demonstrate increased activity from acidic to neutral/alkaline pH, with *S. marcescens* exhibiting a steeper activity rise and higher catalytic performance.

Effects of Temperature

Temperature	*Bacillus subtilis* (280 nm)	*Serratia marcescens* (280 nm)
0	0.02	0.1
Rt	0.07	0.46
37	0.30	0.87

55	0.38	1.24
100	0.05	0.71

Table 13 Effect of Temperature on Casease Activity

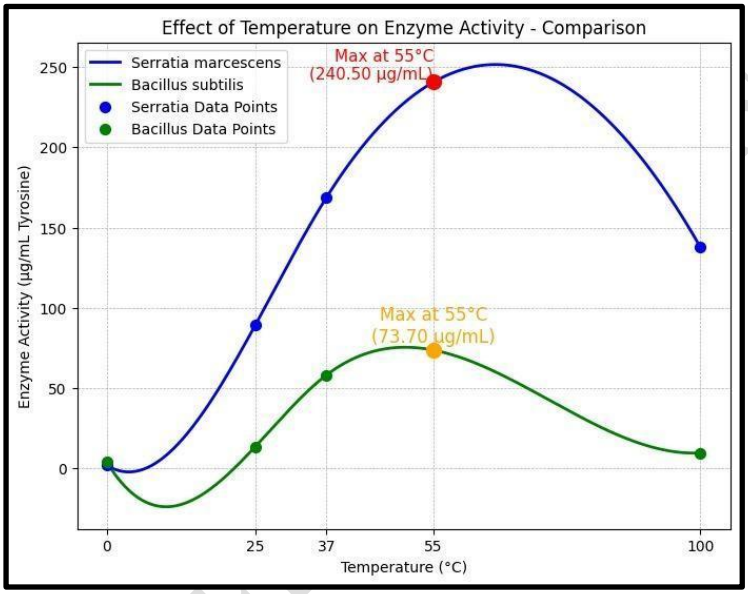

Figure 6 Comparison of enzyme activity of casease *B.subtilis* and *S.marcescens*

The temperature-dependent activity profiles of *Bacillus subtilis* and *Serratia marcescens* caseases both peak at 55°C; however, *S. marcescens* exhibits markedly higher catalytic efficiency, releasing 240.50 µg/mL tyrosine compared to 73.70 µg/mL for *B. subtilis.* While both enzymes show increased activity up to 55°C followed by a decline due to thermal sensitivity, *S. marcescens* consistently maintains superior enzymatic performance across all temperatures.

Effects of Substrate concentration

Substrate concentration (Casein W/V)	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
0	0.0	0.0
0.5	0.06	0.12

1	0.09	0.25
1.5	0.14	0.40
2	0.18	0.58
2.5	0.23	0.74
3	0.27	0.88
3.5	0.31	0.99
4	0.34	1.08
4.5	0.36	1.14
5	0.38	1.18
5.5	0.39	1.21
6	0.40	1.23
6.5	0.41	1.24
7	0.41	1.25
7.5	0.41	1.25
8	0.42	1.25
8.5	0.42	1.25
9	0.42	1.25
9.5	0.42	1.25
10	0.42	1.25

Table 14 Effect of Substrate Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

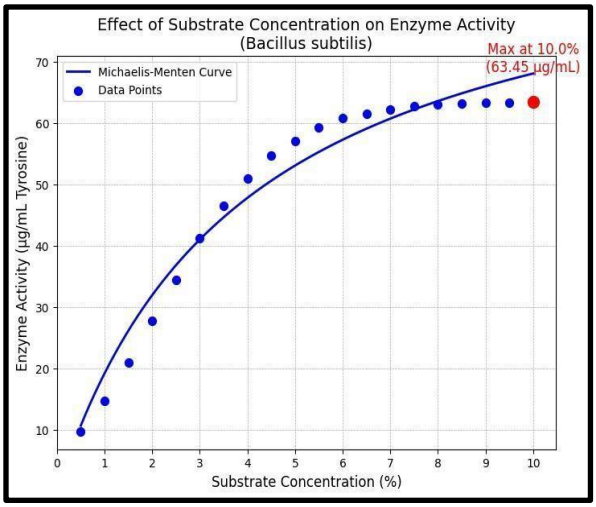

Figure 7a (*Bacillus subtilis*)

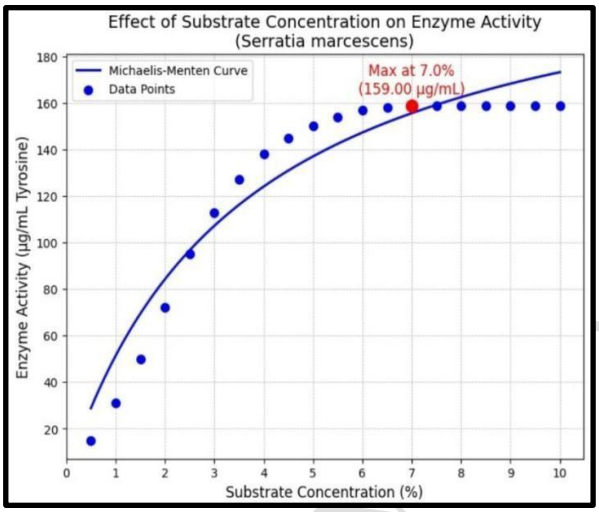

Figure 7b (*Serratia marcescens*)

Figure (7a&7b)Effect of Substrate Concentration on enzyme activity of casease *B.subtilis* and *S.marcescens*

Both *Bacillus subtilis* and *Serratia marcescen*s caseases followed Michaelis-Menten kinetics. B. subtilis reached a Vmax of 63.45 µg/mL tyrosine at 10% substrate, while S. marcescens achieved a higher Vmax of 159.00 µg/mL at 7%, with activity saturating beyond these concentrations.

