Original Research Article

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

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 bacteria isolates to produce protease enzymes was evaluated through primary and secondary screening. After that, 16S rRNA analysis was done to identify the best bacteria 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, temperature, carbon, and nitrogen sources were studied. Others include metal ions, substrate concentration, and agitation speed. The bacteria 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^oC with a pH of 9 after 72h incubation. Starch, gelatin, 1 % substrate concentration, 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].

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Microorganisms that produce protease have been isolated from habitats such as rotten dried fish, hot springs, soil, leather industry effluents [12-15]. It is easier to isolate and screen for microorganisms with desired characteristics since they are found in different environments [16]. 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. MATERIAL AND METHODS

2.1 Microorganisms

The bacteria isolates used in this work were previously isolated from a soil sample in Ado-Ekiti, Ekiti State, Nigeria.

2.2 Screening of Protease-Producing Bacteria

2.2.1 Primary Screening

Slightly modifying the method of Lakshmi et al. [17], skim milk agar was sterilized, and the microorganisms were spot-inoculated on the agar. This was incubated at 37 ^oC for 48 h, after which they were observed for a clear zone, and the diameter of each zone was measured. An uninoculated plate served as a control.

2.2.2 Secondary Screening

The protease production medium (%w/v) consisting of glucose (1.0 g), yeast extract (0.5 g), $MgSO_4.7H_2O$ (0.2 g), Na_2CO_3 (1.0 g), KH_2PO_4 (0.2 g), casein (0.5 g) at pH 8.0 was sterilized. It was inoculated with the standardized inoculum and incubated at 37 ^oC for 48 h. The inoculum was one mL of twenty-four-hour-old culture adjusted to the 0.5 McFarland standard. The medium was centrifuged at 4000 rpm for 20 min while the specific activity of the supernatant was determined [18].

2.3 Identification of the Bacterial Strain

2.3.1 Morphological and Biochemical Characterization

The bacterial strain with the highest specific activity following the secondary screening 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) [19]. 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′- GGTTACCTTGTTACGACTT-3’) [20]. A GeneAmp 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 is (951 bp), with upstream primer nprBcI (59 GTAACAGGAACGAATAAAGTAGGAACTGGTAAAG-39) and downstream primer nprBcII (59-GTTTACAC CAACAGCACTAAATGATTGCTTAAC-39). 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 [21]. The gel electrophoresis was done by slightly modifying the method of Odeyemi *et al.* (2018). A previously prepared 2% agarose gel was used to confirm positive amplification. 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, and the size of the PCR product was 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. This was inoculated with the standardized inoculum, incubated, and centrifuged at 4000 rpm for 20 minutes. The specific activity of the resultant supernatant was determined [18].

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2.6 Enzyme Assay

This was done by a modified method of Prabakaran *et al*. [23]. 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). Enzyme activity was measured at one unit of the enzyme catalyzing one millimole of substrate released per minute under assay conditions. The total protein content was estimated, as stated by Lowry et al. [24], using bovine serum albumin as a standard.

2.7 Optimization of Cultural Parameters for Protease Production

The incubation period, pH, agitation speed, temperature, substrate concentration, and carbon and nitrogen sources were determined for maximum protease production.

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 [25]. 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 using the modified method of Rawway et al. [26]. The carbon in the medium was replaced individually with carbon sources like fructose, galactose, maltose, lactose, sucrose, 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 was studied using the Rawway et al. [26] modified method. 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 modified method of Saggu and Mishra [27] was used to determine the best substrate concentration for protease production. The concentration of casein 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 [28]. The optimum incubation time and pH were used, and an enzyme assay was determined.

3. RESULTS AND DISCUSSION

3.1 Screening of Protease-Producing Bacteria

The skim milk agar showed various degrees of proteolytic activity due to the clear hydrolysis zone. This demonstrated their ability to produce protease enzymes. Those positive for the preliminary screening were subjected to secondary screening. The result is shown in Fig. 1. The lowest protease producer was isolate LS14, with a corresponding activity of 0.014 mmol/min/mL. Isolate LS23B was considered the highest protease producer based on its maximum protease production, where the activity of 0.044 mmol/min/mL was recorded and selected.

