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EVALUATION OF COLLAGENASE ACTIVITY ISOLATED FROM BEEF SAMPLES AND ITS IN-SILICO STRUCTURAL ANALYSIS

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ABSTRACT

Collagenase play a crucial role in various industrial applications, including food processing and biomedical research. This research focuses upon the isolation, purification and in-silico analysis of collagenase producing bacteria. Collagen is an abundant protien in many higher organisms and due to its unique amino acid compostion it can only be cleaved by speicialized proteases like collagenase enzyme secreted by some bacteria such as Baccilus Cereus and Clostrodium Histolyticum. In this research collagenase was purified from beef by repeated streaking method. This research work concludes that the bacterial collagenase displayed best activty at pH 8, with optimum substrate concentration of 2% gelatin and the optimum time of incubation is 48h. The results show that maximum enzyme activity was recorded with Peptone, Maltose, and $MgSO_4$ which were the best sources for nitrogen, carbon, and metal ions respectively. PredictProtein tool was utilized to determine the secondary structure of enzyme and it categorized the protein into 33.26% alpha helices, 16.48% beta strands, and 50.26% loops/turns. Phyre2 and Swiss model were used for determining 3D structure of the enzyme.

KEYWORDS: Collagenase, Beef, Collagen.

INTRODUCTION

In living things, enzymes improve biochemical processes. It is possible to extract them from cells and use them as catalysts for a range of progressively important processes (Dixon & Webb, 1960). The collagenases produced by *Clostridium histolyticum* were the first to be recognised and fully studied. The culture filtrate of *C. histolyticum* may include a mixture of collagenases and other proteinases with potent hydrolytic activity against connective tissue. Because of this, crude preparations derived from this combination are the most widely used enzymatic products and are now the recommended reagents for tissue dissociation studies (Kocholaty et al., 1938).

Collagenase is a proteolytic enzyme, and collagen is the most representative protein among the waste products from leather (Kanth et al., 2008). Without influencing the other proteins, collagenase breaks down natural collagen into tiny peptide pieces. Numerous fields have found use for collagenase, including wound healing, diabetic ulcers, surgery for burns and arterial ulcers, etc.

The intricate structural arrangement of collagen sets it apart. Three parallel polypeptide strands in a left-handed, polyproline II-type (PPII) helical conformation wrap around one another with a stagger of one residue to produce a right-handed triple helix. Gly must appear every third residue due to the tight packing of PPII helices in the triple helix. This repetition is present in all types of collagen, however nonfibrillar collagens have specific locations where it is broken in their triple-helical domain. The amino acids (2S)-proline (Pro, 28%) and (2S,4R)-4-hydroxyproline (Hyp, 38%) are often found in collagen at the Xaa and Yaa sites, respectively (Alipour et al., 2016).

Exogenous collagenolytic enzymes and matrix metalloproteases (MMPs) are essential for the treatment of fibrotic and collagen-related illnesses. Even though the body manufactures MMPs to preserve connective tissue, pathological disorders frequently require medical intervention. Beef can be a source of very potent collagenases that can hydrolyse human collagen without damaging other tissues and working in a wide pH range (Lovejoy et al., 1994).

MATERIALS AND METHODS

Sample collection

Beef sample was collected from shop of beef market from Shadman Market, Lahore The samples was collected by using sterile forceps and was carefully placed in a sterile plastic bag. The sample was brought to laboratory and stored at 4°C for further use.

Isolation and Screening of Collagenase Producing Bacteria

Gelatin agar medium was prepared containing 1g gelatin, 0.5g glucose, 0.1yeast extract, 0.7g dipotassium hydrogen phosphate(K^2HPO^4), 0.2g potassium dihydrogen phosphate (KH^2PO^4), 0.02g magnesium sulphate heptahydrate ($MgSO^4$. $7H^2O$), 0.02g calcium

chloride dehydrate ($CaCl^2 \cdot 2H^2O$) and 1.5g agar. The pH was adjusted at 7.5. The sample was dipped in test tube containing 5 ml distilled water and then spread on gelatin agar medium plates. The plates were incubated at 37°C for 24 hours. After incubation, isolated colonies were streaked on fresh gelatinase agar medium plates. The colonies were screened by TCA solution and clear zones around colonies were observed (Hisano et al., 1989).

Assay of enzyme activity

Submerged fermentation was performed by inoculating colony to test tube containing 5ml of LB media and incubation was done at 37°C for 24 hrs. After 24hrs, 0.1% of inoculum was added in gelatin broth and incubated at 37°C for 48hrs. It was centrifuged at 10000 rpm for 20-25 mins. Enzyme assay was done according to the method of Trans and Nagano. The assay was done for supernatant as well as pellet. The blank (without gelatin) as also run side by side.

