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Optimization of thermostable amylolytic enzyme production from *Bacillus cereus* isolated from a recreational warm spring via Box Behnken design and response surface methodology

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Abstract

This study aimed to find a source for local amylase-producing microbes. Sixteen isolates were obtained from the water samples from the warm spring and characterized based on morphological and biochemical tests. The 16S rRNA molecular identification technique confirmed the most potent isolate as *Bacillus cereus*. The thermophilic property of the bacterium demonstrated that it could withstand temperatures of up to 80 °C. One-factor-at-a-time (OFAT) and Box Behnken Design (BBD) coupled with response surface methodology (RSM) optimization techniques were used to improve amylase production. OFAT established optimal physical parameter conditions as the starch concentration of 5% w/v, inoculum volume of 2% v/v, pH of 8, incubation temperature of 45 °C, and 48 h of incubation, leading to amylase activity of 172.6 U/mL by the isolated *B. cereus*. A quadratic mathematical model with a coefficient of determination (R^2) of 0.9957 was established for the amylase production process. Enhanced amylase activity of 196.02 U/mL was achieved with BBD-RSM under optimal growth conditions of pH of 7, incubation time of 48 h, substrate concentration of 5% w/v of starch, and at 45 °C, a 1.2-fold increase compared to the OFAT method. The *B. cereus* strain isolated from the warm spring was a mildly thermophilic bacterium with the potential for synthesizing amylolytic enzymes with characteristics beneficial for commercial utilization.

Keywords Enzyme, Amylase, Warm spring, Modeling, Optimization

Introduction

Enzymes are proteins that act as catalysts without getting used up in the reaction that they catalyze. Enzymes are critical to the bioprocessing industry because of the mild conditions under which they operate and their high specificity and productivity [1]. The world enzyme market is USD 14.0 billion in 2024 and is projected to reach USD 20.4 billion by 2029 [2]. Amylases are one of the most important enzyme groups of great significance in biotechnology, making up about 30% of the world's market [3]. Amylases are beneficial in food, brewing, fiber, textile, detergent, paper, oil drilling, and pharmaceutical

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industries [4]. Amylases, in particular α-amylase, hydrolyze α-1, 4 glycosidic linkages in starch molecule to yield glucose and maltose [5]. The demand for amylases, especially those from extremophiles, is envisaged to rise swiftly because of their ability to withstand extreme environmental conditions, such as low and high pH and temperature and high salinity [6], thereby fueling market growth. It has been reported that the global requirement for amylase biofuel enzymes is growing primarily due to the soaring cost of fossil fuels [7] and demand for biofuels [8].

Presently, commercially available microbial amylases have almost completely replaced the chemical hydrolysis of starch in the starch processing industry. In the early 1960s, a significant increase in amylase production and utilization was recorded when amylase from *Bacillus subtilis*, amylases and glucoamylase from *Aspergillus niger* were used to replace acid catalysis in industrial production of dextrose from starch [9]. About 60% of commercial enzymes have been produced and secreted by the various *Bacillus* strains [10], placing them among the most significant industrial enzyme producers [9]. Most members of the *Bacillus* species are known to be able to synthesize amylolytic enzymes, some of which their thermostability [11], together with their optimum activities in a wide pH range and high productivity on various carbon sources, contribute to their potential to dominate the enzyme industry.

Warm and hot water springs have been reported to harbor myriad groups of microbes, and different biogeography, geological history of the location, and physicochemical conditions influence phenotypic differences of microorganisms in such environments [12]. It is imperative to research such environments for their microbial diversity from the viewpoint of biotechnological application and conservation [13]. Literature is rich in reports on diverse enzyme biosynthesis from microbes isolated from water [12, 14–16] and soil [12, 17, 18] collected from hot springs worldwide. Amylolytic enzymes with stable activity at temperature > 60 °C have been produced from bacteria isolated from hot springs [14, 15, 19]. This class of enzymes are vital to bioprocessing industry where high temperatures are employed.