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). Fig. 2 depicts the amplification of a specific primer for

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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. Researchers have reported *Bacillus* species for protease production [13-15, 29]. Some authors reported the presence of protease genes in *Idiomarina* sp. C9-1, *Psychrobacter* sp. strain 94-6PB, *B. cereus,* and *B. velezensis* [30-33]. The ability of the organisms to hydrolyze the substrate is clear evidence that their genome harbors the relevant gene.

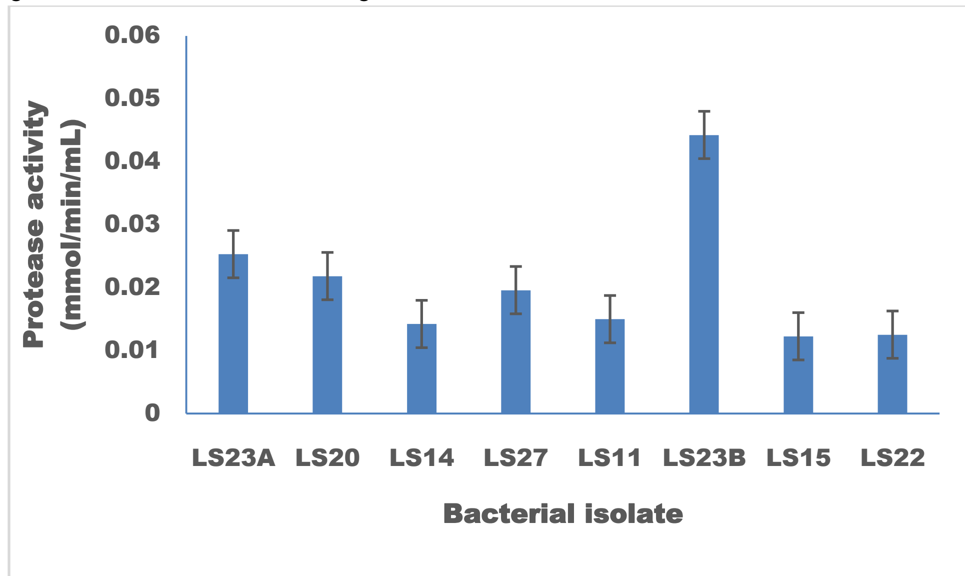

Fig. 1. Secondary screening for protease production by bacterial isolates

Chart 1: 16S rRNA gene sequence of isolate LS23B

GGGGGGGACTACCATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAG
TAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACC
AAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCA
CCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTC
GGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAA
GCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG
CGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGATCTG

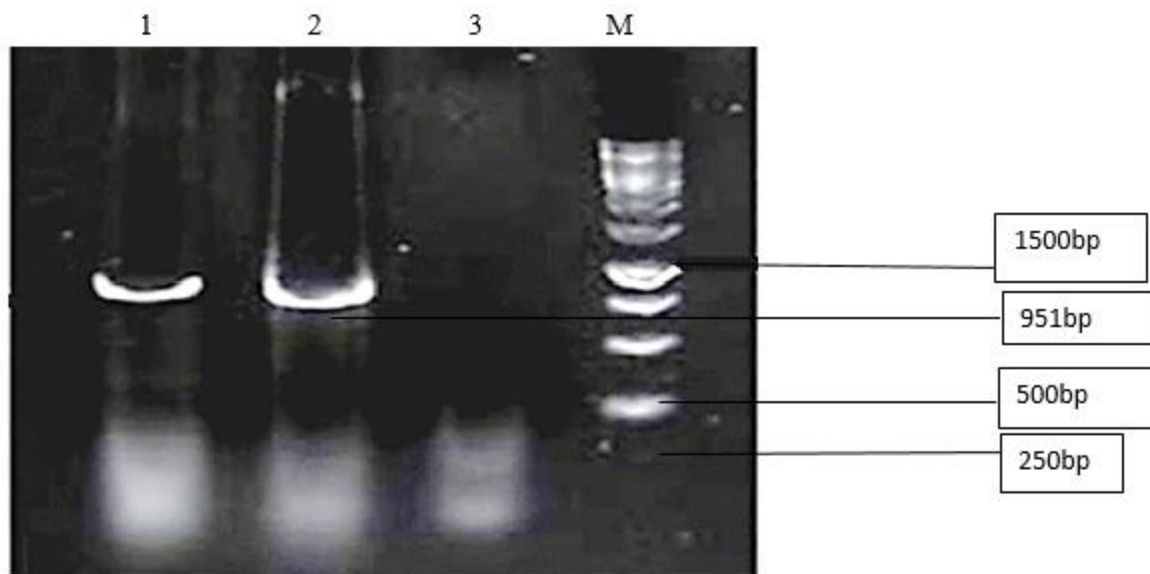

Fig. 2. PCR Amplification of protease (*npr*) gene primer of LS23B. Key: 1: Positive control; 2: Isolate LS23B; 3: Negative control; M: Molecular marker.

3.3 Optimization of Parameters for Protease Production

3.3.1 Temperature

This study produced protease between 25 °C and 45 °C, followed by a slight decrease at 50 °C. The maximum protease activity was obtained at 40 °C (Fig. 3). 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 °C can be attributed to the influence of environmental factors. At 55 and 60 °C, significant activities of approximately 88 and 79 % were observed, respectively. Some results were found to be consistent with our findings. Similar trends were reported for *Bacillus subtilis* [34], *Paenibacillusdendritiformis* [35], and *Bacillus aerius* [36]. Other studies have also reported that the protease production by *Bacillus* sp. [37] and *Bacillus thuringiensis* [38] were enhanced at 35 °C and 37 °C, respectively. This is closely related to our findings. The temperature preference for each microorganism differs, influencing their growth rate and enzyme production. The incubation temperature affects biological processes such as protein denaturation, enzyme secretion and inhibition, and the rate of microbial growth [39].

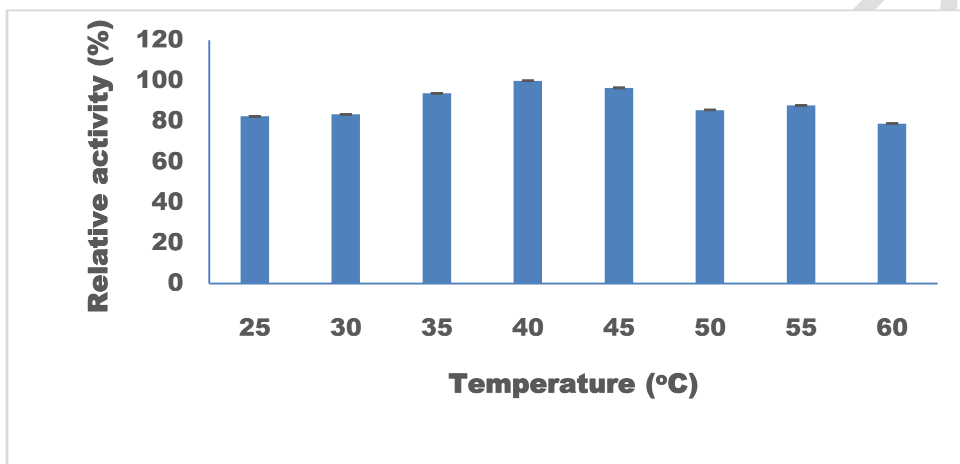

Fig. 3. Influence of temperature on protease production

3.3.2 pH

In this study, maximum protease production was observed at alkaline pH 9, beyond which there was a slight decline (Fig. 4). At acidic pH values of 5 and 6, protease activities of 78 and 88 % were retained, respectively. Activity at pH 10 revealed that 94 % of protease activity is retained. This shows that protease production by *Bacillus cereus* LS23B requires alkaline pH. This conforms with earlier reports where *Bacillus amyloliquefaciens* [14], *Paenibacillusdendritiformis* [35], *Bacillus* sp. [40], *Bacillussubtilis* PTCC 1254 [41], and *Bacillussubtilis* [42] maximally produced protease enzyme at pH 9. Variations in pH could be due to the charge distribution of substrates and enzyme molecules, which invariably determines the catalytic process [43]. In addition, enzyme synthesis is often promoted by the availability and transfer of nutrients across the bacterial membrane [44].

