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# Molecular Characterization and Optimization of Alkaline Protease Production by *Bacillus cereus* LS23B

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

The study aimed to determine the conditions leading to maximum protease production using submerged fermentation and detecting the presence of protease genes in the bacteria. This is necessary to meet the increasing demand for protease enzymes in the industrial market. The ability of the bacterial isolate to produce protease enzymes was evaluated through primary screening. After that, morphological characterization, biochemical tests, and 16S rRNA analysis were done to identify the bacterial strain. To confirm the presence of the gene-encoding enzyme, the protease (*npr*) gene primer was amplified using a polymerase chain reaction. The pH, incubation duration,

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temperature, carbon, and nitrogen sources were studied. Others include metal ions, substrate concentration, and agitation speed. The bacterial strain has 100% similarity to *Bacillus cereus* A9, while the protease encoding gene was confirmed with positive bands of 951bp. The enzyme was optimally produced at 40 $^{o}$C with a pH of 9 after 72 h incubation. Starch, gelatin, 1 % substrate concentration, 1mM $K^{+}$, and agitation speed of 160 rpm fully supported protease production. The presence of the npr gene in the isolate was confirmed. Also, the optimization study reveals that *Bacillus cereus* LS23B can be used in large-scale protease production, which may be used in different biotechnological applications.

*Keywords: Alkaline protease; Bacillus cereus; screening; protease production; culture conditions.*

## 1. INTRODUCTION

Over the years, people have used enzymes daily, highlighting their intrinsic originality as a class of natural catalysts [1]. Proteases are hydrolytic enzymes that perform various physiological tasks in living creatures, including cell differentiation, signaling, inflammation, proliferation, death, hormone processing, protein turnover, gene expression, and blood coagulation [2-4]. Plants, animals, and microbes such as bacteria and fungi all produce proteases. The commercial need for proteases produced by plants and animals is not being met [5]. Furthermore, the US Food and Drug Administration has confirmed the safety of microbial proteases [6,7]. This has heightened interest in using proteases of microbial origin in industrial operations. Different microbial strains are known to produce extracellular protease, among which bacteria species have stood out. Among the bacterial sources, *Bacillus* has been described as the most prominent genus [1]. Actinomycetes, fungi, and bacteria, with a percentage distribution of 8, 11, and 81 %, respectively, have been reported to produce alkaline protease [8]. Aside from *Bacillus* sp., other alkaline protease-producing bacteria have also been reported. The pH range of bacterial alkaline proteases is between 8-12 [9]. Microbial proteases are favorably positioned since they are not affected by climate variations, which invariably means less investment in terms of land utilization and a rise in production rates [10]. The use of microbial strains, especially bacteria species, also makes their genetic manipulation much easier because of the organisms' short generation time and simple genetic makeup [11]. Microorganisms that produce protease have been isolated from habitats such as rotten dried fish, hot springs, soil, leather industry effluents, and industrial wastewater [12-16]. It is easier to isolate and screen for microorganisms with desired characteristics since they are found in different environments [17]. Protease production using submerged fermentation has been reported for *Bacillus circulans* [12] and *Bacillus* sp. DEMO5 [13] and *Bacillus cereus* [15]. There is a need for optimization of several parameters that would boost enzyme production because these microbial strains have distinct growth characteristics. In addition, the demand for protease enzymes in the industrial market keeps increasing, and new strains must be isolated with novel properties. This work is aimed at evaluating cultural and nutritional parameters for maximum enzyme production and also affirming the presence of the gene coding for protease synthesis in the bacterial isolate.

## 2. MATERIALS AND METHODS

### 2.1 Microorganisms

The bacterial isolate used in this work was previously isolated from a soil sample in Ado-Ekiti, Ekiti State, Nigeria.

### 2.2 Screening of Protease-Producing Bacteria

By modifying the method of Lakshmi et al. [18], skim milk agar was sterilized, and the microorganism was spot-inoculated on the agar. This was incubated at 37$^{o}$C for 48 h, after which the plate was observed for a clear zone, and the diameter of the zone was measured.

### 2.3 Identification of the Bacterial Strain

#### 2.3.1 Morphological and biochemical characterization

The bacterial strain was identified using morphological characterization and biochemical tests such as gelatin hydrolysis, catalase, citrate utilization, oxidase, and starch hydrolysis.

#### 2.3.2 Identification using bacterial DNA amplification and sequencing

The genomic DNA of the bacteria strain was recovered and purified according to the manufacturer's instructions using a DNA isolation kit (Promega, USA). The 5′ ends of the 16S rDNA gene were amplified using universal primers (forward primer (8-F) 5′-AGAGTTTGATYMTGGCTCAG-3′) and reverse primer ((1942R) 5′- GGTTACCTTGTTACG ACTT-3') [19]. A GeneAmp polymerase chain reaction (PCR) system 9600 (Applied Biosystems) was used in performing the PCR using the following: Taq Polymerase (1 µL), 10 pM concentrations of forward and reverse primers (1 µL each), sterile deionized water (27 µL), PCR buffer containing dNTPs and $MgCl_2$ (8 µL), and DNA template (2 µL) for a total reaction volume of 40 µL. One (1) cycle at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min, and a final extension at 72 °C for 10 min comprised the cycling program. The PCR product was visualized and subjected to Sanger dideoxy sequencing. The sequence obtained was compared to the GenBank nucleotide database using the essential local alignment search tool (BLAST).