Fermentation conditions for maximum enzyme production from microorganisms vary widely depending on the strain, even though there are similarities in their pattern of growth and enzyme profiles. In performing a cost-effective and economically viable industrial process for the production of enzymes from microorganisms, optimization of nutritional and physicochemical parameters, such as pH, temperature, incubation time, etc., is critical for maximizing their growth and enzyme yield [20]. Growth medium composition: inoculum age, pH, temperature, nitrogen source, and carbon source are among various physicochemical parameters that affect the yield and properties of enzymes produced under these conditions [21, 22]. Typically, the one-factor-at-a-time (OFAT) method is used to evaluate the effect of each factor on enzyme production. However, the method is defective because the interaction of factors cannot be determined. Hence, statistical approaches such as Box Behnken Design (BBD) and Central Composite Design (CCD) combined with response surface methodology (RSM) are employed nowadays to circumvent the problem with OFAT [23–25]. For instance, α-amylase activity from *Bacillus cereus* isolated from hot water from Hamma Lake-Gabes in Tunisia, increased by 141.6-fold (0.06 to 8.5 U/mL) when a full factorial design was compared with OFAT optimization methods [21]. In another study on *B. licheniformis* WF67, CCD was used to obtain the optimal conditions of beef extract of 8.31 g/L, soluble starch of 12.55 g/L, incubation time of 50 h, and pH of 6.33, leading to 6.633 U/mL compared to 3.486 U/mL from OFAT [26]. Using CCD, Ojha et al. [27] obtained amylase activity of 4.16 U/mL with the optimal conditions of peptone of 5%, starch of 10.25%, incubation time of 50 h, and pH of 7.3, compared to 3.2 U/mL from OFAT.

This current study aimed to isolate, identify, produce, and optimize the amylase enzyme production from the Ikogosi warm spring, known for its recreational, culinary, and drinking purposes. The study isolated amylase-producing microbes from the warm water spring and selected the best strain for amylase enzyme production. Also, the isolated strain was characterized, and growth conditions were optimized for maximum amylase activity.

Experimental

The warm spring where water samples were collected is located at longitude 7°30′N–7°45′N and latitude 5°00′E–5°15′ on the outskirts of the agrarian town of Ikogosi-Ekiti in Southwestern Nigeria.

Collection of water samples

Water samples from the warm spring were aseptically collected with sterile sample bottles. These samples were collected at three different points designated as the upper, middle, and lower parts of the spring. The average distance between the points of collection was 1, 5, and 3 m, respectively. The average temperature at the point of collection was 37 °C. The samples were aseptically transported to the laboratory at the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, for microbial isolation and analysis.

Methods

Isolation of amylase-producing microbes

For this purpose, 1 mL of each of the water samples was dispensed into the nutrient broth composed of 0.5% peptone, 0.3% beef/yeast extract, 1.5% agar, and 0.5% NaCl [28] and incubated at 45 °C in a thermostatic incubator for 72 h. A loopful from the turbid broth was streaked on the agar plates and incubated at 45 °C. Pure cultures were achieved at this temperature and subsequently subjected to an amylase test on starch agar plates.

Screening for amylase-producing isolates

Isolates were cultured on the nutrient medium agar plates with 1% soluble starch and incubated at 45 °C for 24 h. Afterward, the plates were flooded with 1% Lugol's iodine solution, and a clear zone formed around the colony, indicating starch hydrolysis and, hence, a positive test for amylase production [29].

Amylase activity assay

For the amylase production, the microbial cells of the isolate were cultivated in the medium broth with 1% soluble starch and incubated for 24 h in an incubator shaker at 45 °C and pH of 7.0 [14]. The cultivation broth was centrifuged at 5000 rpm for 10 min using (ZM200, Germany) centrifuge to obtain the supernatant, which was filtered to collect the crude enzyme [30]. The amylase assay was carried out by employing the DNS method. The reaction mixture of 0.5 mL of the crude enzyme extract and 0.5 mL of starch solution was incubated at 45 °C in a water bath for 15 min. After which, 1 mL of 3,5-dinitrosalicyclic (DNS) solution was added to the reaction mixture to determine the reduced sugars released [31]. One unit (U/mL) of amylase activity is defined as the amount of enzyme (amylase) required to liberate 1 µmol (0.18 mg equivalence) of reducing sugar (D-glucose) from starch per minute under the assay conditions [32].

Growth condition effect investigation

Individual growth parameters, including temperature, incubation time, pH, and carbon and nitrogen sources, and their effects on the isolate were investigated using the OFAT method. The production medium used was composed of peptone of 20 g, $MgSO_4.7H_2O$ of 1 g, K_2HPO_4 of 3 g, and soluble starch of 10 g per liter of distilled water. The pH of the fermentation media was adjusted to 7 with 1N HCl/NaOH.

Temperature and thermostability effects on amylase activity The temperature effect on growth and thermo-tolerance for amylase activity on the isolate was investigated by incubating it for 24 h from 35 to 80 °C. For thermos-tolerance, the OD at each temperature was measured by a spectrophotometer at 600 nm. For the temperature effect on amylase production for each temperature in nutrient broth supplemented with 1% soluble starch, 1 mL of the fermented broth was withdrawn at 24 h and assayed for amylase activity as described in Sect. "Amylase activity assay".