Effects of Enzyme concentration

Enzyme concentration (X)	*Bacillus subtilis* (280nm)	*Serratia* marcescens (280nm)
0.1	0.24	0.46
0.5	0.34	0.83
1	0.42	0.87
2	0.76	1.52
5	1.23	2.34

Table 15 Effect of Enzyme Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

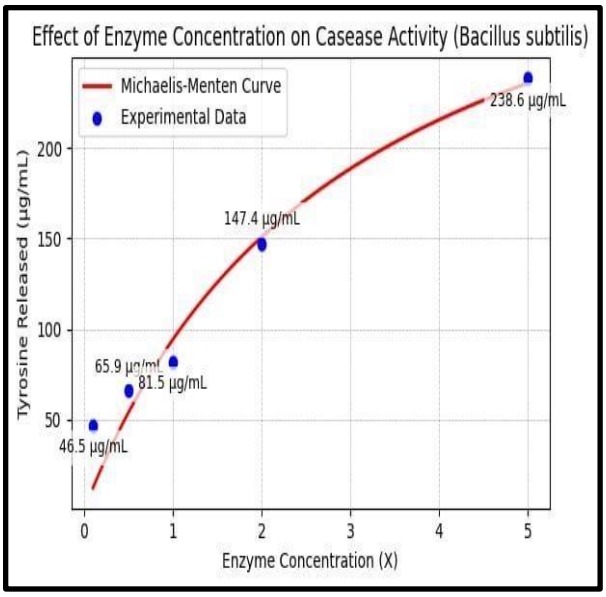

Figure 8a (*Bacillus subtilis*)

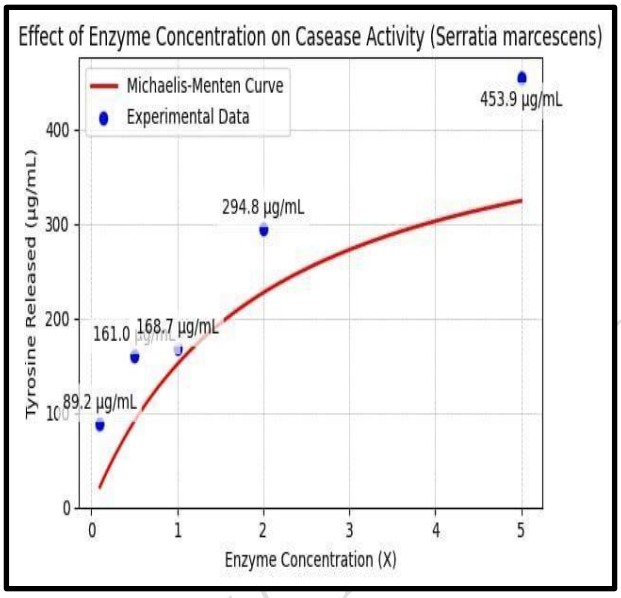

Figure 8b (*Serratia marcescens*)

Figure (8a & 8b)Effect of Enzyme Concentration on enzyme activity of casease *B.subtilis* and *S.marcescens*

The effect of enzyme concentration on casease activity of *Bacillus subtilis* and *Serratia marcescens* followed typical Michaelis-Menten kinetics, with tyrosine release increasing sharply at lower enzyme concentrations and gradually plateauing at higher concentrations, indicating substrate saturation. For *B. subtilis*, tyrosine release ranged from 46.5 µg/mL at 0.1X to 238.6 µg/mL at 5.0X enzyme concentration, whereas *S. marcescens* showed a higher activity, ranging from 89.2 µg/mL to 453.9 µg/mL under the same conditions, reflecting its greater catalytic efficiency

3.9 The kinetic parameters of casease-catalyzed casein hydrolysis, including Km and Vmax, to assess enzyme efficiency and substrate interaction.

Substrate concentration (W/V)	Enzyme activity of *Bacillus subtilis* (Tyrosine/mcg/ml)	Enzyme activity of *Serratia marcescens* (Tyrosine/mcg/ml)
0	0	0
0.5	9.75	15
1	14.70	31
1.5	21.00	50
2	27.75	72
2.5	34.50	95
3	41.25	113
3.5	46.50	127
4	51.00	138
4.5	54.75	145
5	57.00	150
5.5	59.25	154
6	60.75	157
6.5	61.50	158
7	62.25	159
7.5	62.70	159
8	63.00	159
8.5	63.15	159
9	63.30	159
9.5	63.30	159
10	63.45	159

Table 16 Effect of Substrate Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

