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Optimization of *B. cereus* PW4 alpha amylase production by OVAT technique

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Abstract

Amylase enzymes are used in a variety of industries. Palm wine was screened for amylase producing microorganisms by starch iodine method. Isolate PW4 was discovered to be the most effective isolate and was identified as *Bacillus cereus* PW4 (OK384566) by sequencing the 16S rRNA gene. The one-variable-at-a-time strategy was used to optimize α-amylase production parameters, including pH, temperature, incubation duration, inoculum volume, nitrogen source, and metal ion as factors. The enzyme activity was increased to 141.91 U/ml (5.8-fold) in the optimized medium (pH 7, 35 °C, 72 h, 3 % inoculums, 1 % NH_4NO_3 and 1 % $FeCl_3$) from 24.0 U/ml in the un-optimized medium. Data of the optimal condition will aid in the scaling up of amylase production and other industrial activities.

Keywords: Amylase; Hydrolysis; industrial enzymes; Bacillus; Optimization

1. Introduction

Amylase is one of the most important enzymes in modern biotechnology, with applications in clinical, medicinal, pharmaceutical, and analytical chemistry [1]. The Enzyme degrades starch to release glucose, maltose, and malto-oligosaccharide combinations by attacking the 1,4-glycosidic bonds of starch [2] [3]. Alpha amylase is found in a variety of organisms, including plants, animals, and microorganisms. Among these sources of amylase, microbial amylases have several advantages, including an easy production, optimization process, time and space efficiency, and cost effectiveness [4] [5].

Bacillus is a large and diverse genus of human-economically important bacteria. Protease from *Bacillus megaterium*, the world's largest (length 4 mm, diameter 1.5 mm) known bacterium, is frequently utilized in the detergent business [6]. *B. licheniformis* produces alkali-tolerant protease used in detergents [6], whereas *B. licheniformis* generates lipase [7], which is used in the food sector. Since it maintains its native folded state, i.e., its natural unaltered state during the various harsh bioprocess engineering steps and secretes proteins directly into the extracellular medium to facilitate their easy extraction and purification, *Bacillus* sp. is a suitable candidate for commercial enzyme production [8] [5].

The commercially available α-amylase enzyme was derived primarily the from *Bacillus* sp. [9]. Bacillus species such as *B. licheniformis, B. stearothermophilus, B. flavothermus,* and *B. megaterium* have been reported as thermostable alpha-amylase producers due to their heterogeneity and ability to adapt to diverse environments [10]**.** The commercial α-amylase producers have a wide range of properties (thermostability, acid-tolerance and so on), including *Aspergillus* sp., *Streptomyces* sp. and *Bacillus* sp. (including *B. subtilis, B. stearothermophilus, B. licheniformis,* and *B. amyloliquefaciens* [11] to [15].

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Metabolite synthesis and microbial growth are primarily determined by the nutrient content of the culture medium and the conditions under which it is grown [16]. A bioprocess's standardization is impossible without optimizing the media components and cultural factors. Furthermore, optimizing medium components and process parameters is critical for maximizing microbial metabolite synthesis while minimizing production costs [17]. One of the most important approaches utilized for enzyme overproduction in large quantities is medium manipulation and optimization of various parameters [18]. The variables responsible for enzyme production by microorganisms were generally optimized using the one factor at a time (OFAT) approach [19].

The goal of the present study was to screen for alpha amylase producing bacteria and optimize its production ability by one-variable-at-a-time method.

2. Material and methods

2.1. Sample collection

Palm wine samples were obtained from vendors in Rivers State, Nigeria. The samples were collected using sterile containers and sent to the laboratory for analysis.

2.2. Isolation

Ninety milliliters of sterilized saline water was used to serially dilute 10 ml of samples in the range of 10^1 to 10^{10}. Nutrient agar medium (NA) was used to culture serially diluted samples via spread plate method. Sterilization of the culture medium was done at 121 °C, 15 min 15 psi**.** The plates were incubated for 24-48 h, at 37 °C. The isolates were purified using the streaking method and stored on slants (NA with 2% starch) at 4 °C in the refrigerator until needed for analysis.

