

# Caseinase Production and Media Optimization from *Bacillus subtilis*

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**Abstract.** Caseinase is involved in the breakdown of milk protein casein and converts casein into smaller simple sugars which can be easily utilized by the body for the production of ATP and Fat. Casein can be an instant energy source to the body and involves in muscle building. Caseinase enzyme can be extensively used at the industrial scale for Milk, Textile, Dairy, Paper industry and several other medical purposes. In view of the importance of caseinase, the current research deals with the isolation and identification of caseinase producing bacteria from soil. This is followed by the production of enzyme and its purification. The study also includes its kinetic characterization using the parameters Temperature, pH as well as Carbon and Nitrogen Sources. The organism which was isolated from soil and capable of producing the caseinase enzyme was identified to be *Bacillus subtilis* based on the Biochemical tests and 16S rRNA sequencing result. The optimal carbon and nitrogen sources were identified to be Glucose and casein respectively. Regarding the optimal conditions, the suitable temperature for maximum enzyme production was found to be 40 °C and pH was 9. When the organism was cultured under the optimal condition using casein as a nitrogen source and glucose as the carbon source, at 40 °C and pH 9, 1590 ng/mL of enzyme production was estimated.

**Keywords:** Caseinase, *Bacillus subtilis*, kinetic characterization, 16S rRNA sequencing

## 1. Introduction

Casein is the major protein of the milk constituting for about 90% of its composition. It has extensive nutritional value. This is the major protein present in the dairy products like cheese, paneer etc. Caseinase, a natural exoenzyme secreted by several bacteria, hydrolyzes the casein protein and converts it to simple soluble form. Several groups of bacteria are known for their ability to release the enzyme caseinase thus utilizing the casein protein [1]. Several studies were conducted even at the genetic level to extract the gene encoding for caseinase activity. microbial proteases as industrial catalysts offer advantages over the use of conventional chemical catalysts for economically viable, exhibiting high catalytic activity, high degree of substrate specificity, and can be produced in huge amounts etc. [2]. Bacteria appear to be relatively simple life forms. In fact, they are bodies with an incredible degree of adaptation, which involves complexity. Many bacteria multiply to very high speeds, and different species may use as food a huge variety of organic substances [3].

One such study was carried on by Blaschek and Solberg, who published their work in The Journal of Bacteriology [4]. According to their research *Clostridium perfringens* strain ATCC 3626B was cured of caseinase activity at a high frequency after treatment with acriflavine dye (2.5%) or elevated temperature growth (9.1%). The team has done a genetic analysis to isolate the plasmid encoding for caseinase activity. Another study was related to the hemolytic activity of Bacterial species, a fish pathogen *Aeromonas salmonicida*, based on its caseinase enzyme. This study was conducted by Titball and published in the Journal of Infection and Immunity [5]. In this study, it was analyzed that the mutants of this bacteria which were incapable to utilize the casein protein also lost their ability to perform haemolysis of horse blood, showing an association between the two genes.

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In most of the studies caseinase gene can be used as a marker for the identification of various gene traits that occur together with this gene. Esp gene carriage in enterococci occurs together with the caseinase gene. This special property has been used to develop a PCR independent assay for esp gene based on the study of caseinase gene [6]. This study was reported in AIP Conference Proceedings 1571, 202 (2013). For the identification of these caseinase positive traits casein agar has been employed. Organisms like *Streptomyces*, *Pseudomonas*, and *Actinomadura* spp. which can hydrolyse casein will produce a clear halo in the surrounding medium [7], indicating the utilization of casein from the media.

## 2. Materials and methods

### 2.1. Sample Collection

Since soil is known to possess a wide variety of bacteria in which the possibility of obtaining the required bacteria is high, it is selected as the source for the isolation of organisms [8]. Those organisms that are cable of utilizing the casein protein by releasing caseinase are required for the study. Sub surface soil has been collected from the ground near the root system of the plants. The soil is later subjected for serial dilution and plating.

### 2.2. Bacterial Isolation

The 10 fold dilution technique [9] can be used for serial dilution. Nutrient agar medium containing the basic carbon source as glucose, minerals and micronutrients as NaCl and yeast extract, amino acids in the form of peptone was prepared for plating using the standard composition. Agar-Agar was used as a solidification agent. Spread plate technique was to be employed to obtain all species of bacteria onto a common plate as isolated colonies. The spread plates were incubated for 24 h at 37°C in an incubator for the microbial growth. For obtaining the pure culture 100 mL of Nutrient Agar media was once again prepared and autoclaved. From the master plate four morphologically different colonies were picked and inoculated on four petri plates by quadrant streaking [10].