The reaction mixture was composed of 0.3 ml of 0.2% gelatin in water, 0.2 ml of 150 mM Tris-HCl (pH 7.5) containing 12 mM $CaCl^2$, and 0.1 ml of enzyme. The reaction mixture was allowed to incubate at 30 ºC for 30 minutes before being stopped with the addition of 0.6 ml of 0.1 M HCl. The free amino acids were measured by Ninhydrin method (Rosen, 1957).

Identification of strain

The culture obtained was needed to identified. Different biochemical tests were performed such as Gram Staining and Endospore Staining.

Optimization of different conditions

To find the optimal activity of bacteria produced from beef was determined by optimizing different environmental and other factors such as pH, temperature, time of incubation, effect of metal ion, effect of carbon sources, effect of substrate concentration and effect of nitrogen sources (Koocheki et al., 2009).

Effect of pH

The effects of different pH ranges on enzyme actiity were measured at pH4, pH5, pH6, pH7,and pH8. 0.1M NaOH and 0.1M HCl were added to the pH to adjust the levels of acidity and alkalinity (Lima et al., 2009).

Effect of temperature

The impact of various temperatures on bacterial growth was analysed in 5 ml of culture medium at 25°C, 30°C, 37°C, 40°C, 45°C, and 50°C. Next, bacterial activity was recorded that produced collagenase (Lima et al., 2009).

Time of incubation

Examination was done to see how the incubation duration affected the output of bacterial growth at 24, 48, 72, and 96 hours. Various growth rates were noted at different incubation durations in 5 ml of broth (Lima et al., 2011).

Effects of metal ion

By adding 5 ml of broth along with the 1% metal ion, the different metal ions, such as calcium chloride, sodium chloride, manganese chloride, magnesium sulphate, and iron sulphate, were optimized. After the culture was inoculated at 37°C for 24 hours at 120 rpm, the medium was autoclaved and placed in a shaking incubator (Bhagwat et al., 2015).

Effects of carbon sources

By adding 5ml of broth along with 1% carbon sources, the effects of several carbon sources, such as glucose, maltose, lactose, sucrose, and starch, were optimised. Following a 24-hour incubation period at 120 rpm and 37°C, the medium was autoclaved and placed in a shaking incubator (Muralidhar et al., 2001).

Effect of nitrogen sources

By adding 5 ml of broth along with 1% nitrogen sources, the effects of various nitrogen sources, such as beef extract, yeast extract, casein, ammonium hydrogen citrate, and peptone, were optimised. After the culture was inoculated at 37°C for 24 hours at 120 rpm, the medium was autoclaved and placed in a shaking incubator (Hamdy, 2008).

Effect of substrate concentration

The impact of varying gelatin concentrations on the proliferation of the collagenase enzyme was measured by including 5 ml of broth with 0.5%, 1%, 1.5%, and 2% concentrations of gelatin. Enzyme activity values were recorded (Bhagwat et al., 2015).

Bioinformatics analysis

After obtaining the bacterial genome sequence, it was translated into a protein sequence using a sequence massager and translate tool. Predict protein tool was used to obtain the secondary structure of the protein and its composition was examined. Phyre2 and Swiss Model were used to get the 3D structure of the protein and the percentage of alpha and beta strands in the protein (Rani & Pooja, 2018).

RESULTS

Screening of collagenase producing bacteria

After the streaking of isolated coloies on gelatinase agar medium, it was flooded with TCA. Clear zones containing collagenase-producing bacteria were visible against the medium when plates were flooded with TCA solution.

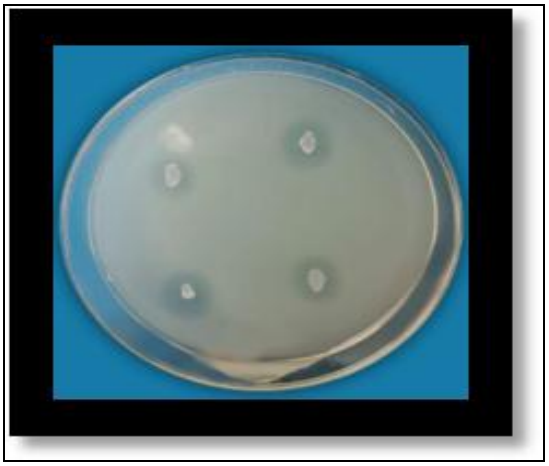

Figure 1: Collagenase-producing bacteria reveal the clear zone by TCA.

Bacterial Isolates

The bacterial colonies were obtained from gelatin agar plates and streaked using a sterilized loop. Pure bacterial isolates were produced using the streaking method, as seen in the image below.

Figure 2: Pure colony of bacteria obtained after 2nd time streaking.

Identification of bacteria strain

Gram Staining

The purple color after gram staining indicated gram-positive bacteria. The glass slide's color was examined under a microscope, and the results are displayed.