Table 1 Range of values of independent variables

Independent Variables	Units	Low (+ 1)	Mid (0)	High (− 1)
pH	–	4	7	10
Incubation period	h	24	48	72
Substrate concentration	%	3	5	7

Incubation time effect on amylase activity The incubation time effect on the isolate was cultured at 45 °C and pH 7.0 for 60 h. Thus, 1 mL of the fermented broth was withdrawn every 6 h and assayed for amylase activity as described in Sect. "Amylase activity assay".

Carbon and nitrogen source effects on amylase activity Four carbon sources (maltose, glucose, starch, and sucrose) and five nitrogen (yeast extract, peptone, casein, ammonium nitrate, and potassium nitrate) were evaluated for amylase production. The isolate was cultured at 45 °C and pH 7.0 for 48 h using 1% of each carbon and 2 g/L of each nitrogen source. Samples were taken from each experiment and assayed as described in Sect. "Amylase activity assay".

pH effect on amylase activity The pH effect on the amylase activity was carried out by preparing different media with a pH range of 4—10. The isolate was cultured at 45 °C for 48 h, and samples were taken from each experiment and assayed for amylase activity as described in Sect. "Amylase activity assay". The best pH obtained was subsequently used for further studies.

Inoculum size effect on amylase activity The inoculum size effect on amylase activity by the isolate was investigated. The inoculum size for inoculating the broth was varied from 1 to 3 mL. The isolate was cultured at 45 °C and pH 8.0 for 48 h. Samples were taken from each experiment and assayed for amylase activity as described in Sect. "Amylase activity assay".

Substrate concentration effect on amylase activity The substrate concentration effect on amylase activity from the isolate was investigated using 1–5% starch. The isolate was cultured at 45 °C and pH 8.0 for 48 h. Samples

Table 2 Biochemical characterization of the isolates from the warm spring water samples

Species	Gram reaction	Catalase	Indole mobility	Citrate	Nitrate reduction
Klebsiella edwarsii	–	+	– – –	+	+
Pseudomonas aeruginosa	–	+ +	– + –	–	+ +
Klebsiella oxytosa	–	+ +	+ – –	–	+ +
K. pneumoniae	–	+ + +	– – –	–	+ +
K. pneumoniae	–	+	– – –	–	+
Sarcina flava	+	+ +	– – –	–	–
K. pneumoniae	–	+ + +	– – –	+	+
P. aeruginosa	–	+ +	– + –	+	+ +
K. Edwarsii	–	+	– – –	–	+
K. Edwarsii	–	+	– – –	–	+
Rhodotorula sp.	+	+	– – –	–	–
K. Edwarsii	–	+ + +	– – –	–	+
B. cereus	+	+ + +	– + –	–	+ +
K. pneumoniae	–	+ + +	– – –	+	+ +
K. edwarsii	–	+	– – –	+	+ +
K. pneumoniae	–	+	– – –	–	+

Fig. 1 Detection of amylase activity of isolate on starch agar plate

were taken from each experiment and assayed for amylase activity as described in Sect. "Amylase activity assay".

Conventional characterization of isolates

Pure culture of the isolate with the largest clear zone after flooding with iodine was cultured at 45 °C, and then identified by morphological characteristics, microscopic appearances, and biochemical tests [33]. The isolate was characterized by the Gram staining technique [34]. Various biochemical tests, such as endospore formation, motility, catalase, oxidase, citrate, and indole tests, were performed according to methods earlier described in the literature [35–39].

Molecular characterization of isolate

PCR amplification and 16S rRNA sequencing The extraction of the total genomic DNA was carried out following the manufacturer's instructions. Amplification of the 16S rRNA gene was conducted by using a pair of

Table 3 Morphological and biochemical characteristics of the selected amylase-producing bacterial isolate

Cells shape	Gram reaction	Spore formation	Oxidase	Catalase	Nitrate reduction	Citrate utilization	
+	+	+	–	+	+	–	
Indole	**Motility**	**H_2S production**	**Glucose**	**Maltose**	**Sucrose**	**Mannitol**	**Lactose**
-	+	–	+	+	+	–	+