### 2.4 Detection of Protease Gene

The DNA region encoding the protease gene used was (951 bp), with upstream primer npr BcI (59GTAACAGGAACGAATAAAGTAGGAACTGG TAAAG-39) and downstream primer npr BcII (59-GTTTACACCAACAGCACTAAATGATTGCTTAA C-39) [20]. A hot start cycle of 94 °C for 5 min and 80 °C for 4 min was used to initiate the PCR program. This was followed by one cycle of 94 °C for 2 min, 64 °C for 1 min, and 72 °C for 2 min; 30 cycles of 94 °C for 30 sec, 64 °C for 30 sec, and 72 °C for 45 sec; and a final extension at 72 °C for 10 min. A previously prepared 2% agarose gel was used to confirm positive amplification for the gel electrophoresis. The wells were loaded with 10 µL of each PCR product and a one-hundred base pair (bp) DNA ladder. The gel was electrophoresed, visualized, and photographed. The sizes of the PCR products were then estimated.

### 2.5 Production of Enzyme

The medium consisting of (%, w/v): $MgSO_4.7H_2O$ (0.01 g), $FeSO_4$ (0.01 g), $K_2HPO_4$ (0.1 g), casein (0.5 g), glucose (0.5 g), peptone (0.5 g) at pH 7 was sterilized. The sterile medium was inoculated with one mL of twenty-four-hour-old culture adjusted to the 0.5 McFarland standard. This medium was incubated at 37 $^oC$ for 24 h after which it was centrifuged at 4000 rpm for 20 min. Thereafter, the resulting supernatant was used as the crude enzyme.

### 2.6 Enzyme Assay

This was done by a modified method of Prabakaran et al. [21]. The substrate was 1 % casein in 0.1 M phosphate buffer at pH 7.0, while the supernatant was used as an enzyme source. One mL of each was pipetted into a test tube, and the reaction mixture was incubated at 50 $^oC$ for 30 min. After that, 3 mL of cold trichloroacetic acid (TCA) was used to terminate the reaction. This was centrifuged at 4000 rpm for 20 min, and the resulting supernatant was used as the crude enzyme. The supernatant was mixed with 2.5mL of 0.5 M $Na_2CO_3$, vortexed, and incubated for 20 min. This was followed by adding 0.5mL of Folin Ciocalteu's phenol reagent, and the absorbance was read at 660 nm using a spectrophotometer (Model 752). Protease activity was measured as the amount of enzyme required to release one micromole of tyrosine per minute.

### 2.7 Optimization of Cultural Parameters for Protease Production

The effects of the incubation period, pH, agitation speed, temperature, substrate concentration, and carbon and nitrogen sources were determined for maximum protease production. The production medium and incubation conditions earlier stated were used in carrying out the optimization studies.

#### 2.7.1 Temperature

The influence temperature of protease production was studied by varying the incubation temperature of the basal medium at 25, 30, 35, 40, 45, 50, 55, and 60 $^oC$. This was incubated for 24 h; the assay was done as previously stated.

#### 2.7.2 pH

The production medium was adjusted to different pH (5-10) to study the impact of pH on protease production. After incubating for 24 h, the assay was carried out as previously stated.

#### 2.7.3 Incubation period

The incubation time was determined by incubating the flasks containing the production

medium separately at different hours, such as 12, 24, 36, 48, 60, 72, 84, and 96 h. Subsequently, the optimum temperature, incubation period, and pH were adjusted accordingly.

#### 2.7.4 Carbon sources

The influence of carbon sources on protease production was studied by individually replacing the carbon in the medium with carbon sources like fructose, galactose, maltose, lactose, glucose, and starch (0.5% w/v). After that, the assay was determined as previously stated.

#### 2.7.5 Nitrogen sources

The effect of organic and inorganic nitrogen sources on protease production was studied. The nitrogen sources in the medium were replaced individually with the following: sodium nitrate peptone, urea, ammonium chloride, gelatin, potassium nitrate, yeast extract, and ammonium sulphate (0.5% w/v).

#### 2.7.6 Substrate concentration

The best substrate concentration for protease production was studied by varying the concentration of casein in the medium. The concentrations were varied between 0.25 and 2.5% w/v, and the enzyme assay was determined.

#### 2.7.7 Agitation rates

In addition, the agitation speed of the fermentation medium was set at 100, 120, 140, 160, 180, and 200 rpm. The optimum incubation time and pH were used, and an enzyme assay was determined.

#### 2.7.8 Metal ions

The optimum metal ion for enzyme production was selected by adding 1 mM of the metal ions ($HgCl_2$, $BaCl_2$, KCl, $FeCl_2$, $MgCl_2$, NaCl, $CaCl_2$) into the production medium, and the enzyme activity was estimated.