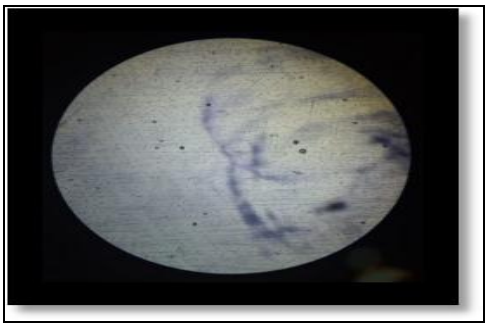

Figure 3: Visual representation of gram staining under microscope.

Endospore Staining

The test was carried out with the chemicals Malachite and Safranin. The bacteria reacted with the chemicals and gave bright green colonies which confirmed the presence of endospores in bacteria.

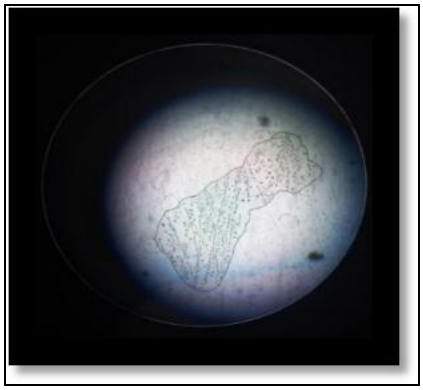

Figure 4: Visual Representation of endospore staining under microscope.

Optimization of Conditions for The Production of Collagenase Enzyme

The effects of different temperatures, incubation periods, pH levels, metal ions, carbon sources, and nitrogen sources on the crude collagenase enzyme's activity were determined.

Effect of pH

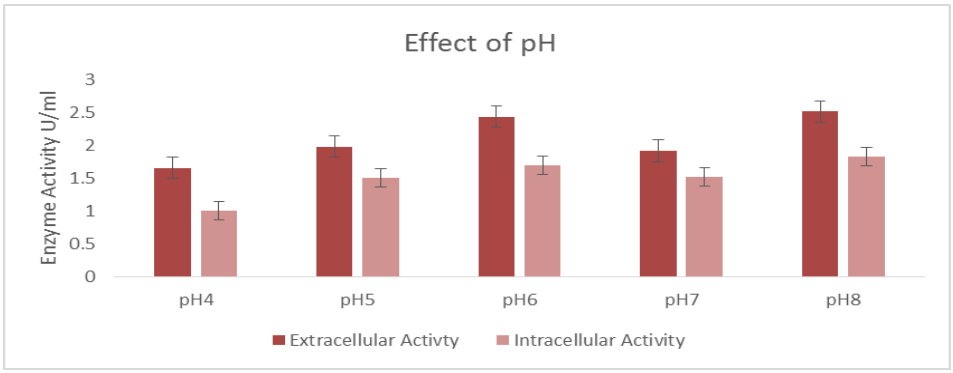

Figure 5: Graphical representation of different pH ranges. In this range bacteria at pH 8 show high enzyme activity as compared to other range.

Effect of time of incubation

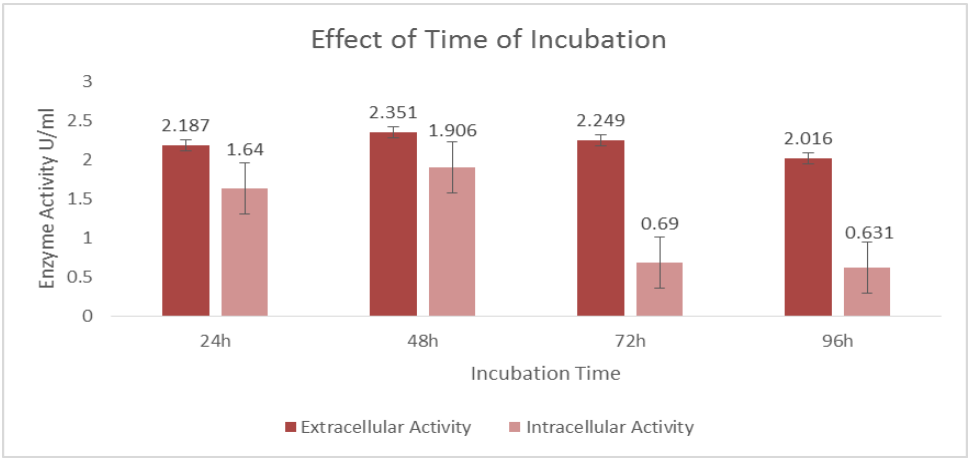

Figure 6: Graphical representation of effect of time of incubation on enzyme activity. The graph indicates that maximun activity is recorded at incubation time of 48h.