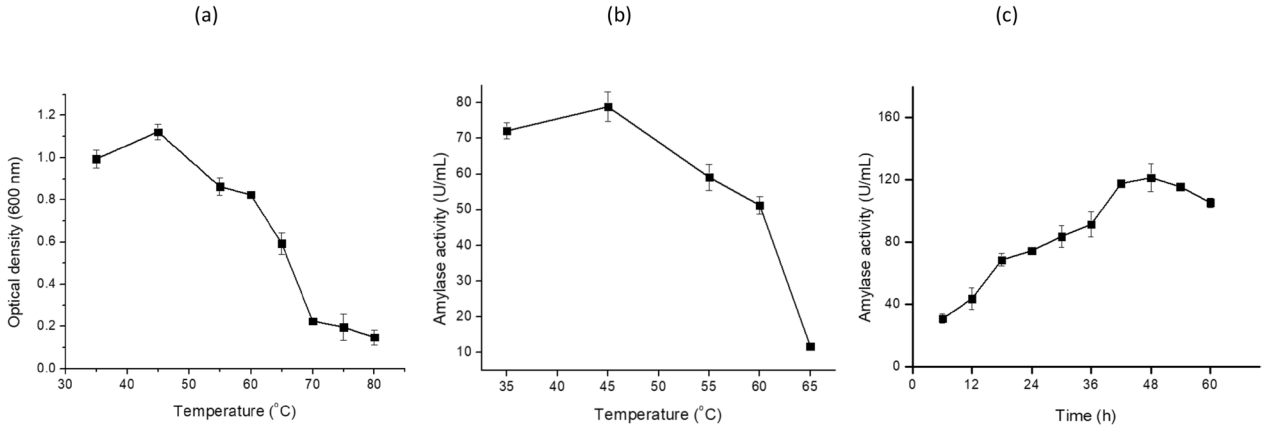

Fig. 2 Temperature effect on growth of the isolate (**a**), and the temperature (**b**) and incubation time (**c**) effects on amylase production (**C**)

universal primers 1492R (5′- TACGGYTACCTTGTT ACGACT T-3′) and the domain bacteria-specific primer 27F (5′- AGAGTTTGATCMTGGCTCAG-3′). All PCR reactions were carried out following the manufacturer's protocols of One Taq (New England Biolabs, USA) under the following conditions: initial denaturation for 5 min at 94 °C, 35 cycles of denaturation for 1 min at 92 °C, annealing for 1 min at 58 °C, extension for 1 min at 72 °C; followed by final extension for 10 min at 72 °C. The PCR products were separated by gel electrophoresis at 100 V for 30 min on 1X TAE and analyzed by staining with EZ vision under UV light on a trans-illuminator. The purified PCR products were then sequenced.

Phylogenetic analyses The nucleotide sequences generated were determined and assembled using Molecular Evolution Genetic Analysis (MEGA) X [40]. The sequence was compared with those available in GenBank using BLAST search (http://www.ncbi.nlm.nih.gov/blast) to retrieve homologous sequences from the database). All sequences were aligned with the CLUSTALW algorithm in BioEdit 7.2.6.1 (https://www.genome.jp/toolsbin/clustalw) [41].

Optimization of amylase production by statistical approach

Based on the outcome of the OFAT investigation, optimization was carried out with the combination of Box-Behnken Design (BBD) and RSM for three selected factors: substrate concentration, pH, and incubation period, to determine their individual and interactive effects on amylase yield, while keeping other factors constant. The experimental design consisted of a block with 17 conditions of three variables (A, B, and C) at three levels (−1, 0, and + 1). Table 1 shows the ranges (minimum and maximum) of the independent variables. Each experiment was carried out in triplicates, and the average amylase activity was reported.

Statistical analysis of the model

Regression equation analysis of Design-Expert 10.0.1 was employed to evaluate the response surface model. Multiple regressions were utilized to fit the coefficient of the polynomial model of the response and correlate the response variable to the independent variables. A second-order model was utilized to analyze this relationship. Test of significance and analysis of variance (ANOVA) was employed to analyze the quality of the fit of the model. Multiple regressions were employed to fit the coefficient of the polynomial model of the response to correlate the response variable to the independence factors. The fitted quadratic response model is given in Eq. (1).

$$Y = a_0 + \sum_{i=1}^{k} a_i X_i + \sum_{i=1}^{k} a_{ii} X_i^2 + \sum_{i<j}^{k} a_{ij} X_i X_j + e \quad (1)$$

where Y is the response variable (amylase yield), a_0 is the intercept value, a_i (i = 1, 2... k) is the first-order model coefficient, a_{ij} is the interaction effect, and a_{ii} represents the quadratic coefficients of X_i, and e is the random error.

Process variables optimization and model validation

The model equation obtained for the process was solved statistically by regression analysis using the Design Expert. The predicted optimal conditions for amylase production was validated in the laboratory. The experiment was carried out in triplicate, and the enzyme activity was estimated following the protocol described earlier.