2.3. Screening for amylase activity

For preliminary screening (Plate assay), the bacterial colonies were cultured on modified nutrient agar (2% starch added) at 37 °C for 48 h. Their starch degradation was validated by application of iodine to the culture plates, which revealed clear zones around the amylase producing (positive) colonies after flooding with iodine solution. The hydrolysis ratio was determined by the ratio of the zone of clearance to colony diameter. Higher hydrolysis ratio was indicative of higher amylase activity, so organisms with these features were selected.

2.4. Production of α-amylase

The amylase was produced in modified medium (25 ml) containing (g/l) soluble starch, 20; peptone, 10; yeast extract, 4; NaCl, 0.5; $MgSO_4$, 0.5; $CaCl_2$, 0.2. at pH 7. The basal medium was sterilized for 15 min. at 121°C and 15 psi. One milliliter of each amylolytic bacteria 24 h nutrient broth culture (A600 0.8, $1.8x\ 10^8$ cfu/ml) was injected into 90 ml of basal medium and incubated at 37 °C for 72 h. At 24 h intervals, the flask was tested for growth (A600), α-amylase concentration, and reducing sugar concentration (540 nm). The samples were collected in triplicate, and the cells were separated (10,000rpm; 20 min) in a refrigerated centrifuge at 4 °C. The supernatant served as crude enzyme for enzyme assay.

2.5. Enzyme assay

Evaluation of enzyme activity was done by dinitrosalicylic acid method (estimation of the reducing sugar at 540 nm). 0.5 ml of Starch (1 % w/v in 100mM phosphate buffer; pH 7.0) was incubated with 0.5 ml crude enzyme at 50 °C for 10 min. The *α*-amylase activity unit (U) is the quantity of enzyme that produce 1 µmol of reducing sugar (glucose) per minute (mol/min).

2.6. Identification of amylolytic bacteria

Molecular characterization of the most potent isolate was done as described in Hasan et al. [21]. The characterization based on their morphological and molecular characteristics. The bacterial isolates' 16S rRNA was sequenced using primers [27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5' CGGTTACCTTGTTACGACTT-3')]. Each sequence's similarity was determined by running it through NCB1 BLAST (https://www.ncbi.nlm.nih.gov/blast/). Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) was used to create multiple sequence alignments using the best matched sequences, and MEGA was used to create phylogenetic trees (version 7). The sequences were entered into the GenBank database and assigned an accession number.

2.7. Optimization of cultural condition for by one-variable-at-a-time

The basal medium composition and cultural conditions of the bacterial isolates were optimized by varying the incubation time (1-5 days), pH (3, 5, 7, 9, 11), temperatures (25, 35, 50 °C), inoculum volume (1, 3, 5, 7, 9, % v/v), nitrogen sources (yeast extract, potassium nitrate, sodium nitrate, ammonium nitrate, and urea (1 %, w/v), metal ions (1 %, w/v) ($FeCl_2$, KCl_2, NH_4Cl_2, $ZnCl_2$). The fermentation set up was incubated at 37 °C, and samples were taken every 24 h for enzyme activity.

3. Results and discussion

3.1. Characterization and screening of bacterial amylase

Plate assay by starch iodine and dinitrosalicylic acid technique was used to determine amylase activity of the bacterial isolates. As the primary goal was to optimize the amylase production process, the isolate PW4 that displayed the highest amylase activity in the plate assay was chosen. The organism was a Gram-positive rod based on Gram's reaction. Molecular characterization of the isolate showed close relatedness with *B. cereus*. The sequence was submitted to GenBank, and the accession number received (OK384566).

3.2. Optimization of α-amylase production

Figure 1 shows the effect of incubation period on amylase production by isolate PW4 (*B. cereus*). After 72 h, the amylase activity of PW4 (141.91 U/ml) decreased steadily, according to the trend of amylase activity measured in the starch-supplemented medium. When the microbial mass was compared to the amylase activity, it was discovered that the activity was higher during the logarithmic growth phase (Figure 1B)

To determine the optimum initial medium pH for amylase production, *B. cereus* was grown in starch medium at different pH levels ranging from 3 to 11, while all other growth variables remained constant. Although *B. cereus* produced a significant amount of amylase at pH 5, the highest amylase yield (19.8 U/ml) was obtained at pH 7. (Figure 2). It was found that incubating the medium at various temperatures ranging from 25 °C to 50 °C followed by an amylase assay provided the best results for amylase production by the strain PW4. When the temperature reached 35 °C, amylase synthesis peaked (Figure 3).