### 2.8. Statistical Analysis

The data presented in this study represent an average value of triplicate readings except pH and carbon sources which are presented as average of duplicate readings. Thereafter, the standard deviation was calculated.

## 3. RESULTS AND DISCUSSION

### 3.1 Screening of Protease-Producing Bacteria

The skim milk agar showed proteolytic activity with a clear zone of hydrolysis of 15 mm. This demonstrated the bacteria's capability of producing protease enzymes.

### 3.2 Identification and Gene Detection of Protease-Producing Bacteria

Morphological examination showed that the bacterium had a smooth colony surface, a cream color, a medium colony size, and an undulating margin. Microscopic examination showed the isolate as Gram-positive rod-shaped and could utilize glucose, maltose, and fructose. Further investigation revealed that the isolate was positive for motility, catalase, starch, gelatin, and citrate utilization. On NCBI BLASTn, the 16S rRNA gene sequences of isolate LS23B were compared to closely related 16S rRNA sequences. Isolate LS23B was identified as *Bacillus cereus* LS23B, having a similarity of 100 % with *Bacillus cereus* A9 accession number KT598357.1 (Chart 1). Researchers have reported *Bacillus* species for protease production. Jafari et al. [16] identified *Bacillus subtilis* PTCC 1254 isolated from wastewater as a potent protease producer. Hashmi et al. [14] also identified *Bacillus subtilis* S1 and *Bacillus amyloliquefaciens* KSM12 as the two highest protease-producing microbial strains from soil habitats. In addition, Kumari and Premila [22] identified two different protease-producing *Bacillus* species from dairy industrial soil. This confirms the observation of Kuebutornye et al. [23] who noted that *Bacillus* species are effective in producing microbial proteases compared to other microbial strains. Fig. 1 depicts the amplification of a specific primer for the *npr* gene by protease-producing bacteria. *Bacillus cereus* LS23B amplified the primer, and positive bands of 951bp confirmed the presence of the *npr* gene. The presence of protease genes was also confirmed by some researchers. For instance, Perfumo et al. [24] identified a protease-encoding gene in *Psychrobacter* sp. strain 94-6PB. Liu et al. [25] also confirmed the presence of the protease gene in *B. velezensis* isolated from Daqu. The ability of the organisms to hydrolyze the substrate is clear evidence that their genome harbors the relevant gene.

## 3.3 Optimization of Parameters for Protease Production

There is a need to optimize both physical and nutritional parameters to achieve a high yield of enzymes.

### 3.3.1 Temperature

There was a gradual increase in enzyme production from 25 $^{o}$C up to 45 $^{o}$C, followed by a slight decrease at 50 $^{o}$C. The maximum protease production was obtained at 40 $^{o}$C with an activity of 3.07 U/mL (Fig. 2). This shows the bacterium's mesophilic nature, and an increase in temperature led to a gradual decrease in enzyme activity. A slight reduction at 50 $^{o}$C can be attributed to the influence of environmental factors. At 55 and 60 $^{o}$C, lower activities were observed which could be attributed to denaturation at higher temperatures. This was consistent with some findings. Khatoon et al. [26] and Niyomukiza et al. [27] reported that optimum protease activity was attained at 40 $^{o}$C for the *Bacillus* strain and *Bacillus aerius*, respectively. In addition, Kumari and Premila [23] reported a maximum enzyme production at 40 $^{o}$C (7.33 U/mL) by *Bacillus* sp. JKSP1. A closer temperature of 37 $^{o}$C (88.14 U/mL) enhanced the protease activity of *Bacillus subtilis* PTCC1254 isolated from wastewater as reported by Jafari et al. [16]. However, a lower temperature of 20 $^{o}$C enhanced the protease production of *Bacillus* sp. HM49 [28] while a temperature of 45 $^{o}$C (155.3 U/mL) enhanced the protease activity of *Bacillus* sp. mar64 [29]. The temperature preference for each microorganism differs, and this influences their growth rate and enzyme production, which could serve as a reason for this variation. The incubation temperature affects biological processes such as protein denaturation, enzyme secretion and inhibition, and the rate of microbial growth [30].

### 3.3.2 pH

Although there was an increase in protease production from pH 5 to pH 10, the highest enzyme activity was observed at alkaline pH 9, with an activity of 2.89 U/mL beyond which there was a slight decline (Fig. 3). At acidic pH values of 5 and 6, enzyme activities of 2.32 and 2.63 U/mL were observed, respectively. At pH 10, the enzyme activity was 2.83U/mL. This shows that protease production by *Bacillus cereus* LS23B requires alkaline pH. The finding suggests that the enzyme could be used for industrial processes since alkaline proteases are more preferred. This finding is supported by other authors. Jafari et al. [16] and Kotb et al. [29] reported pH 9 as the optimal pH for protease production by *Bacillus subtilis* PTCC1254 and *Bacillus* sp. mar64 isolated from soil samples. Hashmi et al. [14] also confirmed optimum protease production by *Bacillus subtilis* S1 and *Bacillus amyloliquefaciens* KSM12 isolated from soil at pH 9. However, pH 7 [22], 7.5 [26], and 8 [28] fully enhanced the production of protease by different *Bacillus* species isolated from various soil samples. Variations in pH could be due to the charge distribution of substrates and enzyme molecules, which invariably determines the catalytic process [31]. In addition, enzyme synthesis is often promoted by the availability and transfer of nutrients across the bacterial membrane [32].