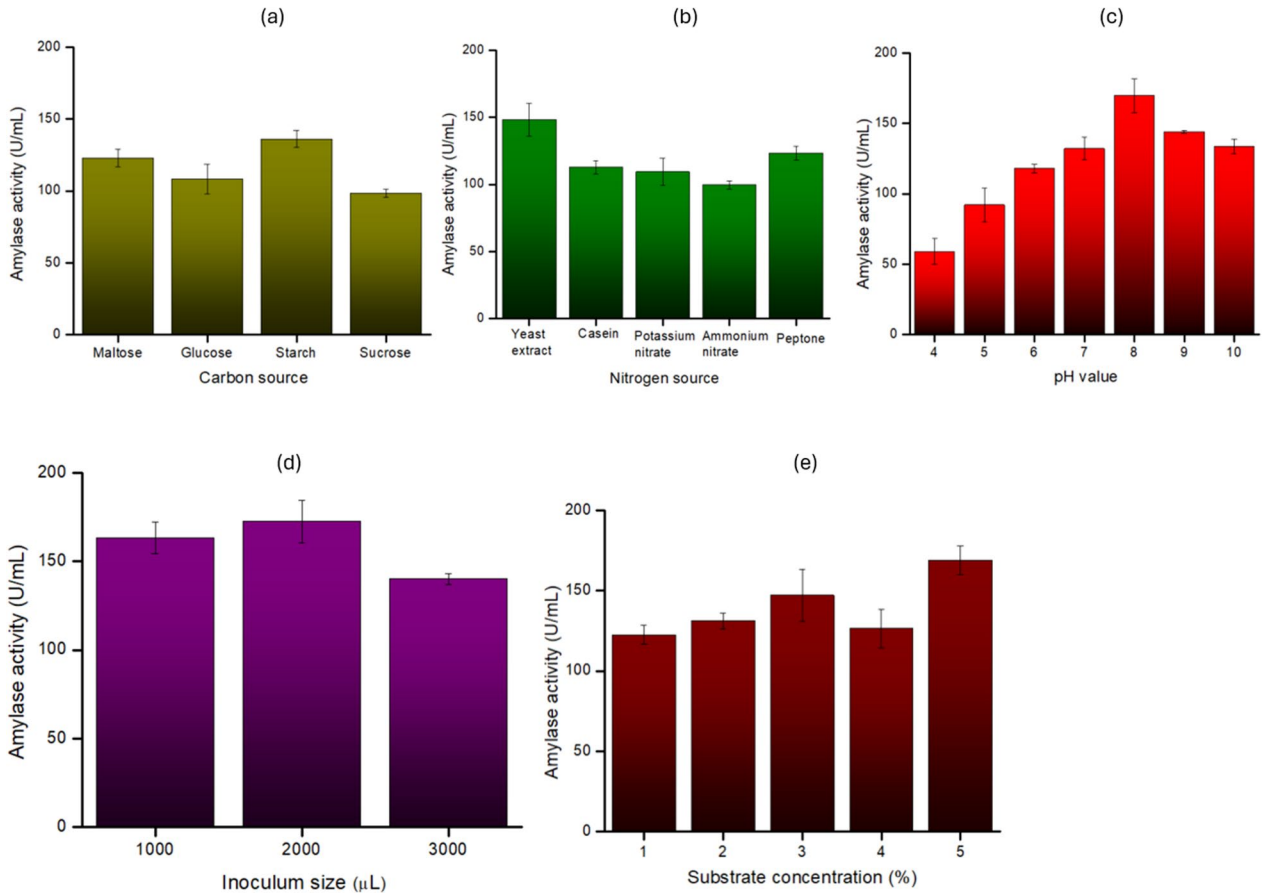

Fig. 3 Effects of growth parameters (**a**) carbon source, **b** nitrogen source, **c** pH, inoculum size, and substrate concentration on amylase production

Results and discussion

Isolation and identification of amylolytic bacteria strain

Visible growth was observed from the sample taken from the upper part of the spring. Plates on which growth was observed were kept for further use, while those with no visible growth were discarded. Five of the sixteen strains isolated from the warm spring water samples (Table 2) showed positive extracellular amylase production. Only one strain showed zone clearance ≥ 10 mm on the starch agar plates after flooding with Lugols' iodine solution, signifying the ability of the microorganisms for a relatively high amylase activity (Fig. 1). The isolate was identified as *B. cereus* based on various tests itemized in Table 3.