Effect of Nitrogen Sources

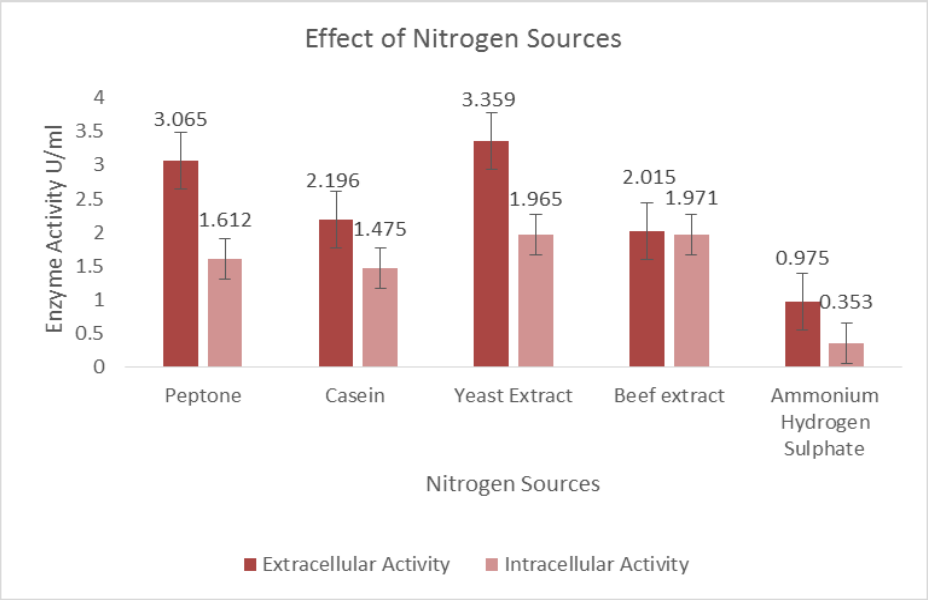

Figure 7: Graphical representation of enzyme activity at different nitrogen sources. In these sources, yeast extract shows high enzyme activity as compared to other sources.

Effect of carbon sources

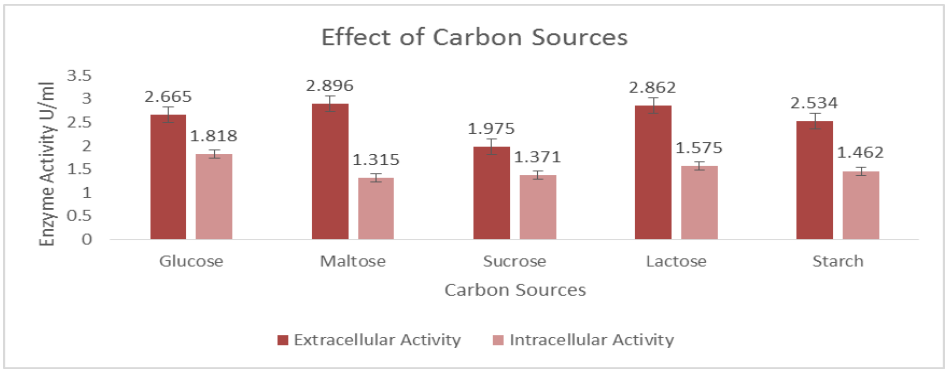

Figure 8: Graphical representation of enzyme activity at different carbon sources.

Effect of metal ions

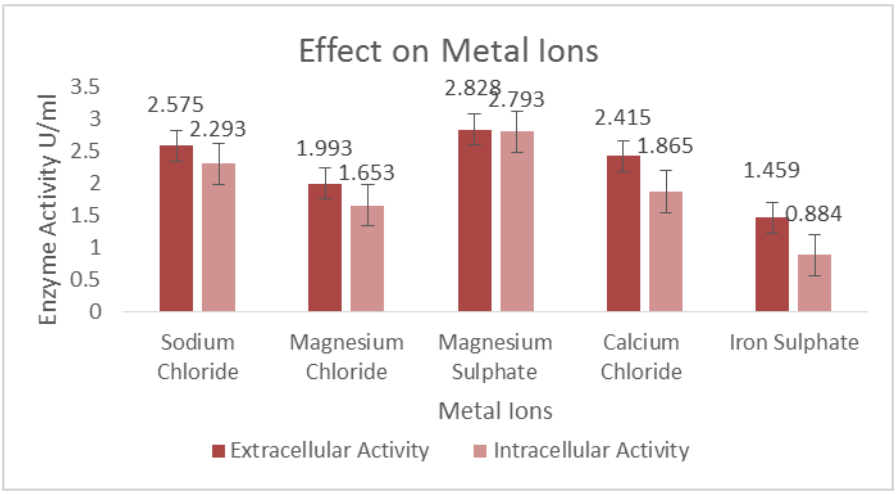

Figure 9: Graphical representation of enzyme activity with different metal ions. Highest enzyme activity is recorded by magnesium sulphate.

Effect of Substrate Concentration

The highest extracellular activity was shown at 2% which was2.953 U/ml and highest intracellular activity was also at 2% which was 2.490 U/ml.