### 2.3. Screening for Caseinase Production

Once all the bacteria have been isolated as pure cultures they need to be tested for the caseinase activity. The special media have to be used for this purpose. The media would contain all the components similar to nutrient agar media except for the carbon source. Rather than using Glucose as the carbon source. Casein has to be used in this screening [11], Abou El-Hassayeb and Abdel Aziz explained that the addition other sources of carbon except starch in the production medium caused remarkable decreases in protease production by *B. subtilis* [12]. Only those organisms which can utilize casein as the carbon source can grow in the screening media, as here is no other simple carbon source which can be utilized. Carbon source plays important role in the enzyme production and different bacteria utilized different C-source for their growth and metabolism [13]. Thus the media would allow the growth of selectively those organisms which are capable of producing caseinase. Thus a screening media plate would contain only those selected bacteria which possess the required quality which is tested for. These bacteria are to be further subcultured so as to obtain pure cultures of the positive bacteria, Positive caseinolytic activity was detected by the formation of a turbidity zone (white halo) surrounding the well (12 mm or more) [14].

### 2.4. Identification of Caseinase Producing Bacteria

The basic protocol and the first step for the identification of any bacterial species is the Gram's staining [15]. The bacterium that has been selected for its capability to produce caseinase enzyme has to be identified for further study. Gram staining was performed for the sample and the results were analyzed. Furthermore, Bergey's Manual [16] has been used for the various biochemical tests that have to be performed in series for the identification of the bacteria. Based on the nature of gram's stain the bacteria can be subjected for various biochemical tests. The biochemical tests performed for caseinase

positive bacteria were Starch Hydrolysis Test [17], Voges Proskauer test [18], simmon's citrate test [19] and growth in 6.5% NaCl. All the results are recorded before processing for the next test in the series.

## 2.5. Molecular Characterization for Bacterial Identification

Following procedure was adopted for Bacterial identification by 16S rRNA Sequencing:

### 2.5.1. Extraction of DNA from Bacteria

The cell suspension was made from the pure culture for DNA extraction. In this protocol lysis buffer containing Tris HCl, EDTA, NaCl, proteinase K and SDS [20] is initially added to break down the cell wall and expose the internal cell contents outside. Centrifugation helped to separate the cell contents from one another. Ethanol precipitation assisted in the separation of DNA from the solution. The separated DNA is further purified by ethanol wash and preserved at 4°C.

### 2.5.2. Agarose Gel Electrophoresis

In order to confirm the presence of DNA and its quality the separated DNA was subjected to electrophoresis in 0.8% Agarose Gel [21]. Here the tracking dye used was Bromophenol blue [22] and Ethidium bromide [23] was used for visualizing the DNA under UV. The gel was run for 30 min at 100 V and observed under the UV Trans illuminator.

### 2.5.3. Amplification and Sequencing

In order to identify the bacteria at the molecular level amplification was performed using polymerase chain reaction [24] to obtain amplicons which can be used for sequencing.

**Note:** The primers used for the amplification of 16S rRNA gene.

- F      AGA GTT TGA TCC TGG CTC AG
- R      ACG GCT ACC TTG TTA CGA CTT

**PCR Conditions which are required for amplification:**

1 cycle: 94°C for 5 min (Initial denaturing)

35 cycles: 94°C for 30 s (denaturing)

55°C for 30 s (annealing)

72°C for 30 s (extension)

1 Cycle: 72°C for 7 min (final extension)

Amplified PCR product was subjected to electrophoresis using 1% Agarose in TAE buffer and 1 kb ladder as marker and visualized by staining with ethidium bromide. 3730XL ABI sequencer, an automated sequencing device [25] based on capillary electrophoresis has been used for reading the sequence of the amplicons provided.

### 2.5.4. BLAST for Sequence Identification

The similarity search of the obtained sequence was done using BLAST. With the user entered sequence the tool performs a pair wise similarity search based on Needleman - Wunsch algorithm and the sequence is compared against a set of sequences from the database. This comparison with the database sequences yield an insight to the identity of the sequence.

## 2.6. Media Standardization for Enzyme Production

In order to analyze the best media composition for the maximum yield of enzyme various media compositions have been tested. The caseinase producing bacteria was inoculated in media of different carbon sources, the enzyme produced was obtained after centrifugation, OD was measured in spectrophotometer at 595 nm and its concentration was determined by plotting a graph against standard OD vs enzyme concentration. Carbon sources tested were Glucose, Mannitol, Fructose, Lactose and Sucrose. In each case the enzyme produced was further evaluated for its concentration determination. The results are further graphically represented and compared. Similarly a comparative study of various

nitrogen sources on enzyme production was undertaken. Different nitrogen sources tested were Casein, Peptone, Sodium Nitrate, Urea and Ammonium Sulphate. The production of enzyme requires an optimum standard temperature of incubation and *pH*. In the current work the optimum temperature and *pH* required were studies by keeping their varying values.