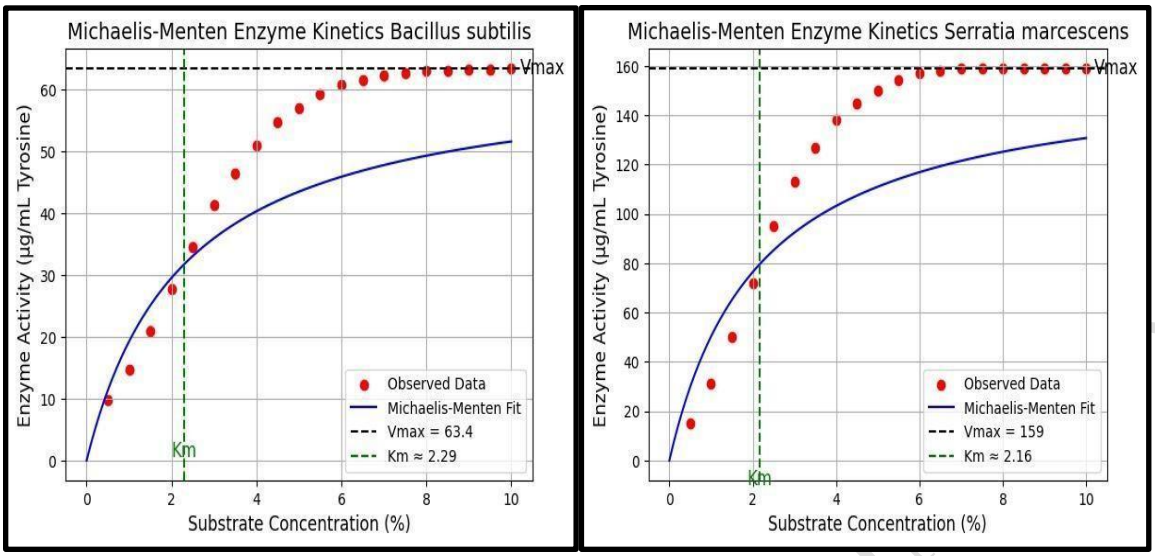

Figure 9 a (*Bacillus subtilis*) Figure 9 b (*Serratia marcescens*)

Figure (9a & 9b) Michaelis-Menten plot representing the enzyme kinetics of casease from *B. subtilis* and *S. marcescens*

The enzyme kinetics of casease-mediated casein hydrolysis in *Bacillus subtilis* and *Serratia marcescens* were analyzed using the Michaelis-Menten model. Both enzymes exhibited typical Michaelis-Menten behavior, showing a clear dependence of activity on substrate concentration. *B. subtilis* casease displayed a maximum velocity (Vmax) of 63.4 µg/mL of tyrosine with a Michaelis constant (Km) of 2.29%, indicating moderate substrate affinity, whereas *S. marcescens* casease exhibited a higher Vmax of 159 µg/mL and a Km of 2.16%, reflecting more efficient catalysis and strong substrate affinity even at low concentrations. These results highlight the superior catalytic efficiency of *S. marcescens* casease and provide a basis for evaluating potential inhibitory effects of chemical agents for industrial applications.

3.10 Effect of Chemical Agents on Casease Activity in *Bacillus subtilis* and *Serratia marcescens*

Chemical Agents	*Bacillus* subtilis (280nm)	*Serratia marcescens* (280 nm)
Control	1.11	1.49
1 N NAOH	1.03	1.16
1N HCL	1.13	0.99
OH	1.06	1.36
Glycerol	1.09	1.21

H2O2	0.71	1.09

Table 17 Effect of Chemical Agents on Casease Activity in *B. subtilis* and *S. marcescens*

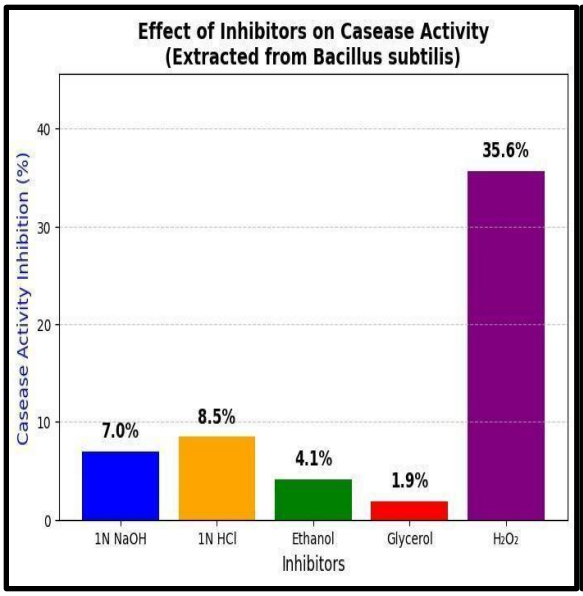

Figure 10 a (*Bacillus subtilis*)

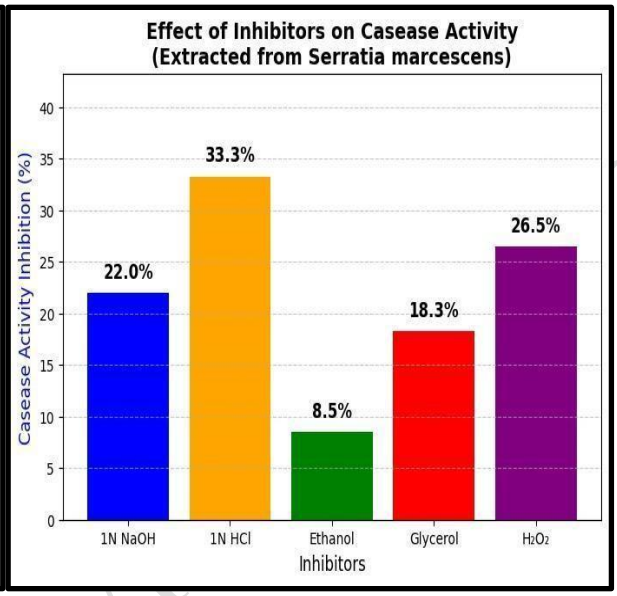

Figure 10 b (*Serratia marcescens*)