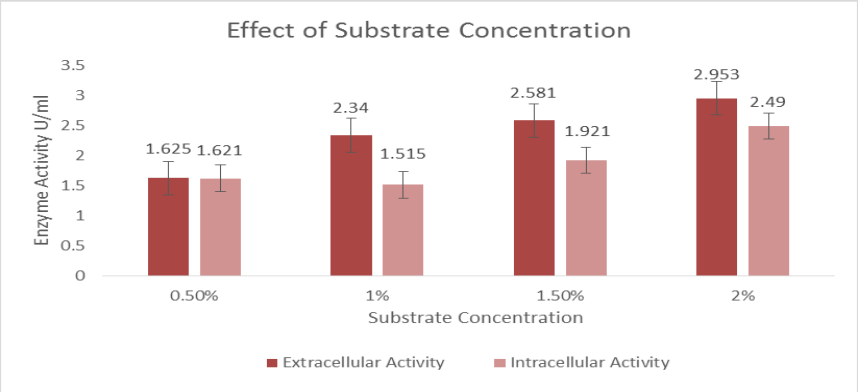

Figure 10: Graphical representation of enzyme activity with different substrate concentrations. Maximum activity was recorded at concentration of 2%.

Scanning electron microscopy

The image shows a scanning electron microscope (SEM) image at 5,020x magnification, with a width of 22.77 micrometers.

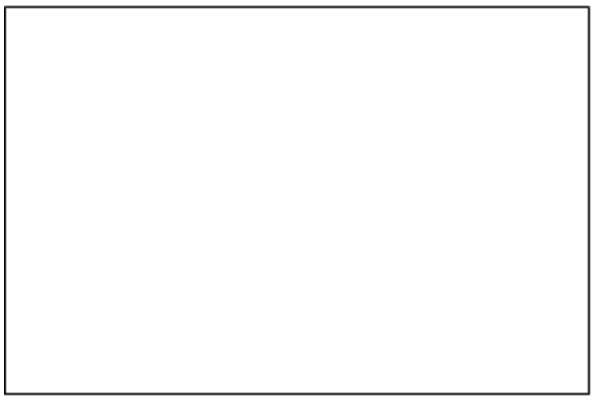

Figure 11: SEM image showing at5,020x magnification.

Bioinformatics analysis

FASTA Sequence from NCBI

Figure 12: FASTA sequence of Bacillus cereus strain R75E collagenase gene, complete codons.