Among the nitrogen sources studied, ammonium nitrate provided the highest enzyme activity (63.0 U/ml) (Figure 4). The effects of sodium nitrate and yeast extract on amylase production were the least. Amylase production was also affected by inoculum concentration; amylase activity increased with inoculum concentration, peaking at 3 % (82.160 U/ml) (Figure 5). Sodium chloride, zinc chloride, iron II chloride, calcium chloride, and potassium chloride were the metal ions investigated (Figure 6). Potassium chloride, sodium chloride, and ammonium chloride all stimulated growth and enzyme production; however, iron II chloride (87.08 U/ml) was the best for amylase production, while zinc chloride (6.835 U/ml) had the least enzyme activity.

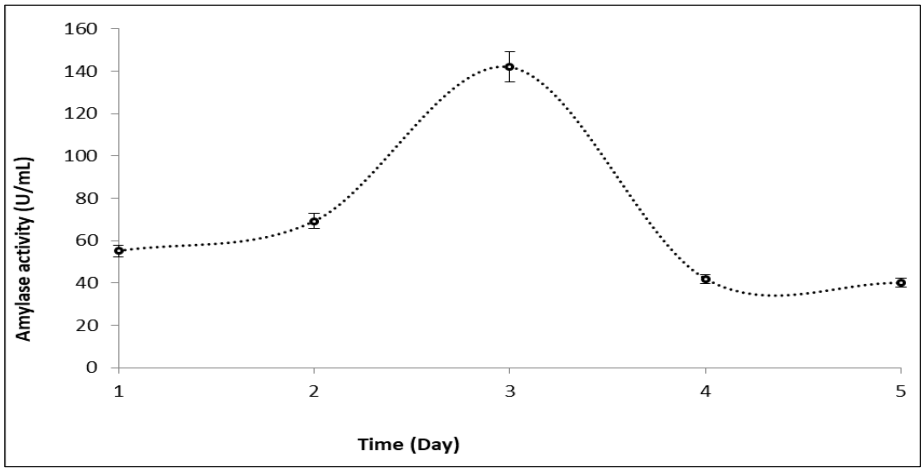

Figure 1 Effect of incubation time on *B. cereus* PW4 α-amylase activity

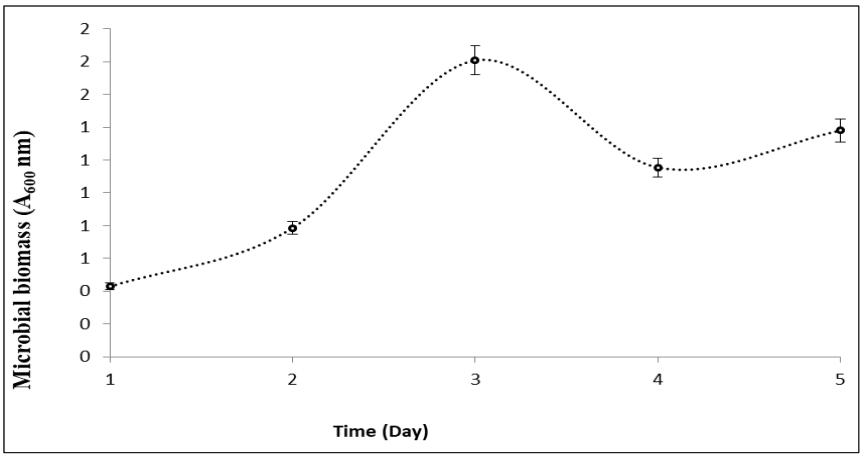

Figure 1B Effect of incubation time on *B. cereus* PW4 microbial mass

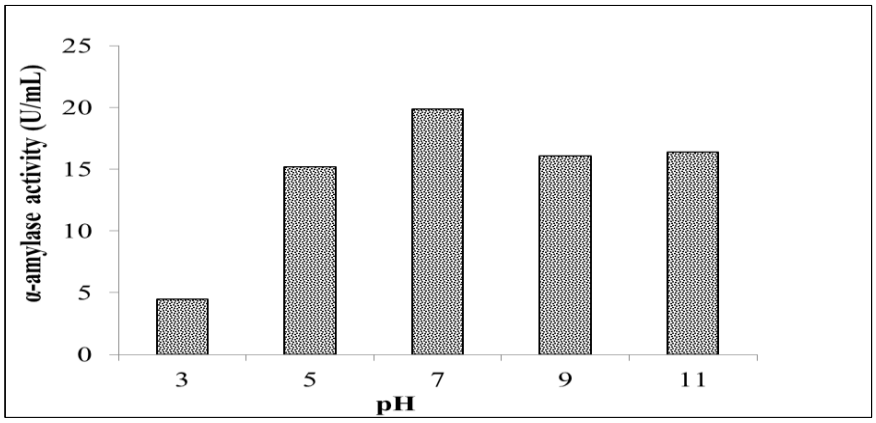

Figure 2 Effect of pH on B. cereus PW4 α-amylase activity

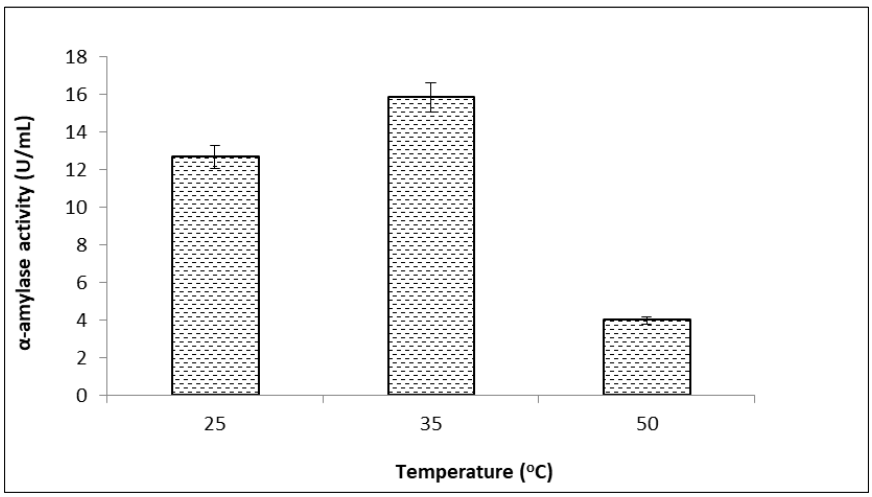

Figure 3 Effect of temperature on *B. cereus* PW4 α-amylase activity

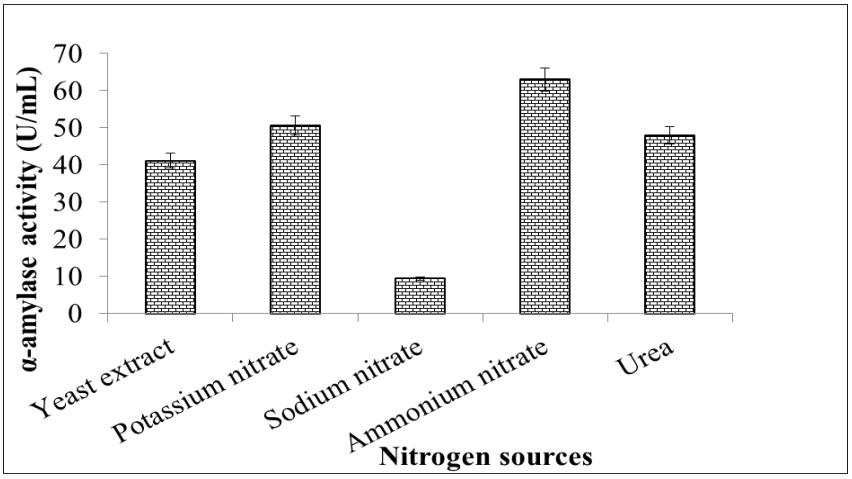