Figure (10a & 10b) Effect of inhibitors on casease activity of *B. subtilis* and *S. marcescens*

The effects of various inhibitors on casease activity from *Bacillus subtilis* and *Serratia marcescens* were evaluated, revealing differential sensitivities. For *B. subtilis*, hydrogen peroxide (H_2O_2) caused the highest inhibition (35.6%), while 1N HCl (8.5%), 1N NaOH (7.0%), ethanol (4.1%), and glycerol (1.9%) showed progressively lower effects, indicating susceptibility to oxidative stress and extreme pH. In contrast, *S. marcescens* casease was most inhibited by 1N HCl (33.3%), followed by H_2O_2 (26.5%) and 1N NaOH (22.0%), whereas glycerol (18.3%) and ethanol (8.5%) had minimal impact, reflecting a higher tolerance to organic compounds but sensitivity to acidic and oxidative conditions.

3.11 To assess whether inhibitors act as irreversible or reversible inhibitors based on their impact on enzyme activity.

Chemical Agents	*Bacillus subtilis* (280 nm)	Inhibition Type	*Serratia marcescens* (280 nm)	Inhibition Type
Control	1.11	No	1.49	No
1 N NAOH	1.03	Mild	1.16	Mild
1N HCL	1.13	Increase activity	0.99	Inhibition
OH	1.06	Mild	1.36	Mild
Glycerol	1.09	Minimal	1.21	Minimal
H2O2	0.71	Strong	1.09	Moderate

Table 18 Assessment of type of Inhibition of Enzyme Activity by Chemical Agents

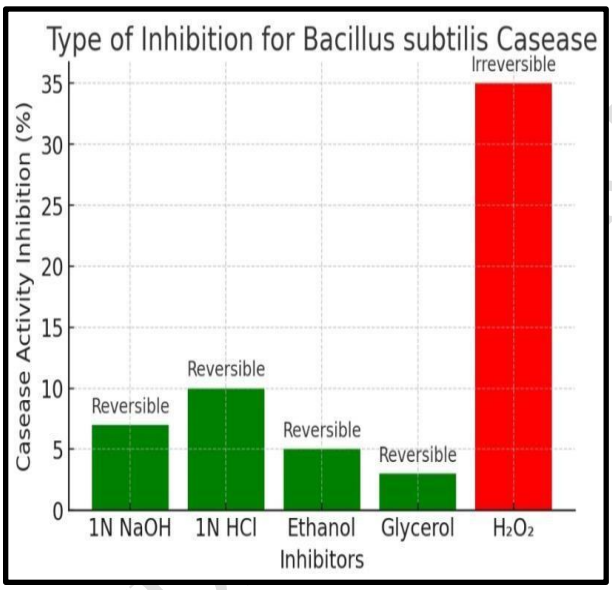

Figure 11 a (*Bacillus subtilis*)

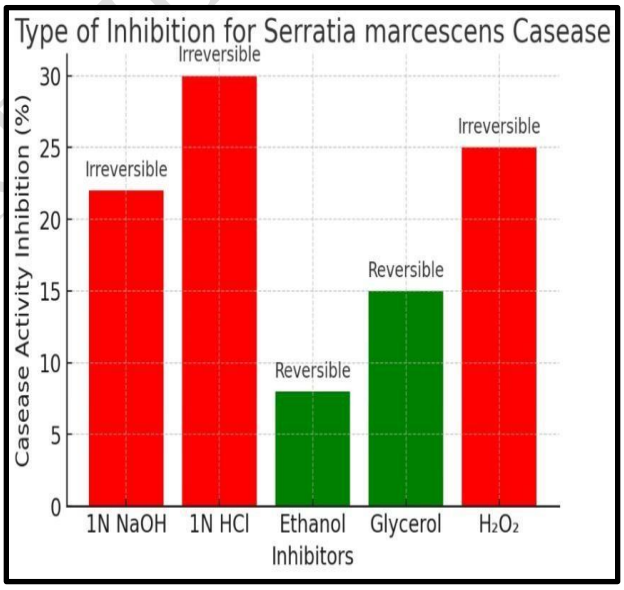

Figure 11 b (*Serratia marcescens*)

Figure (11 a & 11b) Inhibition exhibited by different inhibitors on casease activity of *B. subtilis* and *S. marcescens*

The casease enzyme from *Bacillus subtilis* showed maximum inhibition with H_2O_2 (35.6%) due to oxidative damage. Moderate inhibition was observed with 1N HCl (8.5%) and 1N NaOH (7.0%), indicating partial reversible effects. Minimal inhibition was recorded with Ethanol (4.1%) and Glycerol (1.9%), suggesting reversible interaction without structural damage. Overall, *Bacillus subtilis* casease exhibited greater resistance to pH changes but was highly sensitive to oxidative stress. And The casease enzyme from *Serratia marcescens* showed maximum inhibition with 1N HCl (33.3%) and H_2O_2 (26.5%), indicating possible irreversible

inhibition due to denaturation and oxidative damage. Significant inhibition was also observed with 1N NaOH (22.0%), reflecting sensitivity to alkaline conditions. In contrast, lower inhibition was recorded with Glycerol (18.3%) and Ethanol (8.5%), suggesting reversible inhibition likely through weak interactions without permanent enzyme damage.