Massager triplets

ATG AAC AAG AAA TCA AAG ATC AAT AAA GTG ATG CTT AGC ATT AGT ACA ATG GCT TTA TCG TTA GGC GCA CTT
CAA ACT CAT GCA GCA GCG GAA GAA AAA GTA CCG TAT AAC GTG TTA AAA ACG AAA CCG GTT GGA ATT GAA AAG
TCG GTA GAT GAA GTT GGA CAT ATT TCA AAA GTT GAT GAA ACT TTA TCA TTT CAA GAA CGT TTA AAA GTA GGA
GAT TTT TCA CAG CGA CCA GCA TCT ATT ACG AAG AAA ACT GCA GTA AAG CAG GTT AAA GAA AGC TAT TCA ATG
GCT GAT TTA AAC AAA ATG AAT GAC CAA GAA TTA GTT GAA ACG TTA GGC AGT ATT AAA TGG CAC CAA ATT ACA
GAC TTA TTC CAG TTT AAT GAA GAT GCA AAG GCT TTT TAT AAA GAT AAA GGA AAA ATG CAA GTC ATT ATA GAT
GAA TTA GCT CAT AGA GGT AGT ACA TTT ACG AAA GAT GAT TCA AAA GGA ATT CAA ACG TTT ACT GAA GTG CTG
CGT TCA GCT TTT TAT CTT GCA TTT TAT AAT AGT GAA TTA AGC GAC TTA AAT GAA AGA AGC TTC CAG GAT AAA TGT
TTA CCT GCT TTA AAA GCA ATC GCA AAA AAT CCA AAC TTT AAG CTT GGT ACA GTT GAA CAA GAT ACA GTC GTA
TCT GCA TAC GGT AAA TTA ATT AGT AAT GCT TCA AGC GAT GTT GAA ACG GTT CAA TAC GCA TCG AAT ATT TTA
AAG CAA TAC AAT GAT AAT TAT ACT ACT TAT GTA AAT GAT CGA ATG AAG GGA CAA GCA ATA TAC GAT ATT ATG
CAA GGT ATT GAC TAT GAT ATA CAG TCG TAC TTA ACT GAG GCT CGT AAA GAA GCG AAT GAA ACG ATG TGG TAT
GGA AAA GTA GAT GGG TTT ATT AAT GAA ATA AAT CGT ATT GCT CTT CTA AAT GAA GTA ACG TCA GAA AAT AAA
TGG CTC GTT AAT AAT GGA ATT TAT TTT GCT AGC CGT TTA GGG AAG TTT CAT AGC AAT CCA AAT AAA GGA TTA
GAG GTT GTT ACA CAA GCA ATG CAT ATG TAC CCG CGC TTA AGT GAA CCG TAT TTT GTT GCG GTA GAA CAA ATT
ACA ACA AAT TAT AAT GGT AAA GAT TAT AGC GGG AAT ACA GTA GAT TTA GAG AAA ATA CGT AAA GAA GGA AAA
GAG CAA TAC TTA CCA AAA ACG TAT ACA TTC GAC GAT GGA TCA ATT GTG TTC AAA ACA GGA GAT AAA GTA TCA
GAA GAA AAA ATT AAG AGA CTA TAT TGG GCT GCG AAG GAA GTA AAG GCA CAG TAT CAC CGT GTA ATT GGA AAT
GAC AAA GCG TTA GAG CCA GGA AAT GCG GAT GAT GTA TTA ACG ATC GTA ATT TAT AAT AGT CCA GAT GAA TAT
CAG TTA AAT AGA CAA TTG TAT GGA TAT GAA ACA AAC AAC GGT GGA ATT TAT ATT GAA GAG ACA GGT ACA TTC
TTT ACA TAT GAG CGT ACA CCA GAG CAA AGT ATT TAT AGT TTA GAA GAG TTA TTC CGT CAT GAA TTT ACT CAT TAT
CTG CAA GGG AGA TAT GAA GTT CCT GGT TTA TTT GGA AGA GGA GAT ATG TAT CAA AAT GAA AGG CTA ACT TGG
TTC CAA GAA GGA AAT GCA GAG TTT TTC GCA GGA TCT ACT CGT ACG AAT AAC GTT GTA CCA AGA AAG AGT ATA
ATT AGC GGA TTA TCA TCT GAT CCT GCA AGC CGT TAT ACA GCA GAG CGT ACA CTA TTT GCT AAA TAC GGT TCT
TGG GAT TTC TAT AAT TAC TCG TTC GCA TTG CAG TCT TAC TTA TAT ACG CAT CAG TTT GAA ACA TTT GAT AAA ATT
CAA GAT TTG ATT CGT GCG AAT GAC GTG AAA AAT TAT GAT GCA TAT CGT GAA AAT CTA AGT AAA GAT CCT AAG
TTA AAT AAA GAG TAT CAA GAG TAT ATG CAG CAG TTA ATT GAT AAT CAA GAT AAA TAT AAT GTA CCG GAA GTA
GCA GAT GAT TAT TTA GCT GAA CAT GCA CCG AAA TCG TTA ACT GAA GTG AAA AAA GAA ATT AGT GAT ACG TTG
CCT ATG AAT GAT ACA AAA ATG ACA AAA CAT AAT TCT CAA TTC TTT AAT ACA TTT ACA TTA GAA GGT ACG TAT
ACA GGT AGT GTC ACA AAA GGT GAA TCA GAA GAT TGG AAA GCA ATG AGT AAA AGA GTA AAT GAA TCT TTA GAA
CAA TTG GCG CAA AAA GAA TGG AGT GGC TAC AAA ACT GTT ACA GCA TAC TTC GTC AAT TAT CGT GTG AAT AGC
TCA AAT GAA TTT GAA TAT GAT GTA GTC TTC CAT GGA ATC GCA AAA GAT GAT GGA GAA AAT AAA GCT CCA ACG
GTT AAT ATA AAT GGC CCT TAT AGC GGT CTT GTA AAA GAG GGA ATT CAA TTT AAA AGT GAT GGC TCA AAC GAT
GAA GAT GGA AAA ATT GTT TCT TAT TTA TGG GAA TTT GGA GAT GGA AGC ACA AGT GCA GAA GTG AAT CCA GTA
CAT GTA TAT GAA AGA GAA GGT TCT TAT AAA GTA TCG TTA AGA GTA AAA GAT GAT AAG GGA AAA GAG AGC AGA
AGC GAA ACA ACT GTT ACG ATT AAA GAT GGA AGT TTA ACA GAA TCA GAA CCA AAT AAT CGT CCA GAG GAA GCA
AAT CGT ATC GGG CTA AAT AGT ACG ATA AAA GGT AGT CTT ATT GGC GGG GAC CAC ACT GAT GTT TAT ACA TTT
AAT GTA GCA TCA GCG AAA GAT ATC GAC ATT TCT GTT TTA AAT GAG TAT GGA ATT GGG ATG ACA TGG GTA CTT
CAC CAT GAA TCA GAT ATG CAA AAT TAT GCA GCT TAC GGT CAA GCT AAT GGG AAT CAT ATA GAA GCA AAA TTT
AAT GCA AAA CCA GGT AAG TAT TAC TTG TAT GTA TAT AAA TAT GAT AAT GGC GAT GGA ACA TAC GAA TTG TCA
GTA AAA TAA

Figure 13: Massager triplets of collagenase sequence using "Massager triplet" tool.