Figure 4 Effect of nitrogen source on *B. cereus* PW4 α-amylase activity

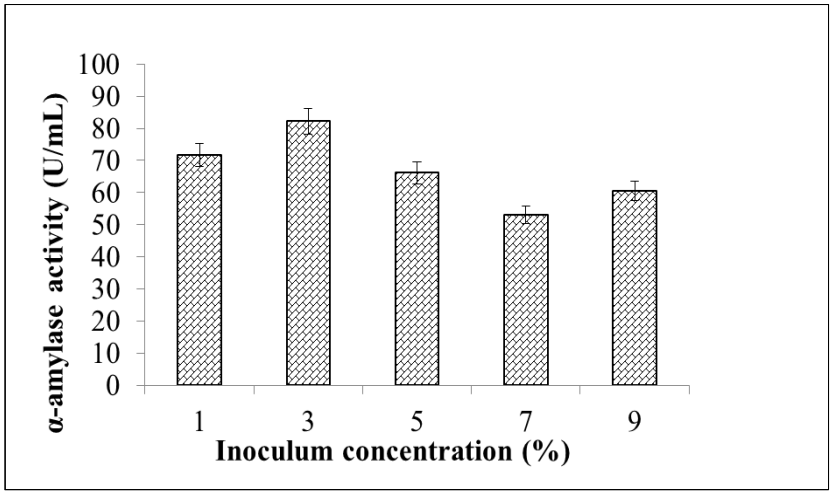

Figure 5 Effect of inoculum concentration on *B. cereus* PW4 α-amylase activity

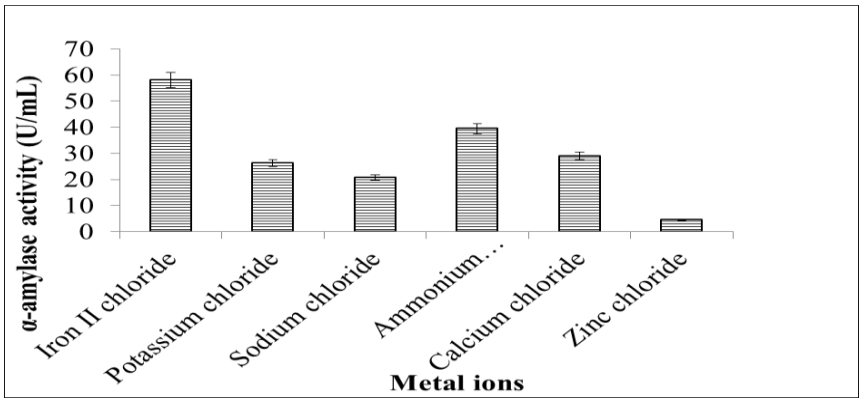

Figure 6 Effect of various metal ions on B. cereus PW4 α-amylase activity

4. Discussion

The most potent isolate in terms of amylase production was PW4 hence it was identified to specie level as *Bacillus cereus* (OK384566) and used for optimization studies. Researchers have utilized the starch iodine technique to check for starch-degrading microbes in a variety of materials (soil, fermentation broth, and digestate) [20] [5]. Hasan et al. [21] assessed amylase activity of *Penicillium* sp. and *Aspergillus versicolor* isolated from soil by halo zone formed from application of iodine on starch agar plates. Several studies have shown that amylase-producing microbial strains have been isolated from a variety of sources such as water [22], sugar industry's press mud soil [6], hot springs [23]. From garden soil samples, amylase producing *B. cereus* amy3 was found [10]. A novel bacterium, *Bacillus aryabhattai* KIIT BE-1 isolated from biodigester