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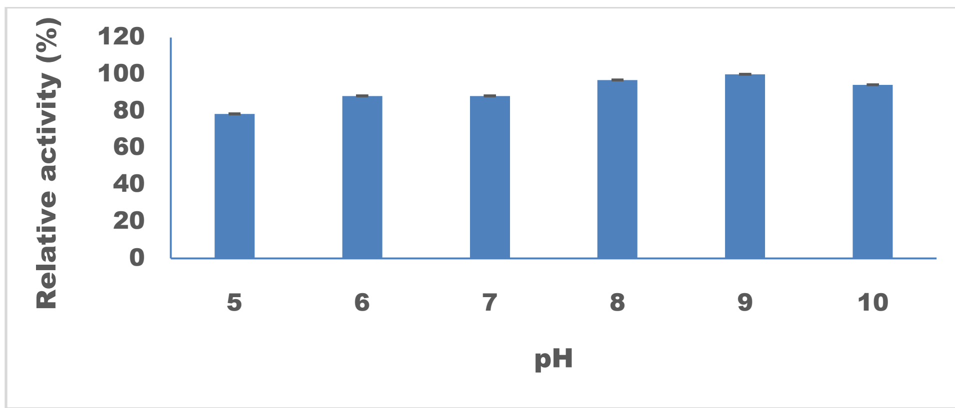

Fig. 4. Influence of pH on protease production

3.3.3 Incubation Period

The curve shows a gradual increase in protease production from 70 % activity at 12h to 100 % activity at 72 h, where the enzyme attained its total activity. Beyond the optimum, the protease activity gradually decreased, reaching a low point of 74.95 % at 96 h. This could have been caused by the medium becoming depleted of nutrients or hazardous substances being released into the fermentation medium (Fig. 5). In a similar investigation, an incubation period of 72 h enhanced protease production by *Bacillus* sp. DEMO5 [13]; *Bacillus amyloliquefaciens* [14], and *Bacillus nakamurai* [45].

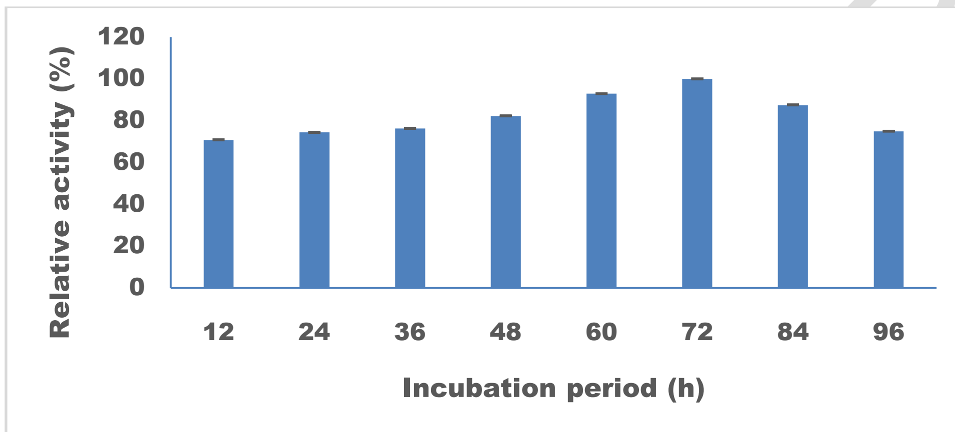

Fig. 5. Influence of incubation period on protease production

3.3.4 Carbon Sources

Nutritional parameters such as carbon source significantly affect enzyme synthesis. This investigation validated the role of carbon sources as effective inducers of protease activity. Protease production was enhanced when starch (0.107 mmol/min/mL) was added to the medium (Fig. 6). Starch was metabolized faster than other carbon sources. This was closely followed by fructose (0.091 mmol/min/mL) and lactose (0.072 mmol/min/mL). Contrarily, galactose and glucose inhibited protease production, so they cannot be utilized as carbon sources. In a related study, supplementing starch as a carbon source to the medium significantly increased protease yield by *Bacillus* sp. DEMO5 and *Bacillus amyloliquefaciens* [13, 46]. Patil and Kurhekar [47] found that carbon source had no positive effect on protease production by *Bacillus isronensis* strain KD3. Carbon sources are essential because they aid growth, energy production, biosynthesis, and other cellular processes [27, 48].