Codons and their respective amino acids

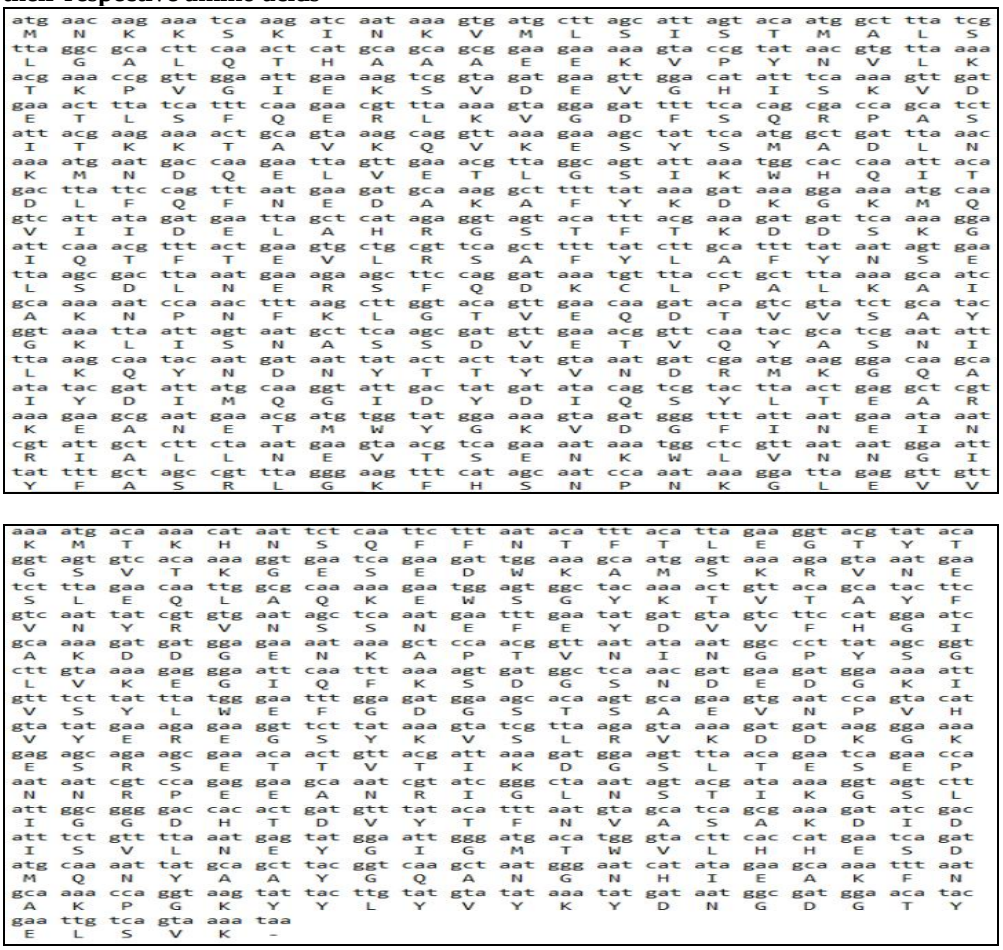

Figure 14: Codons and there representive amino acids using the Expassy tool.

Protein sequence of Bacillus cereus strain R75E collagenase gene

MNKKSKINKVMLSISTMALSLGALQTHAAAEEKVPYNVLKTKPVGIEKSVDEVGHISKVDETLSFQERLKVGDFSQRPASITKKTA
VKQVKESYSMADLNKMNDQELVETLGSIKWHQITDLFQFNEDAKAFYKDKGKMQVIIDELAHRGSTFTKDDSKGIQTFTEVLRSA
FYLAFYNSELSDLNERSFQDKCLPALKAIAKNPNFKLGTVEQDTVVSAYGKLISNASSDVETVQYASNILKQYNDNYTTYVNDRM
KGQAIYDIMQGIDYDIQSYLTEARKEANETMWYGKVDGFINEINRIALLNEVTSENKWLVNNGIYFASRLGKFHSNPNKGLEVVTQ
AMHMYPRLSEPYFVAVEQITTNYNGKDYSGNTVDLEKIRKEGKEQYLPKTYTFDDGSIVFKTGDKVSEEKIKRLYWAAKEVKAQY
HRVIGNDKALEPGNADDVLTIVIYNSPDEYQLNRQLYGYETNNGGIYIEETGTFFTYERTPEQSIYSLEELFRHEFTHYLQGRYEVPG
LFGRGDMYQNERLTWFQEGNAEFFAGSTRTNNVVPRKSIISGLSSDPASRYTAERTLFAKYGSWDFYNYSFALQSYLYTHQFETFD
KIQDLIRANDVKNYDAYRENLSKDPKLNKEYQEYMQQLIDNQDKYNVPEVADDYLAEHAPKSLTEVKKEISDTLPMNDTKMTKH
NSQFFNTFTLEGTYTGSVTKGESEDWKAMSKRVNESLEQLAQKEWSGYKTVTAYFVNYRVNSSNEFEYDVVFHGIAKDDGENKA
PTVNINGPYSGLVKEGIQFKSDGSNDEDGKIVSYLWEFGDGSTSAEVNPVHVYEREGSYKVSLRVKDDKGKESRSETTVTIKDGSLT
ESEPNNRPEEANRIGLNSTIKGSLIGGDHTDVYTFNVASAKDIDISVLNEYGIGMTWVLHHESDMQNYAAYGQANGNHIEAKFNAK
PGKYYLYVYKYDNGDGTYELSVK

Figure 15: Protein sequence of *Bacillus cereus* strain R75E collagenase gene.