Previous studies have shown amylase-producing microorganisms isolated from various sources, including *B. cereus* from hot springs [12], *B. licheniformis* from the soil [42], *B. paramycoides* from brick kiln soil [43]. *B. licheniformis* from a hot spring [15, 44], *Bacillus sp.* from marine environments [45], and poultry feces soil samples [4]. The isolation and identification of microorganisms with excellent amylase-producing ability from new sources provide a platform for enzyme production that is more economically attractive and readily available to meet the world's ever-growing demand [46].

OFAT investigation results on amylase production

Results of the studies on various growth parameters that influence amylase production are presented in this section.

Temperature effect on growth and amylase production

The best temperature for the growth of the *B. cereus* was 45 °C, although it could thrive well between 35 and 65 °C with maximum temperature tolerance of up to 80 °C after which no visible growth was observed (Fig. 2a). The results of the investigation on the effect of temperature on amylase production are represented in Fig. 2b. The plot shows that the best temperature was 45 °C, the same as the best temperature established for the growth of the bacterium. There was insignificant amylase activity beyond 65 °C. The results revealed the mildly thermophilic nature of the isolate with amylolytic activity above

35 °C up to 60 °C followed by a sharp decline with an optimum of 45 °C.

A similar observation was obtained by Krishnan et al. [47], showing maximum amylase activity obtained at 45 °C by three different *bacillus* species (*B. licheniformis, B. firmis, and Paenibacillus glucanolyticus*) using both submerged fermentation and solid-state fermentation methods. Likewise, in a study by Pathania et al. [48], amylase production by *B. amyloliquifaciens* SH8 peaked at 16.07 U/mL at 45 °C under submerged fermentation. Studies have shown the potential of several *Bacillus* species for amylolytic enzyme production, including the *B. cereus* strains. A thermophilic *B. cereus* MK isolated from potato farmland in India produced a thermostable α-amylase at 55 °C under solid-state fermentation [49]. Various optimum temperatures ranging from 35 to 80 °C for amylase enzyme production by a plethora of *Bacillus* species have been reported in the literature [50].

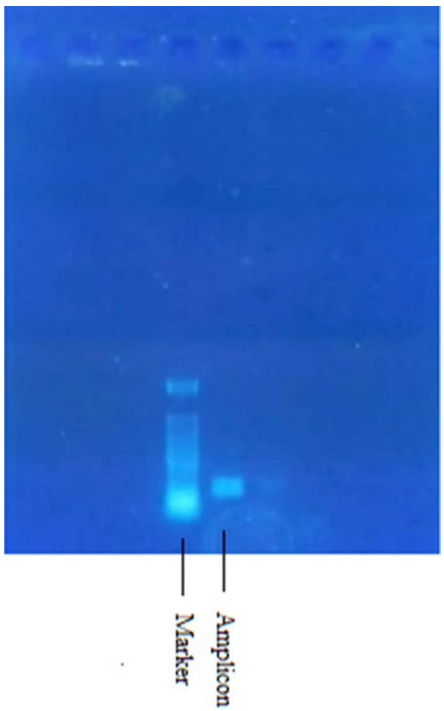

Fig. 4 Amplification of 16S rRNA gene from bacterial isolate on agarose gel containing ethidium bromide

Incubation time effect on amylase production

Figure 2c depicts the results obtained in this study for the effect of incubation time on amylase production. The amylase activity progressively increased from 6 h till it peaked at 48 h of cultivation (Fig. 2c). Maximum activity of 121.5 U/mL was achieved after 48 h, correlating to the stationary growth phase of the isolate. Hereafter, the amount of enzyme produced by the microorganism declined (Fig. 2c). Comparable results have been reported with maximum amylase activity from *B. amyloliquefaciens* after 48 h incubation under submerged fermentation conditions [51]. However, several studies have recorded different time frames for maximum amylase activity by fermentation. For instance, Simair et al. [45] recorded maximum amylase yield after 36 h fermentation, while Unakal et al. [52] and Singh et al. [42] both observed maximum production after 24 h fermentation. Microorganisms generally grow and build up necessary components during the log and lag phases of their growth cycle, while they usually begin the synthesis of secondary metabolites during their resting phase [53].