**Chart 1. 16S rRNA gene sequence of isolate LS23B**

GGGGGGGACTACCATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGG
CGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAG
CTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTG
CCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTC
GGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTA
CCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG
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GGGCTCAACCTGGGATCTG

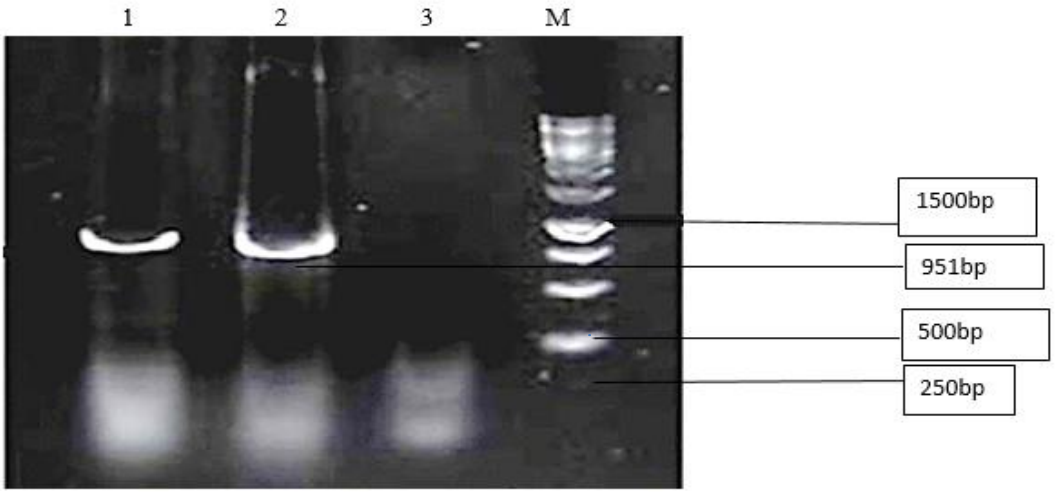


**Fig. 1. PCR Amplification of protease (*npr*) gene primer of LS23B. Key: 1: Positive control; 2: Isolate LS23B; 3: Negative control; M: Molecular marker.**