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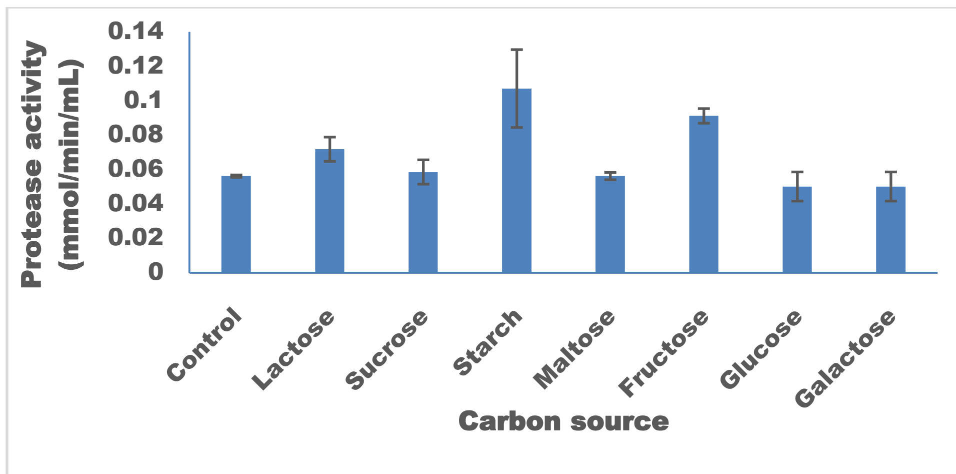

Fig. 6. Influence of carbon source on protease production

3.3.5 Nitrogen Sources

This study revealed that the medium supplemented with gelatin (0.060 mmol/min/mL) enhanced protease production. This was followed by yeast extract (0.036 mmol/min/mL) and peptone (0.023 mmol/min/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. 7). Urea, sodium nitrate, and potassium nitrate all inhibited protease production. In a similar investigation, gelatin was more suitable for synthesizing proteases by *Bacillus subtilis* ASASBT and *Bacillus. circulans* [35, 12]. However, yeast extract was the best nitrogen source for protease production by Isolate Y-46 [49] and *Bacillus cereus* PW3A [50]. Contrarily, supplementing the production medium with a carbon source did not positively affect protease production by *Bacillus isronensis* strain KD3 [47]. 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 [44, 51].

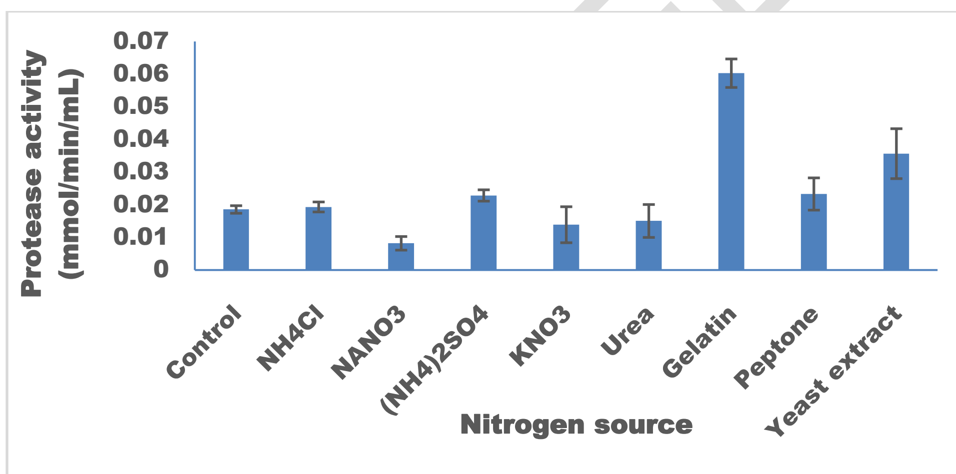

Fig. 7. Influence of nitrogen sources on protease production

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 a gradual increase in the enzyme yield from 0.25 % to 1 %. The enzyme production peaked at 1 % casein concentration with a corresponding protease activity of 0.051 mmol/min/mL. High casein concentrations negativelyaffected protease production. The yield decreased, perhaps due to substrate inhibition or catabolite repression (Fig. 8). This is consistent with previous results where 1 % substrate concentration was reported to influence protease production by some *Bacillus* species [17, 27].