Carbon source effect on amylase production

The results of the different carbon sources on amylase production are displayed in Fig. 3a. The carbon sources tested in this study produced maximum amylase activity of 122.8, 108.1, 136.1, and 98.4 U/mL using maltose, glucose, starch, and sucrose, respectively. This observation shows that the bacterium preferred starch as

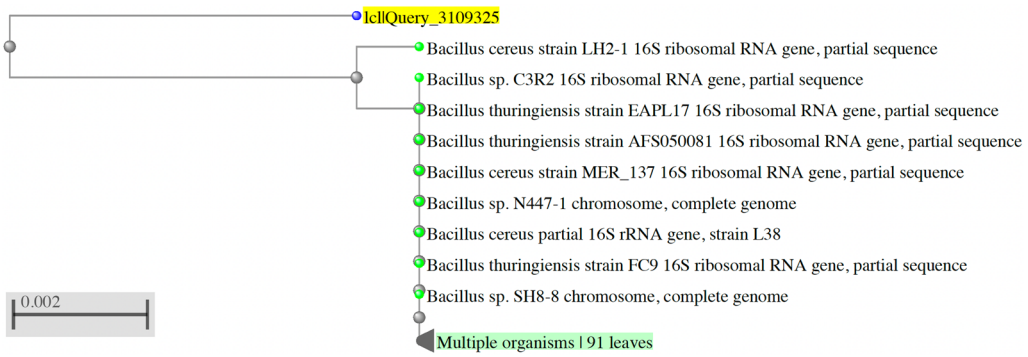

Fig. 5 Phylogenetic tree based on 16S rRNA sequence of isolate and its closest *Bacillus* species

its carbon source for maximum amylase production. Maltose and glucose could serve as good alternatives as they both supported an appreciable yield of amylase compared to starch, while sucrose, on the other hand, is not a desirable substrate for inducing amylolytic enzyme production. In another study, starch and maltose increased amylase activity by *B. licheniformis*, while glucose, sucrose, and fructose gave a lower yield, sucrose being the least [9]. Starch is the primary substrate for the induction of extracellular amylase enzyme [54], although the effect of carbon sources varies widely among many species of amylase-producing bacteria [55].

Table 4 BBD matrix with corresponding experimental and predicted amylase activity by *B. cereus*

pH	Incubation time (h)	Substrate concentration (%)	Actual activity (U/mL)	Predicted activity (U/mL)
4	48	3	13.68	9.72
7	48	5	199.16	192.72
7	48	5	191.11	192.7172
4	72	5	33.07	29.10
7	72	7	178.79	177.33
7	24	3	104.06	105.52
4	24	5	27.99	30.49
10	24	5	13.30	17.27
10	48	3	68.46	63.03
7	48	5	194.43	192.72
10	48	7	65.74	69.71
7	48	5	188.90	192.72
7	48	5	189.99	192.72
7	72	3	84.46	92.40
4	48	7	107.15	112.59
10	72	5	55.25	52.74
7	24	7	138.07	130.13

Nitrogen source effect on amylase production

The effects of the different nitrogen sources on amylase production are displayed in Fig. 3b. The amylase activity observed was 148.2, 112.7, 109.5, 99.5, and 123.3 U/mL using yeast extract, casein, potassium nitrate, ammonium nitrate, and peptone, respectively. The organic nitrogen sources (yeast extract, peptone, and casein) supported higher amylase production than inorganic nitrogen sources (potassium nitrate and ammonium nitrate). Yeast extract gave the best amylase activity, followed by peptone and casein. Many previous studies of *Bacillus* species have reported yeast extract as the best nitrogen source for amylase enzyme production [56, 57]. Lal et al. [9] found potassium nitrate to be the best of all the inorganic nitrogen sources tested, corroborating similar outcomes of this present study.

pH effect on amylase production

Figure 3c indicates that the *B. cereus* can synthesize amylolytic enzyme over a wide pH range (4–10) but, more significantly, above pH 6. The amylase activity recorded at pH 7 and 8 was 132.4 and 169.8 U/mL, respectively. The bacterium behaved alkalophilic. The activity obtained at a pH of 8–10 is higher than those recorded at acidic pH. In various literature, multiple optima pH was observed for amylolytic activities in crude amylase preparations. For instance, a pH of 8 was also recorded as the optimum for

Table 5 Significance level and ANOVA for amylase production process

Source	Sum of squares	df	Mean square	F-value	p-value
Model	78,099.67	9	8677.74	181.86	< 0.0001
A-pH	54.36	1	54.36	1.14	0.3212
B-incubation time	580.61	1	580.61	12.17	0.0102
C-substrate concentration	6000.55	1	6000.55	125.75	< 0.0001
AB	339.83	1	339.83	7.12	0.0321
AC	2313.31	1	2313.31	48.48	0.0002
BC	909.65	1	909.65	19.06	0.0033
A^2	52,298.85	1	52,298.85	1096.00	< 0.0001
B^2	10,054.08	1	10,054.08	210.70	< 0.0001
C^2	1290.47	1	1290.47	27.04	0.0013
Residual	334.02	7	47.72		
Lack of Fit	264.98	3	88.33	5.12	0.0744
Pure Error	69.05	4	17.26		
Cor Total	78,433.70	16			

df degree of freedom

amylase production by *Bacillus* sp. [58, 59], *B. cereus* [60], *B. licheniformis*, and *Paenibacillus glucanolyticus* [47]. Contrasting results were obtained by Halder et al. [61], with an optimum pH of 6.5 for amylase production by *B. cereus* and an optimum pH of 9 by *B. licheniformis* [44]. pH of the fermentation medium plays a crucial role in the growth and enzyme secretion of the cultured microorganisms by inducing morphological changes in the microbial cell [62].