3.12 To evaluate the influence of different metal ions on casease activity.

Ca2+ Con (mM)	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
0.1	0.8482	1.1039
0.5	0.8829	0.9294
1	0.9971	0.9783
2	0.9884	0.8501
5	0.8187	1.0372
10	0.7386	2.0024
Control	1.1068	1.4855

Table 19 Effect of (Ca^{2+}) Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

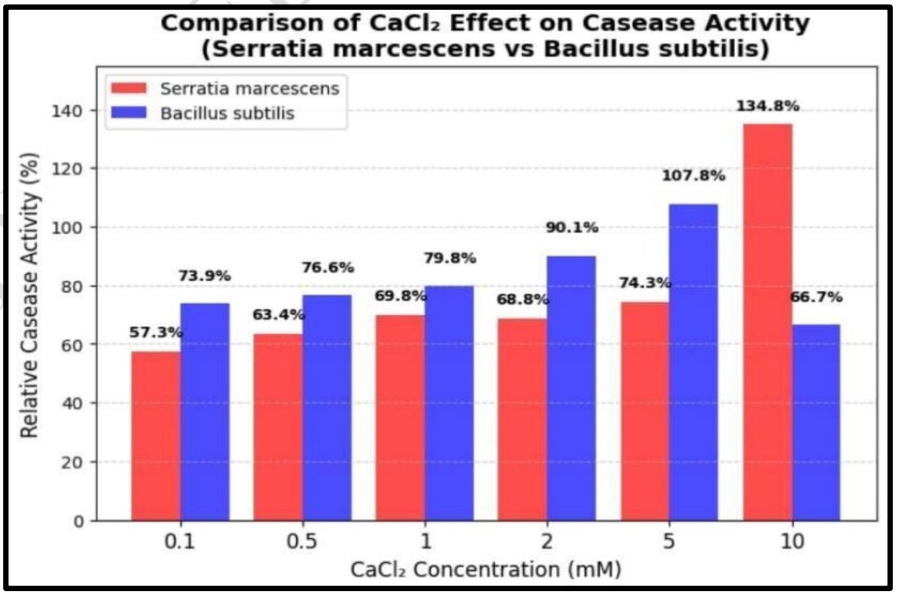

Figure 12 Effect of $CaCl_2$ on Casease Activity in *B.subtilis* and *S.marcescens*

The effect of $CaCl_2$ on casease activity of *Bacillus subtilis* and *Serratia marcescens* showed distinct concentration-dependent responses. In *B. subtilis*, activity increased from 74.0% at 0.1 mM to a maximum of 107.8% at 5.0 mM $CaCl_2$, but declined to 66.7% at 10.0 mM, indicating inhibition at higher concentrations. In contrast, *S. marcescens* exhibited a biphasic pattern with gradual increases from 57.3% at 0.1 mM to 74.3% at 5.0 mM, followed by a marked enhancement to 134.8% at 10.0 mM, suggesting superior tolerance and stimulation by higher $CaCl_2$ levels. Overall, *S. marcescens* casease demonstrated better adaptability and activation in response to increasing calcium concentrations compared to *B. subtilis*.

Cu2+ Con (mM)	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
0.1	0.7994	1.0158
0.5	0.9060	0.8805
1	0.9110	0.9207
2	0.8957	1.6096
5	1.5252	1.9019
10	0.9726	0.8031
Control	1.1068	1.4855

Table 20 Effect of (Cu^{2+}) Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

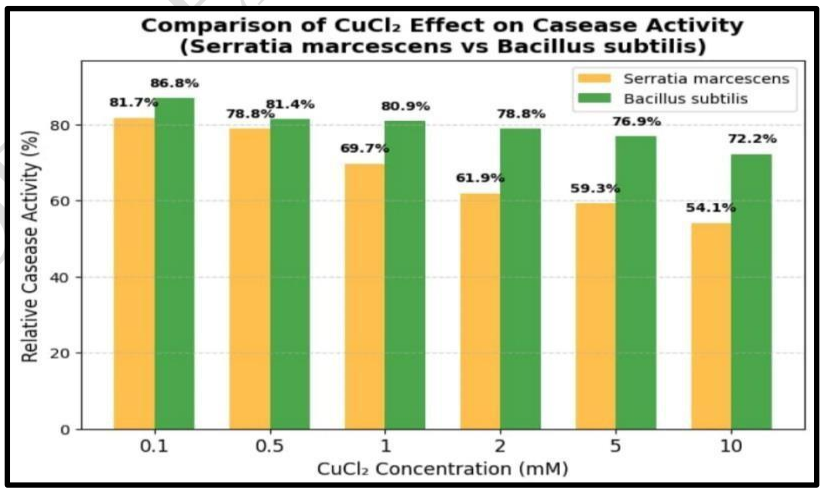

Figure 13 Effect of $CuCl_2$ on Casease Activity in *B.subtilis* and *S.marcescens*

The effect of $CuCl_2$ on casease activity of *Bacillus subtilis* and *Serratia marcescens* showed a concentration-dependent inhibition in both species. *B. subtilis* exhibited the highest activity at 0.1 mM (86.8%), gradually decreasing to 72.3% at 10.0 mM, indicating relative resistance to Cu^{2+} ions. In contrast, *S. marcescens* activity declined sharply from 81.7% at 0.1 mM to 54.1% at 10.0 mM, reflecting higher sensitivity to copper-induced inhibition. Overall, *B. subtilis* casease demonstrated greater tolerance to $CuCl_2$ compared to *S. marcescens*.