## 2.7. Purification of the Enzyme Produced

The protein produced above is crude and contains several impurities which have to be removed. Thus the crude extract has to be subjected for several purification techniques to obtain a pure enzyme. Salt Precipitation was performed by adding Ammonium sulphate [26] upto 80% saturation into the crude enzyme under ice cold conditions and incubated at 4°C overnight. This grabs all the protein into its precipitate form that settles as a salt in the bottom of the beaker. After incubation to collect the pure protein the mixture has to be centrifuged at 10,000 rpm for 10 min. The pellet obtained was subjected for dialysis in activated nitrocellulose membranes kept on magnetic stirrer overnight.

## 2.8. Sodium DodecylSulphate Polyacrylamide Gel Electrophoresis

Molecular weight determination by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 3-mm slab gel.

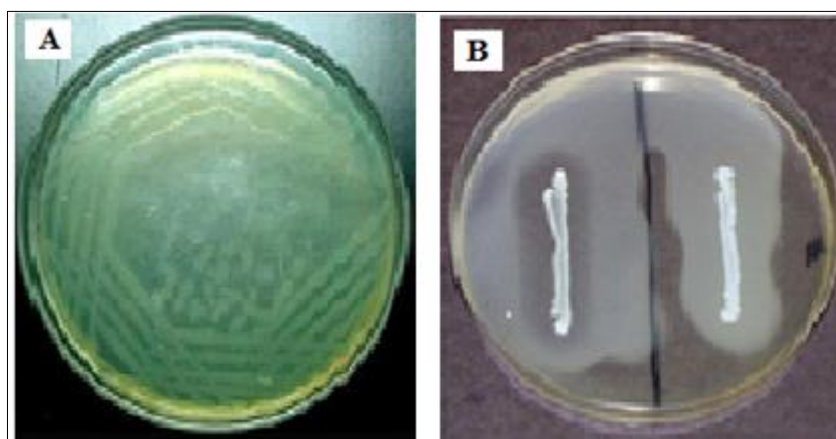
Polyacrylamide separating gel (12%) that consists of 30% acrylamide mixture, 1.5 M Tris-HCl buffer, *pH* 8.8, 10% SDS (w/v), 10% (w/v) ammonium per sulfate and TEMED. It was prepared by mixing 1.68 mL of water, 2 mL of 30% acrylamide mixture, 1.25 mL of 1.5 M Tris-HCl (*pH* 8.8), 50  $\mu$ L of 10 % SDS, 25  $\mu$ L of 10 % ammonium per sulfate and lastly 2.5  $\mu$ L of TEMED to final volume of 5mL.

Stacking gel (4%)- It was prepared by mixing 3.05 mL of water, 665  $\mu$  of 30% acrylamide mixture, 1.25  $\mu$ L of 0.5 M Tris-HCl (*pH* 6.8), 50  $\mu$ L of 10 % SDS, 25  $\mu$ L of 10 % ammonium per sulfate and lastly 5  $\mu$ L of TEMED to final volume of 5 mL. The gels were stained with Coomassie brilliant blue R-250 and destained. The standard proteins with a range of 3-35 KDa weight were used as the marker.

## 3. Results and discussions

### 3.1. Screening for Protease Production

The four pure cultures showed good growth after 24 h of incubation and subjected for screening of caseinase production. Figure 1 shows one of the bacterial isolates showed positive result with a clear transparent zone around the colony. Gelatinase hydrolysis is used as an interchangeable term for caseinase activity [27], where gelatinase is extracellular zinc-endopeptidase that capable for gelatinase hydrolysis, collagen, casein and other active peptides [28].



**Figure 1.** Pure culture and screening results  
**A-** Test culture, *Bacillus* Sample,  
**B-** Demonstration of Negative result in a sample

### 3.2. Biochemical Identification of Bacteria

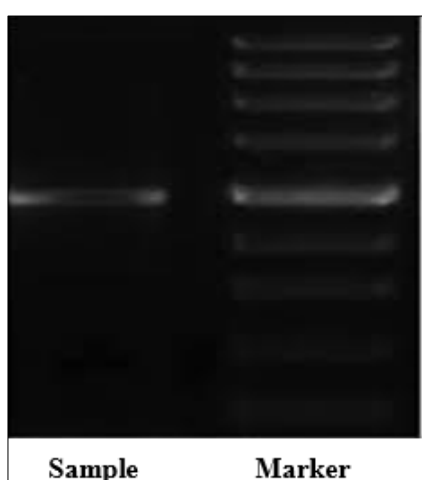
The colony morphology, Gram's staining nature and biochemical tests' response of the positively screened bacteria [29] as shown in the Table 1.