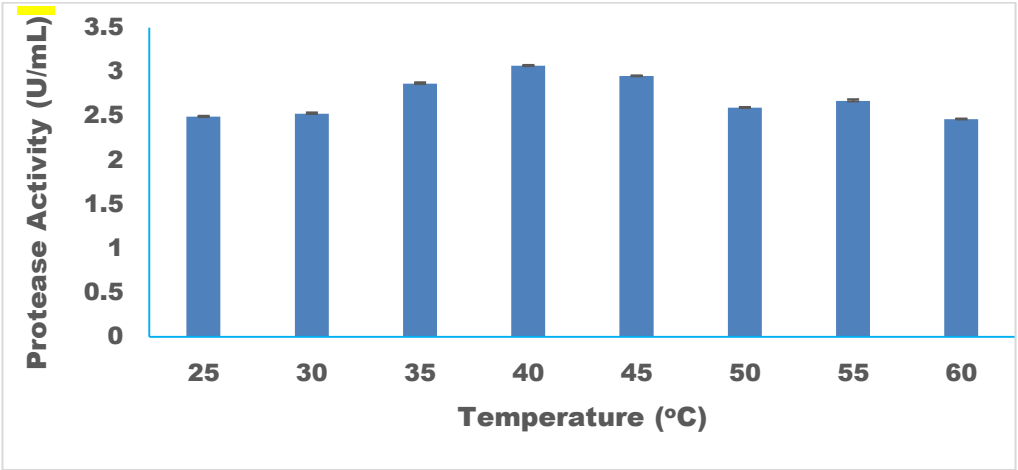


**Fig. 2. Influence of temperature on protease production**

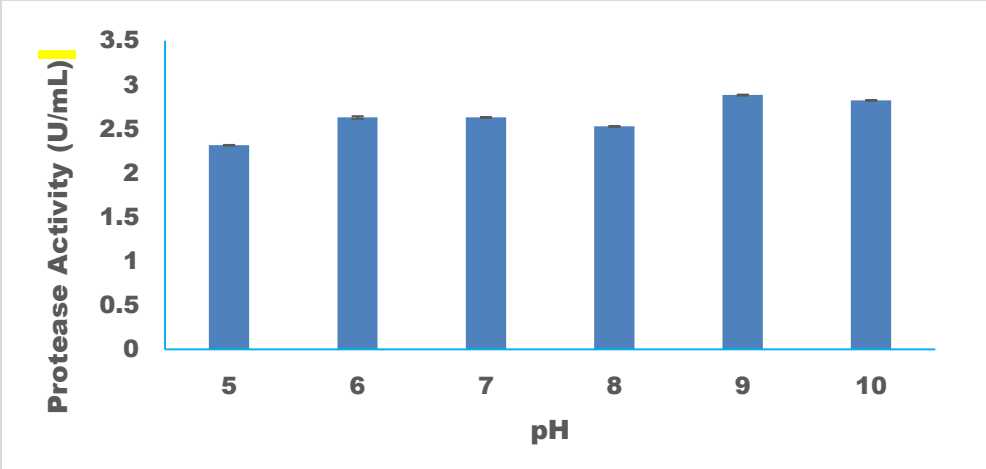


**Fig. 3. Influence of pH on protease production**

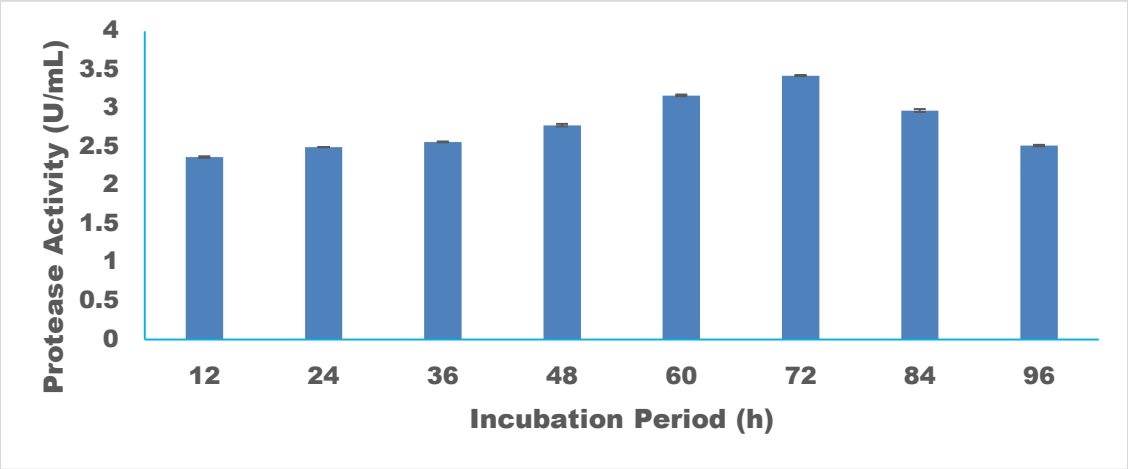


**Fig. 4. Influence of incubation period on protease production**

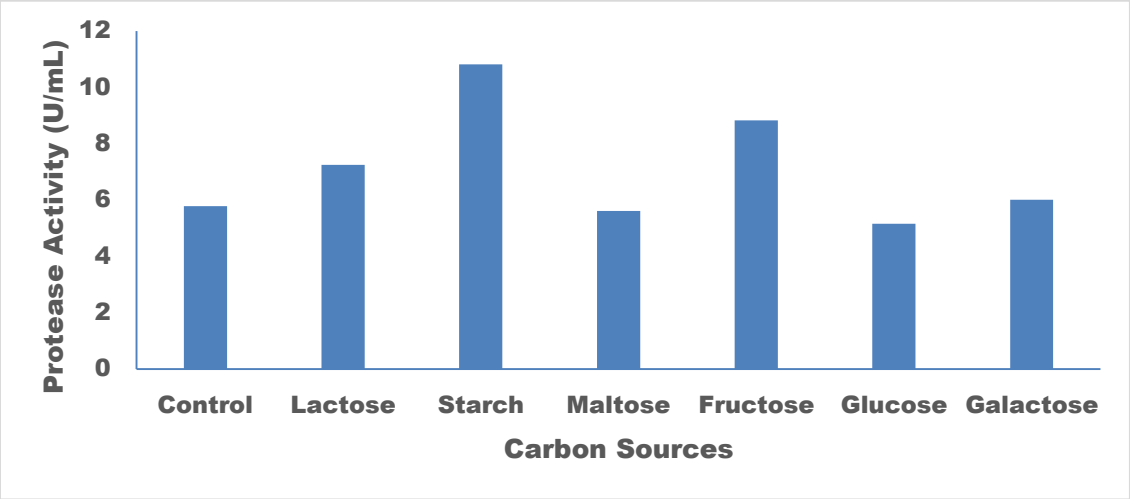


**Fig. 5. Influence of carbon source on protease production**

### 3.3.3 Incubation period

In this study, an increase in the incubation period led to a gradual increase in protease production as shown in Fig. 4. The least activity was recorded at 12 h (2.36 U/mL) of incubation while the optimum production was attained at 72 h of incubation with a corresponding enzyme activity of 3.42 U/mL. Beyond the optimum, the protease activity gradually decreased, with an activity of 2.51 U/mL at 96 h of incubation. This could have been caused by the depletion of nutrients or hazardous substances being released into the fermentation medium. In a similar investigation, an incubation period of 72 h enhanced protease production by *Bacillus amyloliquefaciens* [14], *Bacillus subtilis* PTCC1254 [16], *Bacillus* sp. HM49 [28], and *Bacillus* strain [26]. However, there was maximum protease production by *Bacillus* sp. mar64 at 60 h of incubation [29] while an incubation period of 48 h enhanced protease production by *Bacillus* sp. JKSP1 and *Bacillus subtilis* MJSP2 [22].