digestate, was found to have Amylase activity of 3.0 U/ml [17]. Various species of Bacillus with significant amylase activity have been found in garbage soil, including *B. azotoformans, B. stearothermophilus, B. acidocaldarius, B. subtilis, and B. megaterium* [1]. Several Bacillus species have been shown to produce significant quantity of amylase. Similarly, Pranay et al., [9] reported amylase production by *Bacillus* sp. isolated from garbage dump sites. Saha et al., [1] isolated *Bacillus* sp. T5-12, *B. cereus* MSW, *Bacillus* sp. FJAT- 14266, *B. toyonensis* KK25A, *B. cereus* T10, *Stenotrophomonas* sp. ZJZG10, *B. subtilis* XF-1 and *Pseudomonas* sp. NCCP-1179 from garbage soil.

This study's optimization of amylase production employed the one-variable-at-a-time (OVAT) technique and resulted in an increase in enzyme output of 141.91 U/ml over the previous one when *B. Cereus* PW4 was grown under suboptimal circumstances (24 U/ml). Similarly using OVAT technique amylase production increased from 800 to 3122 IU/ml when *B. cereus* amy3 was grown in amylase production medium (mosambi peel 1.75% (w/v), sodium nitrate 0.09%, Tyrosine 0.09 % 3% v/v inoculum) at pH 7, 37 °C for 48 h [10]