**Table 1.** Biochemical characterization of caseinase producing test bacteria

S. No	Test	Observation
1	Gram's Staining	Gram Positive Bacilli
2	Starch Hydrolysis	Positive for Amylase
3	Voges Proskauer test	Positive
4	Simmon's citrate Test	Positive
5	Growth in 6.5% NaCl	Positive

### 3.3. Amplification and Sequence Similarity

The amplicons obtained for 16S rRNA gene were found to be of approx 1500 bp length as shown in Figure 2.



**Figure 2.** Gel Picture of the amplicon

Figure 3 shows the sequence obtained were identified as of *Bacillus subtilis* strain *DUCC3714* 16S ribosomal RNA gene, partial sequence.

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AAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCTGAATAG
GGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCA
GGCGGTTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTTCATTGGA
AACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCACGTGTAGCGGTGAAA
TGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGTGCTAAGTGTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAA
CGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTG
ACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG.
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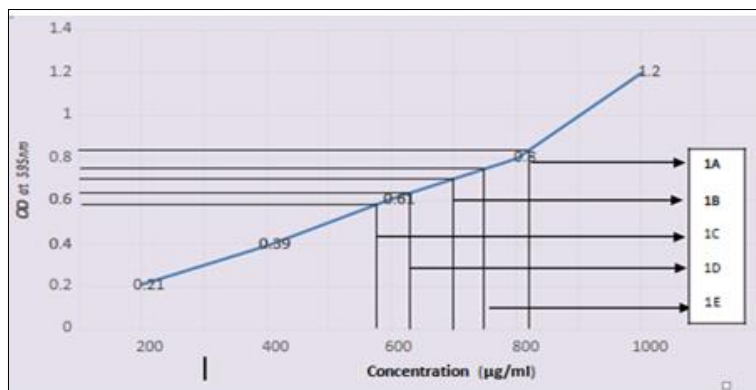
**Note:** The assembled DNA sequence was used to carry out BLAST with the nr database of NCBI.  
 BLAST Reference

Score	Expect	Identities	Gaps	Strand
1035 bits (560)	0.0	560/560(100%)	0/560(0%)	Plus/Plus

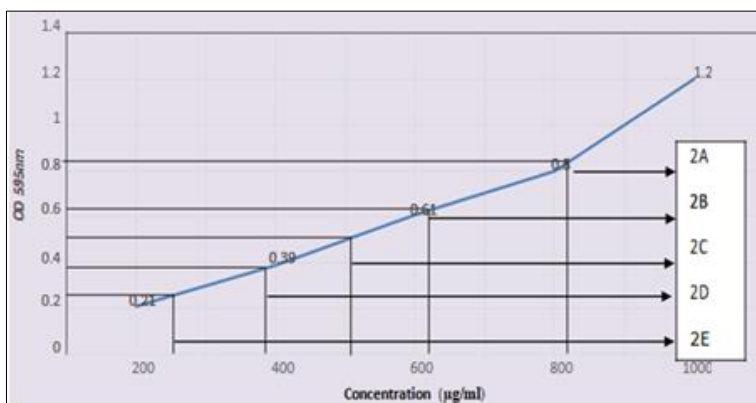
**Figure 3.** Sequence of *Bacillus subtilis* strain *DUCC3714* 16S ribosomal RNA gene, partial sequence

### 3.3. Media Optimization

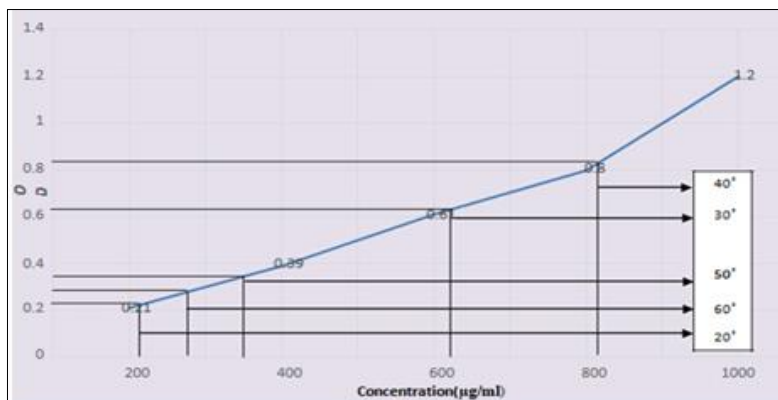
Based on the concentration obtained Figures 4 to 7 it was found that the enzyme yield was highest with Glucose as a carbon source and casein as the nitrogen source indicated by their respective highest OD values. The optimum temperature and pH for the maximum enzyme yield were found to be 40°C and pH 9. Thus, maintaining the above conditions with standard media composition shown in Table 2 would provide the highest yield of caseinase from *Bacillus subtilis* strain DUCC3714.