### 3.3.4 Carbon sources

Nutritional parameters such as carbon source significantly affect enzyme synthesis. The influence of carbon sources on enzyme production was done with different monosaccharides, disaccharides, and polysaccharides. This investigation validated the role of carbon sources as effective inducers of protease activity. Protease production was enhanced when starch (10.81 U/mL) was added to the media (Fig. 5). Starch, a polysaccharide was metabolized faster than other carbon sources. Fructose and lactose had a

corresponding activity of 8.83 U/mL and 7.25 U/mL, respectively. Contrarily, maltose, and glucose inhibited enzyme production which signifies that they cannot be utilized as carbon sources. In a related study, supplementing starch (278.12 U/mL) as a carbon source to the medium significantly increased protease yield by *Bacillus amyloliquefaciens* isolated from a local wastewater treatment plant [33]. In another literature, Shurigin et al. [34] reported starch as the best carbon source for protease production by *B. subtilis* IMRUZ7. This was followed by maltose, galactose, and lactose, while glucose, fructose, and sucrose were not effective. However, maltose [29], fructose [26], galactose [22], and sugarcane bagasse [15] were most effective for protease production by *Bacillus* sp. mar64, *Bacillus* strain, *Bacillus subtilis*, and *Bacillus subtilis* PTCC1254, respectively. In an investigation carried out by Patil and Kurhekar [35], the effects of carbon sources such as maltose, sucrose, glucose, and starch on protease production were tested. The researchers observed that the carbon sources had no positive effect on protease production by *Bacillus isronensis* strain KD3 isolated from dairy industrial effluent. Carbon sources are essential because they aid growth, energy production, biosynthesis, and other cellular processes [36,37].

### 3.3.5 Nitrogen sources

The influence of both organic and inorganic nitrogen sources on enzyme production was studied. It was observed that the media supplemented with gelatin enhanced protease production with a corresponding activity of 16.82 U/mL. This was followed by yeast extract (3.57 U/mL) and peptone (2.26 U/mL). Although some inorganic nitrogen sources such as ammonium chloride and ammonium sulphate enhanced protease production, the production rate was not as high as those obtained with gelatin and yeast extract (Fig. 6). Urea, sodium nitrate, and potassium nitrate all inhibited protease production. This shows that protease activity by *Bacillus cereus* LS23b was increased in the presence of organic nitrogen sources than the inorganic sources. In a similar investigation, gelatin was more suitable for synthesizing proteases by *Bacillus subtilis* ASASBT and *Bacillus circulans* [12,38]. However, yeast extract was the best nitrogen source for protease production by a *Bacillus* strain [26] and *Bacillus cereus* PW3A [39], which agrees with this study. Kumari and Premila [22] reported a maximum protease production by *Bacillus* sp. JKSP1 and *Bacillus subtilis* when peptone (6.89 U/mL) and casein (8.56 U/mL) were supplemented into the medium. Contrarily, supplementing the production medium with nitrogen sources like malt extract, tryptone, ammonium sulphate, and beef extract did not have any effect on protease production by *Bacillus isronensis* strain KD3 [35]. The utilization of nitrogen sources by microorganisms is species-specific due to their genetic differences, which could have contributed to the variation. Also, microbial organisms derive their secondary energy from nitrogen sources [32,40]. Microorganisms can synthesize protein, amino acids, nucleic acids, and cell wall components by utilizing different nitrogen sources [16].

### 3.3.6 Substrate concentration

From the result, each substrate concentration had a different effect on protease production. The effect of substrate concentration on protease concentration showed an increase in the enzyme yield from 0.25 % to 1 %. The enzyme was fully expressed at 1 % casein concentration with a corresponding protease activity of 5.20 U/mL. At lower and higher substrate concentrations above the optimum, enzyme production was decreased. The yield decreased, perhaps due to substrate inhibition or catabolite repression (Fig. 7). This is consistent with some results. Lakshmi et al. [18] reported maximum protease production by *Bacillus licheniformis* at 1% substrate (casein) concentration. Optimization studies of *Bacillus infantis* SKS1 by Saggu and Mishra [36] also reported a substrate concentration of 1% for maximum protease production. In addition, strains of *S. fimicola* S2 and N6, as reported by Naureena et al. [41] enhanced protease production by 1% casein concentration with a corresponding activity of 1.230 U/mL and 1.034 U/mL, respectively.