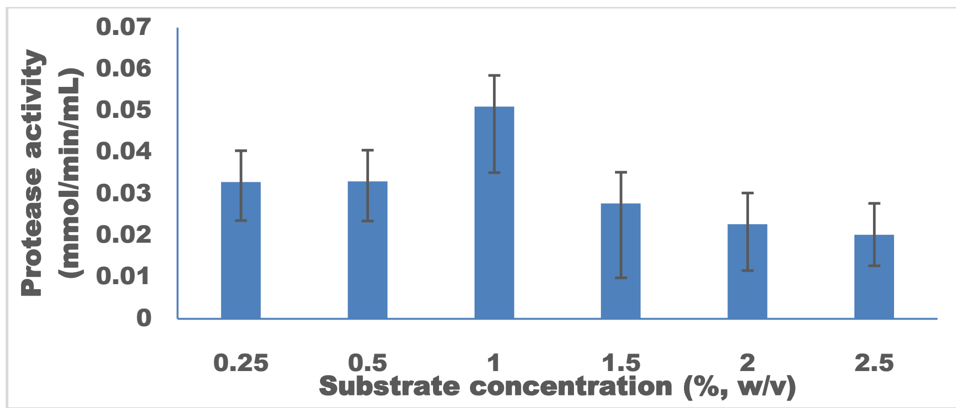

Fig. 8. Influence of substrate concentration (%, w/v) protease production

3.3.7 Agitation Rates

The impact of agitation speed on protease production was examined under static and shaking conditions. The ideal agitation speed for protease production by *Bacillus cereus* LS23B was 160 rpm (0.056 mmol/min/mL). The medium's oxygen and nutrients were probably rapidly dissolved enough to favor the enzyme's synthesis. The static and agitation speeds other than 160 rpm did not enhance protease production (Fig. 9). A previous study revealed that maximum protease production by *Bacillus subtilis* PTCC1254 was attained at 150 rpm, which is close to our findings [41]. In another study, a higher agitation speed of 200 rpm increased the protease synthesis of *Bacillus isronensis* strain KD3 [47].

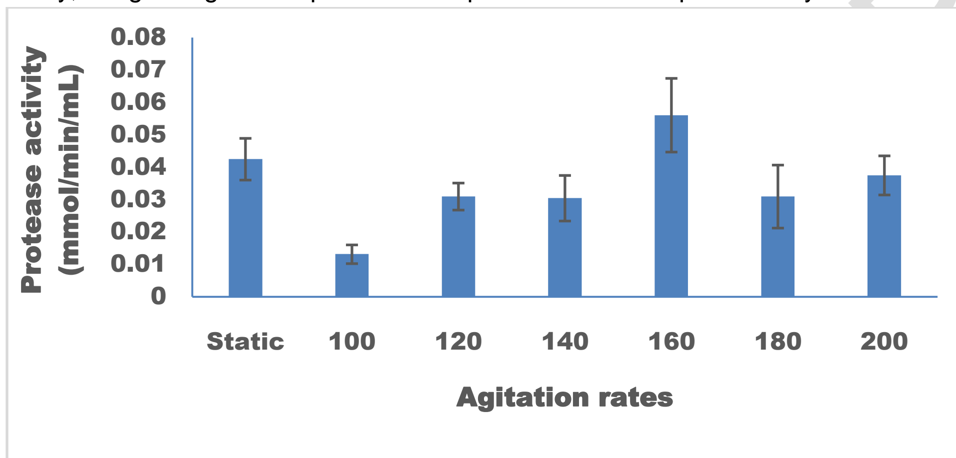

Fig. 9. Influence of agitation rates on protease production

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 and gelatin 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.

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