Fe^2-Con (mM)	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
0.1	0.9114	0.8882
0.5	1.2640	1.2814
1	1.6589	1.3583
2	1.7248	0.8627
5	1.8804	0.9322
10	1.9812	1.0345
Control	1.1068	1.4855

Table 21 Effect of (Fe^{2+}) Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

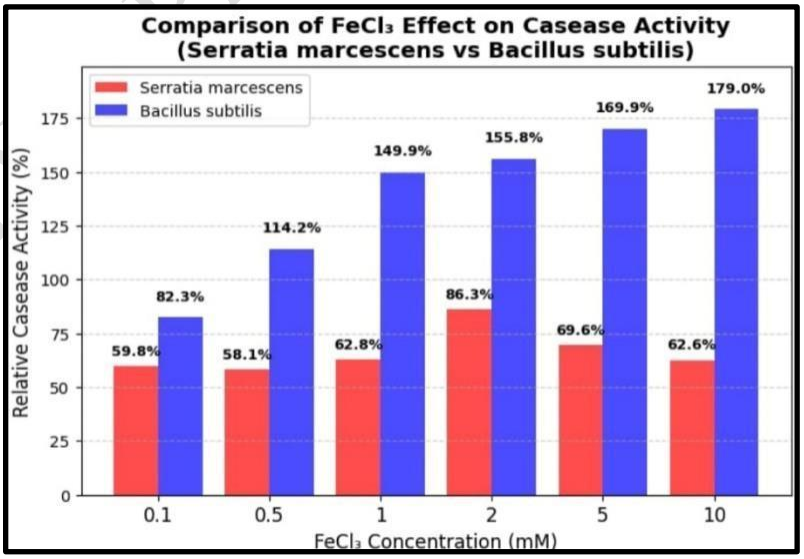

Figure 14 Effect of $FeCl_3$ on Casease Activity in *B.subtilis* and *S.marcescens*

The effect of $FeCl_3$ on casease activity of *Bacillus subtilis* and *Serratia marcescens* showed contrasting responses. In *B. subtilis*, activity increased progressively from 82.3% at 0.1 mM to a maximum of 179.0% at 10.0 mM, indicating strong activation by Fe^{3+} ions. In contrast, *S. marcescens* exhibited a fluctuating pattern, with slight decrease from 59.8% at 0.1 mM to 58.1% at 0.5 mM, followed by a peak at 86.3% at 2.0 mM, and a gradual decline to 62.6% at 10.0 mM, suggesting activation at lower concentrations but inhibition at higher $FeCl_3$ levels.

Mg2+ Con (mM)	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
0.1	1.1129	1.4226
0.5	1.0852	1.6366
1	1.1001	1.3465
2	1.2811	1.3743
5	1.0388	1.3357
10	1.1173	1.2489
Control	1.1068	1.4855

Table 22 Effect of (Mg^{2+}) Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

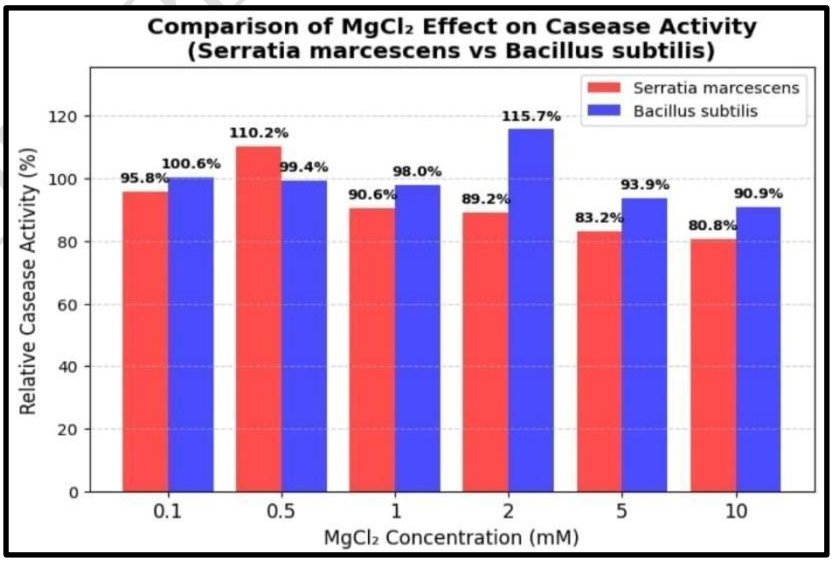

Figure 15 Effect of $MgCl_2$ on Casease Activity in *B.subtilis* and *S.marcescens*

The effect of $MgCl_2$ on casease activity in *Bacillus subtilis* and *Serratia marcescens* showed distinct concentration-dependent patterns. In *B. subtilis*, activity remained near 100% at 0.1–1.0 mM, peaked at 115.7% at 2.0 mM, and declined to 90.9% at 10.0 mM, indicating moderate activation at optimal levels and inhibition at higher concentrations. In *S. marcescens*, activity increased to 110.2% at 0.5 mM, followed by a gradual decline to 80.8% at 10.0 mM, suggesting mild activation at low concentrations and stronger inhibition at higher Mg^{2+} levels. Overall, *B. subtilis* maintained slightly higher activity than *S. marcescens* at elevated $MgCl_2$ concentrations.