### 3.3.7 Agitation rates

The impact of agitation speed on protease production was examined under shaking conditions [Fig. 8]. The ideal agitation speed for protease production by *Bacillus cereus* LS23B was 160 rpm (5.74 U/mL). The increased protease production at 160 rpm shows that the organism needed oxygen for the production of the enzyme. The medium's oxygen and nutrients were probably rapidly dissolved enough to favor the enzyme's synthesis. In a related study, the production of alkaline protease from industrial

wastewater in *Bacillus subtilis* PTCC1254 was investigated and increased protease activity was reported at 150 rpm. A closer agitation speed of 155 rpm was reported to enhance protease activity by *Bacillus subtilis* IMRUZ -7 [34] while Niyomukiza et al. [27] reported that better protease activity was achieved at 180 rpm. In another study, a higher agitation speed of 200 rpm (49±0.058 U/mL) enhanced the protease synthesis of *Bacillus isronensis* strain KD3 while the second best was attained at 150 rpm (26 ±0.32 U/mL) [35].

### 3.3.8 Metal ions

The effect of different metal ions on protease production by *Bacillus cereus* LS23B at 1 mM is shown in Fig. 9. All metal ions enhanced protease production when compared with the control (3.69 U/mL), including $Hg^{2+}$ which has been reported as an enzyme inhibitor. However, $K^{+}$ strongly enhanced protease production with an activity of 5.57 U/mL. This was followed by $Mg^{2+}$ (4.98 U/mL) and $Ca^{2+}$ (4.87 U/mL). Increased protease production by $K^{+}$ shows that it is needed for protease synthesis by *Bacillus cereus* LS23B. Thermal denaturation can be prevented and the active conformation of the enzyme can be maintained at high temperatures due to the presence of either monovalent or divalent cations [42]. A similar study carried out by Asha and Palaniswamy [2] showed that a high level of protease production was observed in the presence of $Mn^{2+}$. $Mg^{2+}$ and $Cd^{2+}$ did not affect protease production while $Hg^{2+}$ completely inhibited the enzyme production by *Bacillus cereus* FT1.