The temperature of 35°C was shown to be ideal for the synthesis of amylase. Most *Bacillus* sp. produce the most amylase when cultured between 35 and 37 °C, according to literature. *B. subtilis* BI19 amylase activity peaked at 37 °C, according to Hasan et al., [21]. Species within the same genus have different optimum amylase production temperatures and pH. Amylase activity in *B. cereus* PW4 was greatest in the alkaline pH range (7-9), with the maximum concentration found at pH 7. Enzyme activity was lowest at acidic pH levels, *B. cereus* is an alkaliphile. Enzyme activity increased from 3.20 U/ml to 4.16 U/ml after fermentation parameter adjustment [17]. Accordingly, Bakri et al. [3], reported maximum amylase production at 37 °C, and pH range of 4 to 6. Maximal amylase production by *Bacillus* sp. was observed at pH 8, 40 °C for 24 h [4]. Bozic et al. [23] reported 37 °C as optimal temperature for amylase production by *Bacillus* sp. Raul et al., [24] reported increase in amylase activity when ammonium sulphate was used as nitrogen source. Ilesanmi et al. [25] discovered that the optimal pH for *Pseudomonas aeruginosa* lipase production was pH 7, while pH 9 was optimal for *B. subtilis* D19 α-amylase production [26]. Hasan et al., [21] identified pH 8 as the optimum for *Bacillus* sp. α-amylase production. Ahmed et al. [27] found that *B. subtilis* strain-MK1 produced the most α-amylase at pH 7, 37 °C, after 3 days.

The different inoculum sizes [1–5 percent (v/v)] were tested for increased amylase production by *B. cereus* PW4. When 1% inoculum was used, enzyme production was determined to be low. As inoculum size increased amylase production increased likewise and peaked at 3 %. Strain amy3 produced amylase best at 3% inoculum volume [10]. According to the study by Almanaa et al., [26], using 5% inoculums resulted in maximum α-amylase production. Dash et al., [28] discovered that 2 % inoculum concentration resulted in the highest α-amylase production. Bacterial population competition for growth-supporting nutrients seems to be responsible for the observed decrease in enzyme production in substrates fermented with higher inoculum concentrations (>5%). For maximum amylase production from *Streptomyces* sp., Al-Dhabi et al., [20] recommends a 1 % inoculum concentration. Ilesanmi et al., [25] found that 4 % inoculum concentration was optimal for *Pseudomonas aeruginosa* lipase production.

Metal ions stimulates enzyme production; chlorides of iron, potassium, sodium, ammonium, calcium, and zinc were used for α-amylase production. *B. cereus* PW4 produced the most α-amylase when exposed to $FeCl_3$ (iron II chloride). The presence of $MgSO_4$ in the cultivation medium was found to be responsible for increased α-amylase production by *B. subtilis* strain-MK1 [26]. When Ca^{2+} was present, *Bacillus* sp. KR1 produced the highest amylase concentration [9].

It is critical for microbial growth and enzyme synthesis that nitrogen sources be in the culture medium. The culture medium was supplemented with 1 % (w/v) of different nitrogen sources, including sodium nitrate, ammonium nitrate, potassium nitrate, and yeast extract, to investigate the influence of nitrogen source on amylase production by *B. cereus* PW4. As demonstrated in Figure 4, ammonium nitrate results in the highest amylase activity of 63.0 U/ml. Saha & Mazumdar [10] reported sodium nitrate as best nitrogen source for production of amylase by *B. cereus* amy3 (2456 U/ml). Following optimization, *B. cereus* had an amylase activity of 538 U/ml [29].

5. Conclusion

Bacillus cereus PW4 was shown to be the top amylase producer (NCBI GenBank Access. No. OK384566). The one-variable-at-a-time was critical in media optimization, with amylase activity increasing. The optimum temperature and pH for amylase activity obtained from this strain were 35 °C and 7.0, respectively. The addition of Iron II chloride as a metal ion and ammonium nitrate as nitrogen source resulted in increased enzyme activity. This study found that the Gram-positive, rod-shaped bacteria can synthesize amylase, which is evidenced in the hydrolysis of starch, which is critical in biotechnology. Amylase activity was shown to be up to 5.8-fold more potent once the fermentation was scaled up, which suggests that commercial production of *B. cereus* amylase for various industrial uses is feasible. An additional investigation to confirm the technological feasibility of mass commercial manufacturing is advised for scaled-up process validation.

Compliance with ethical standards

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Disclosure of conflict of interest

No known conflict of interest exists among the researchers.

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