Zn2+ Con (mM)	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
0.1	0.8279	1.2488
0.5	0.4247	1.1409
1	1.4108	1.2209
2	1.0319	0.9420
5	1.2310	1.0857
10	1.1313	1.2642
Control	1.1068	1.4855

Table 23 Effect of Zn^{2+} Concentration on Casease Activity in *B.subtilis* and *S.marcescens*

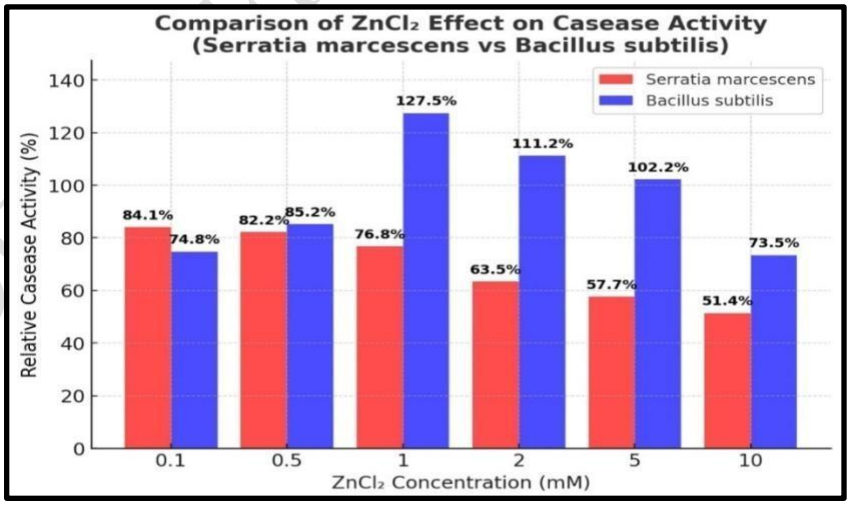

Figure 16 Effect of $ZnCl_2$ on Casease Activity in *B.subtilis* and *S.marcescens*

The effect of $ZnCl_2$ on casease activity demonstrated clear species-specific differences between *Bacillus subtilis* and *Serratia marcescens*. In *B. subtilis*, casease activity initially declined to 74.8% at 0.1 mM $ZnCl_2$ but increased to a peak of 127.5% at 1.0 mM, indicating an activation effect at moderate concentrations, followed by a gradual decrease to 73.5% at 10.0 mM with

further Zn^{2+} increase, suggesting inhibition at higher levels. Conversely, *S. marcescens* exhibited a consistent decline in activity from 84.1% at 0.1 mM to 51.4% at 10.0 mM, reflecting a progressive inhibitory effect of Zn^{2+}. These findings indicate that Zn^{2+} exerts a dual regulatory effect on casease activity in *B. subtilis* activation at optimal concentrations and inhibition at elevated levels while acting as a potent inhibitor in *S. marcescens*, highlighting species-specific differences in enzyme sensitivity to metal ions.

Mn2+ Con (mM)	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
0.1	1.2734	1.0543
0.5	1.1562	1.1268
1	0.9611	1.2459
2	1.3360	1.4172
5	0.9576	1.5745
10	1.1569	1.2123
Control	1.1068	1.4855

Table 24 Effect of Mn^{2+} on Casease Activity in *B.subtilis* and *S.marcescens*

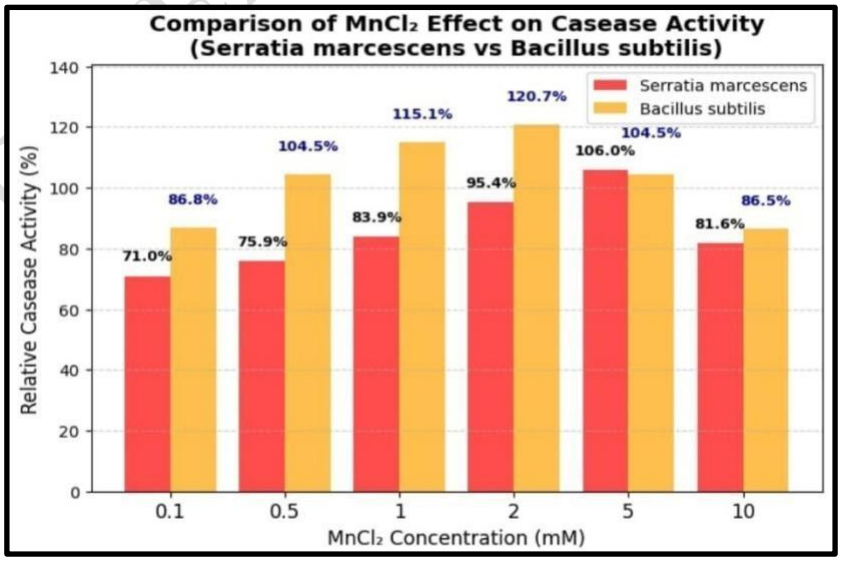

Figure 17 Effect of $MnCl_2$ on Casease Activity in *B.subtilis* and *S.marcescens*

The effect of $MnCl_2$ on casease activity exhibited species-specific biphasic responses in *Bacillus subtilis* and *Serratia marcescens*. In *B. subtilis*, enzyme activity increased from 86.8% at 0.1 mM to a maximum of 120.7% at 2.0 mM, suggesting activation at moderate Mn^{2+} levels, followed by a decline to 86.5% at 10.0 mM, indicative of inhibition at higher concentrations. In contrast, *S. marcescens* showed a gradual enhancement of activity from 71.0% at 0.1 mM to 106.0% at 5.0 mM, beyond which the activity decreased to 81.6% at 10.0 mM. These results suggest that *B. subtilis* casease is more sensitive to Mn^{2+} activation at lower concentrations than *S. marcescens*, while both species experience inhibitory effects at elevated Mn^{2+} levels, emphasizing differential metal ion responsiveness between the two microbial enzymes.