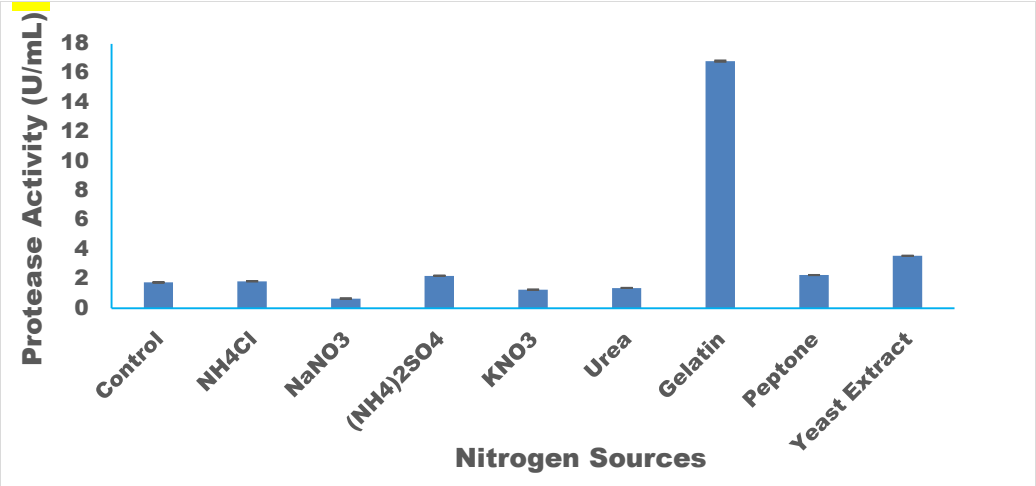


**Fig. 6. Influence of nitrogen sources on protease production**

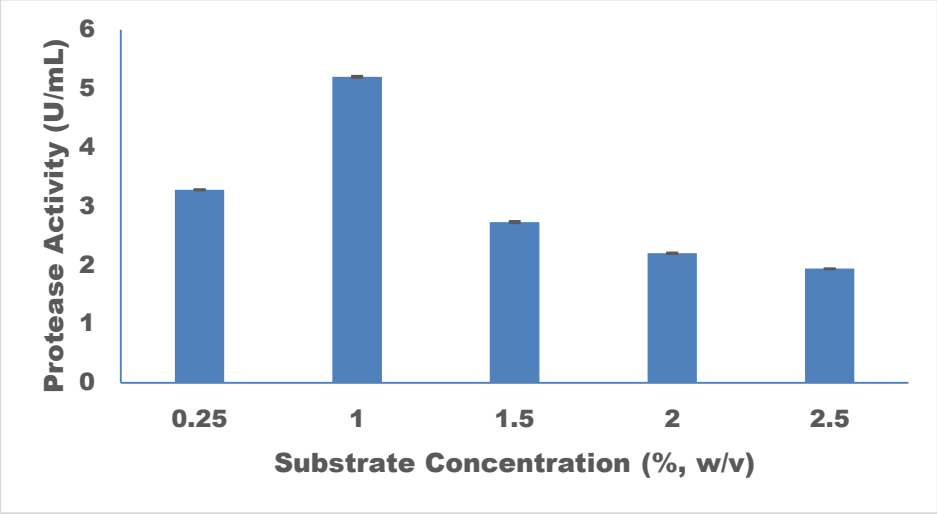


**Fig. 7. Influence of substrate concentration (%, w/v) protease production**

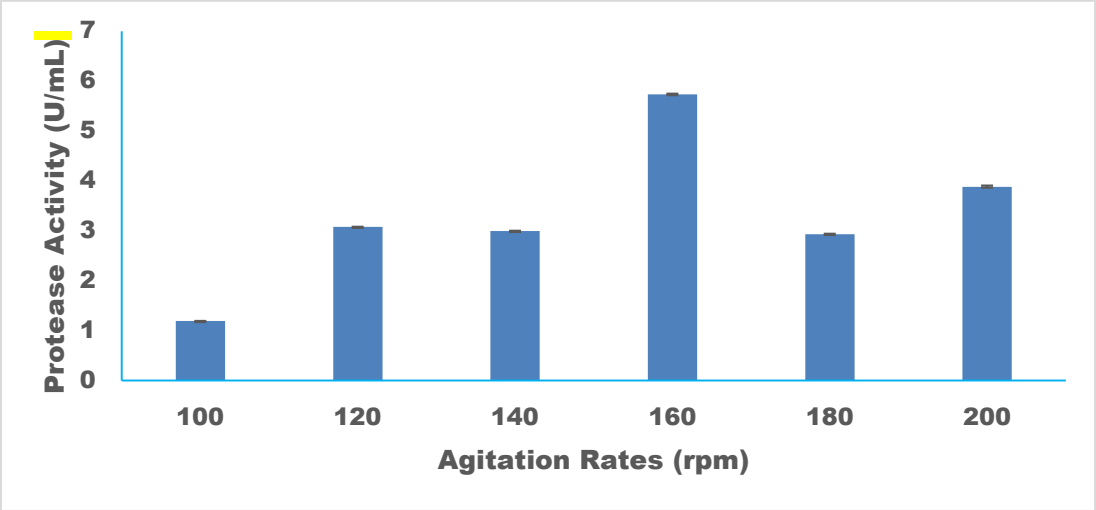


**Fig. 8. Influence of agitation rates on protease production**

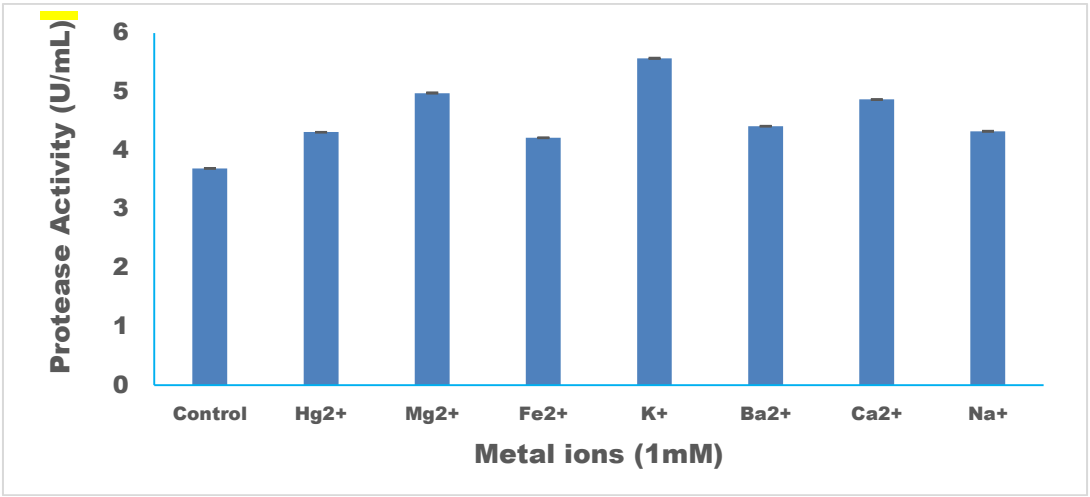


**Fig. 9. Influence of metal ions on protease production**

## 4. CONCLUSION

In this study, the bacteria isolate was identified as *Bacillus cereus* LS23B. Microorganisms for synthesizing enzymes for commercial use must be optimized to the highest degree. This study demonstrated the significant effect of cultural and physical factors on protease synthesis by *Bacillus cereus* LS23B. Maximum protease production was achieved after 72 h of incubation with a casein concentration of 1 %, 160 rpm, pH of 9, and a temperature of 40 $^{o}$C. Starch, gelatin, and $K^{+}$ (1mM) also had stimulatory effects. This will save cost and help in medium composition for large-scale alkaline protease synthesis as the industrial sector's need for hydrolytic enzymes keeps rising. The results obtained in this study suggest that *Bacillus cereus* LS23B is a potential candidate for producing alkaline protease for industrial operations. In addition, studies are needed to determine the potential applications of the protease obtained.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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