Secondary structure prediction

For secondary structure analysis, predict protein was used and the structure of the enzyme was analysed. This tool predicts the presence of alpha helix by symbol H, beta strand by E, coils by C, and loops by L.

The percentage of each type in the protein is given as follows:

Table 1: Predicted secondary structure composition.

Sec structure type	H	E	L
% in protein	33.26	16.48	50.26

Residue composition for protein

This table details the residue composition percentages for various amino acids in a protein. Each cell represents the percentage of a specific amino acid present in the protein structure, denoted by their single-letter codes.

%A: 5.8	%C: 0.1	%D: 6.4	%E: 8.2	%F: 4.2
%G: 6.5	%H: 1.9	%I: 5.1	%K: 8.4	%L: 6.6
%M: 1.9	%N: 6.9	%P: 2.6	%Q: 4.0	%R: 3.3
%S: 7.5	%T: 6.5	%V: 6.4	%W: 1.0	%Y: 6.6

Figure 16: Residue composition of Protein.

DISCUSSION

For research purposes, collagenase-producing bacteria were isolated from beef and allowed to grow on gelatin agar plates. The pure colony was created and cultured for 24 and 48 hours. The colony incubated for 24 hours showed moderate results while higher growth was observed after 48 hours of incubation. The colonies were further purified by the streaking method. Screening was done using TCA, and the zones of hydrolysis were observed, indicating collagenase production. This method is consistent with the work of (Wu et al., 2010), who also utilized gelatin agar for effective isolation and screening of collagenase-producing bacteria, observing clear zones of hydrolysis as a confirmation of enzyme production.

The morphological analysis in our study revealed two distinct colony types: small, non-sticky colonies, and large, sticky colonies. This observation is comparable to the findings of (Thapa et al., 2021), who reported similar morphological distinctions in bacterial colonies isolated for enzyme production, noting that the larger, sticky colonies often correlated with higher extracellular enzyme activity.

The biochemical tests, including Gram staining, identified the bacteria as Gram-positive., (KIME TATH, 2020) also found that Gram-positive bacteria, particularly Bacillus species, are prolific producers of collagenase. The endospore staining in our study further supported the likelihood of Bacillus species presence, which is known for its enzyme production capabilities, corroborating findings by (Shafi et al., 2017).

Our study demonstrated that the maximum collagenase activity occurred at pH 8, with extracellular activity reaching 2.513 U/ml. This finding is supported by the work of (Hamdy, 2008), who reported optimal collagenase activity at slightly alkaline pH levels, specifically around pH 8 to 9. This pH range is conducive to the stability and activity of many collagenases, indicating a common characteristic among different strains.

The highest enzyme activity in our study was observed at 48 hours of incubation, with extracellular activity at 2.351 U/ml. The study reported by swarnalatha showed that maximum collagenase production by *Bacillus subtilis* occurred within 48 to 72 hours of incubation (Shahzad et al., 2015). The incubation period is critical

as it impacts the yield and efficiency of enzyme production.

Our results indicated that peptone and beef extract significantly enhanced enzyme activity, with peptone showing the highest extracellular activity at 3.065 U/ml. These results are consistent with those reported by (Fedoryuk & Shamtsyan, 2014), who noted that organic nitrogen sources like peptone and beef extract were effective in promoting collagenase production in Bacillus species.

Among various carbon sources tested, maltose and glucose were the most effective, with maltose showing the highest extracellular activity at 2.896 U/ml. This is similar to findings by Joo *et al*. (2003), who reported that disaccharides like maltose and monosaccharides like glucose were optimal for collagenase production in Clostridium histolyticum (Fedoryuk & Shamtsyan, 2014).

Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) provided detailed structural and chemical information about the bacterial samples. SEM confirmed the rod shape of the bacteria, identifying them as *Bacillus* sp. Further structural analysis using the Swiss model and Phyre2 tools provided 3D models of the collagenase enzyme. The Swiss model predicted the protein as a monomer with full sequence coverage and a sequence similarity of 0.61 using AlphaFold v2. Phyre2 modeled 69% of the sequence with 100.0% confidence, revealing 24% alpha helices and 18% beta strands in the structure, similar to the approach used by (Rani & Pooja, 2018) to elucidate features of enzyme-producing bacterial strains.

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