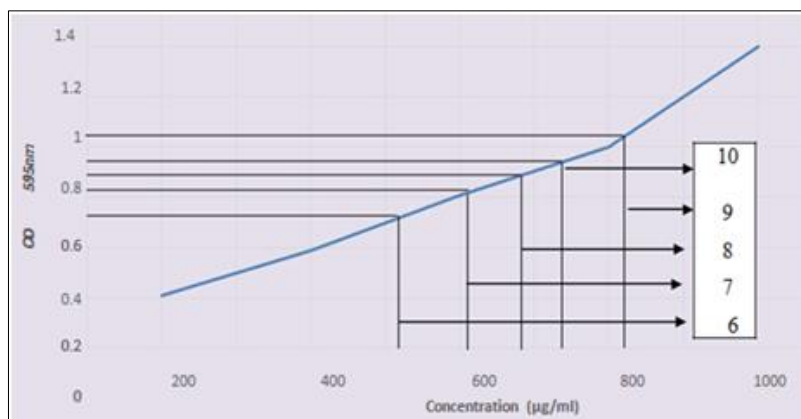
**Figure 4.** Effect of varying carbon sources on enzyme production  
1A=Glucose; 1B= Mannitol; 1C: Lactose; 1D= Sucrose; 1E= Fructose



**Figure 5.** Effect of various nitrogen sources on enzyme Production  
2A: casein, 2B: Peptone, 2C: Sodium nitrate, 2D: Urea, 2E: Ammonium sulphate



**Figure 6.** Effect of various temperature of incubation on enzyme production



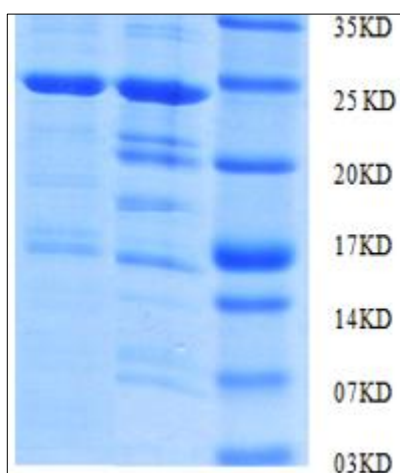
**Figure 7.** Effect of various pH of media on enzyme production

**Table 2.** Standardized media components and conditions for caseinase production from *Bacillus subtilis* strain DUCC3714

S. no.	Constituent	Amount for 100 mL
01	Glucose	0.1gm
03	Yeast extract	0.05gm
03	Casein	0.05gm
04	K <sub>2</sub> HPO <sub>4</sub>	0.01gm
05	MgSO <sub>4</sub>	0.02gm
06	Na <sub>2</sub> CO <sub>3</sub>	0.1gm
07	Distilled water	For dilution upto 100 mL
	Temperature	40 °C
	pH	9

### 3.4. Molecular Weight Determination by SDS

The SDS PAGE picture was shown in Figure 8. Results of our study came resemble with other studies, where *bacillus subtilis* was appeared resemble with strains of *Bacillus thuringiensis* with a variety of enzyme capacities, like protease, amylase, esterase, and chitinases [30].



**Figure 8.** The SDS PAGE picture showing the protein as a thin band in the gel  
 Lane 1: Dialyzed Protein,  
 Lane 2: Crude Protein,  
 Lane 3: Protein marker

#### 4. Conclusions

In the current study a complete analysis of the caseinase enzyme was undertaken which includes, identification of caseinase producers by screening, pure culture preparation, media composition standardization with respect to Nitrogen source and carbon source, standardization of incubation conditions like pH and temperature. The optimum temperature and pH for maximum yield of the enzyme was detected to be 40 °C and pH 9 respectively. The bacteria which were positive for enzyme production was identified using Grams' staining followed by Biochemical characterization and molecular analysis. The bacterium was identified to be *Bacillus subtilis* strain *DUCC3714* using insilico analysis. Thus the above standard composition and incubation conditions can be used for obtaining maximum yield from the bacteria.

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