3.13 The substrate specificity of casease non-casein protein substrates

Substrate	*Bacillus subtilis* (280nm)	*Serratia marcescens* (280nm)
Casein	3.07	3.18
Legumin	2.38	1.87
Glycinin	2.60	2.54
Zein	2.39	2.31
Gelatin	2.58	2.56
Gliadin	2.22	2.33

Table 25 Evaluation of Substrate specificity of casease on non-casein protein substrates

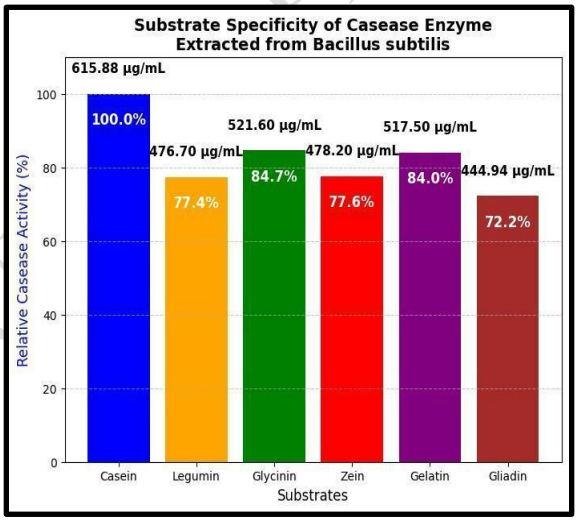

Figure 18 a (*Bacillus subtilis*)

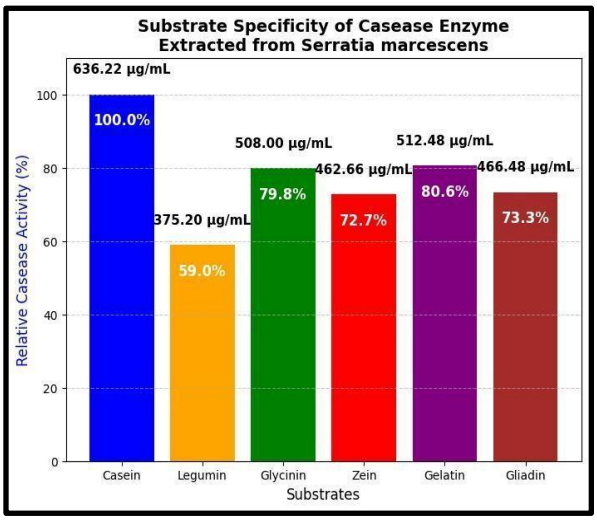

Figure 18 b (*Serratia marcescens*)

Figure (18a & 18 b) Substrate Specificity of casease activity on non-casein protein substrates

The casease enzyme extracted from *Bacillus subtilis* exhibited the highest activity towards casein (100%, 615.88 µg/mL). Among non-casein substrates, gelatin (84.0%, 517.50 µg/mL) and glycinin (84.7%, 521.60 µg/mL) showed comparatively higher activity. Zein (77.6%, 478.20 µg/mL) and legumin (77.4%, 476.70 µg/mL) demonstrated moderate activity, while gliadin exhibited the lowest activity (72.2%, 444.94 µg/mL).

The casease enzyme extracted from *Serratia marcescens* exhibited the highest activity towards casein (100%, 636.22 µg/mL). Among non-casein substrates, gelatin (80.6%, 512.48 µg/mL) and glycinin (79.8%, 508.00 µg/mL) showed higher activity. Zein (72.7%, 462.66 µg/mL) and gliadin (73.3%, 466.48 µg/mL) demonstrated moderate activity, while legumin exhibited the lowest activity (59.0%, 375.20 µg/mL).

CONCLUSION

This study highlights significant variation in casein hydrolysis among bacterial species, identifying *Bacillus subtilis* and *Serratia marcescens* as the most efficient casease producers. Both primarily secrete extracellular enzymes, though *S. marcescens* exhibits delayed but higher overall proteolytic activity compared to *B. subtilis*. The tyrosine standard assay confirmed superior catalytic efficiency in S. marcescens, reflecting enhanced enzyme secretion or activity. Bioinformatic characterization revealed *B. subtilis* casease as subtilisin E, a serine protease employing a Ser–His–Asp catalytic triad, while *S. marcescens* casease corresponds to serralysin, a zinc-dependent metalloprotease containing a HEXXH motif. Despite performing similar functions, their sequence divergence and distinct clustering in phylogenetic trees indicate independent evolutionary paths. Homology models supported conserved structural folds and reliable catalytic frameworks.

Enzyme kinetics demonstrated optimal activity for *B. subtilis* near neutral pH and for S. marcescens under slightly alkaline conditions, both at approximately 55 °C *S.marcescens* achieved a higher Vmax, confirming greater catalytic efficiency. Metal-ion modulation and inhibitor assays indicated species-specific responses, with calcium and magnesium enhancing activity at low levels, while excessive zinc, copper, and manganese produced inhibitory effects.Substrate utilization assays further revealed broad proteolytic potential, particularly toward gelatin and glycinin, suggesting potential for applications in dairy processing, bioremediation, and industrial protein degradation. Overall, *B. subtilis* subtilisin E and *S. marcescens* serralysin represent promising enzymes for future biotechnological and industrial applications due to their stability, activity, and structural adaptability.

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