Inoculum size effect on amylase production

The effect of different inoculum sizes (1000–3000 µL) on amylase production was determined in cultures grown at 45 °C and pH 8.0 for 48 h (Fig. 3d). Increasing the inoculum size affected the amount of enzyme obtained from the fermentation media. When the inoculum size was doubled, amylase activity increased from 163.3 to 172.6 U/mL, representing a 5.7% increase in the amylase activity recorded at 1000 µL inoculum volume (Fig. 3d). Further increase in the inoculum size led to a reduction in the enzyme activity. Sharif et al. [59] reported an optimum inoculum size of 900 µL for amylase production from a *Bacillus* sp. This could be due to the overpopulation of the cells compared to the nutrients available to them. Various levels of inoculum sizes (0.5–10% v/v) have been reported [45, 63].

Substrate concentration effect on amylase production by B. cereus

The effect of starch concentration on amylase activity was examined using peptone as the nitrogen source. The results obtained showed a significant impact on the amylase activity (Fig. 3e). Starch is an important media component for the growth and amylase synthesis of the *bacillus* strain. Therefore, it is crucial to ascertain the quantity required for the maximum of the enzyme. Amylase activity increased continuously as the concentration increased, reaching a maximum of 168.9 U/mL at 5% except at 4% concentration, where a sudden decrease was observed (Fig. 3e). Sharif et al. [59] observed increasing amylase activity from 0.5 to 1.5% substrate concentration, but a reduction occurred at 2%.

Table 6 The model fit statistics

Parameter	Value
R^2	0.9957
Adjusted R^2	0.9903
Predicted R^2	0.9446
Adequate precision	34.5408
Mean	109.04
Standard deviation	6.91
Coefficient of variance, CV (%)	6.34

Molecular characterization of the isolate

The molecular identification of the amylase-producing bacterial isolate was achieved by sequencing part of the 16S rRNA. The amplification of the 16S rRNA was confirmed by agarose gel electrophoresis (Fig. 4). The PCR product of the isolate was gel-eluted and sequenced. This was followed by BLAST analysis of the sequence data of the 16S rRNA of the isolate. The 16S rRNA gene sequence provides an accurate grouping of microorganisms even at the subspecies level; it is considered a powerful tool for rapidly identifying bacterial species [64].

The phylogenetic analysis of the 16S rRNA sequence of the isolates, along with the sequence retrieved from the National Centre for Biotechnology Information (NCBI), was carried out with MEGA X using the neighbor-joining method. The results of the phylogenetic analysis showed distinct clustering of the isolate with *B. cereus* (Fig. 5). The 16S rRNA sequence of the isolate demonstrate high resemblance to members of the genus *Bacillus*, showing 98.88% similarity with *Bacillus cereus* strain LH2-1 (GenBank Accession number HM003220), *Bacillus sp.* C3R2 (GenBank Accession number GQ228624), *B. thuringiensis* strain EAPL17 (GenBank Accession number JX500188), *B. thuringiensis* (GenBank Accession number OP986676), and *B. cereus* strain MER_37 (GenBank Accession number KT719715). The sequence analysis of 16S rRNA showed very high identity to *B. cereus*, confirming the results of the morphological and biochemical tests on the isolate (Table 3).

Optimization of amylase production by *B. cereus* using BBD

To maximize the amylase enzyme production by the *Bacillus* isolate, BBD was employed to investigate the individual and interactive effects of the three factors selected from the preliminary optimization with the OFAT method. These factors, initial pH, incubation time, and substrate concentration being the most significant factors affecting enzyme production in the submerged fermentation, were considered the independent variables, and their effects on amylase production were established.

Model fitting and statistical analysis

Table 4 shows the BBD experimental plan with the observed and predicted responses for the experiments carried out in the laboratory. The effect of the variables (initial pH, incubation time, and substrate concentration) on amylase activity (U/mL) was fitted by the second-order polynomial regression equation given by Eq. (2).

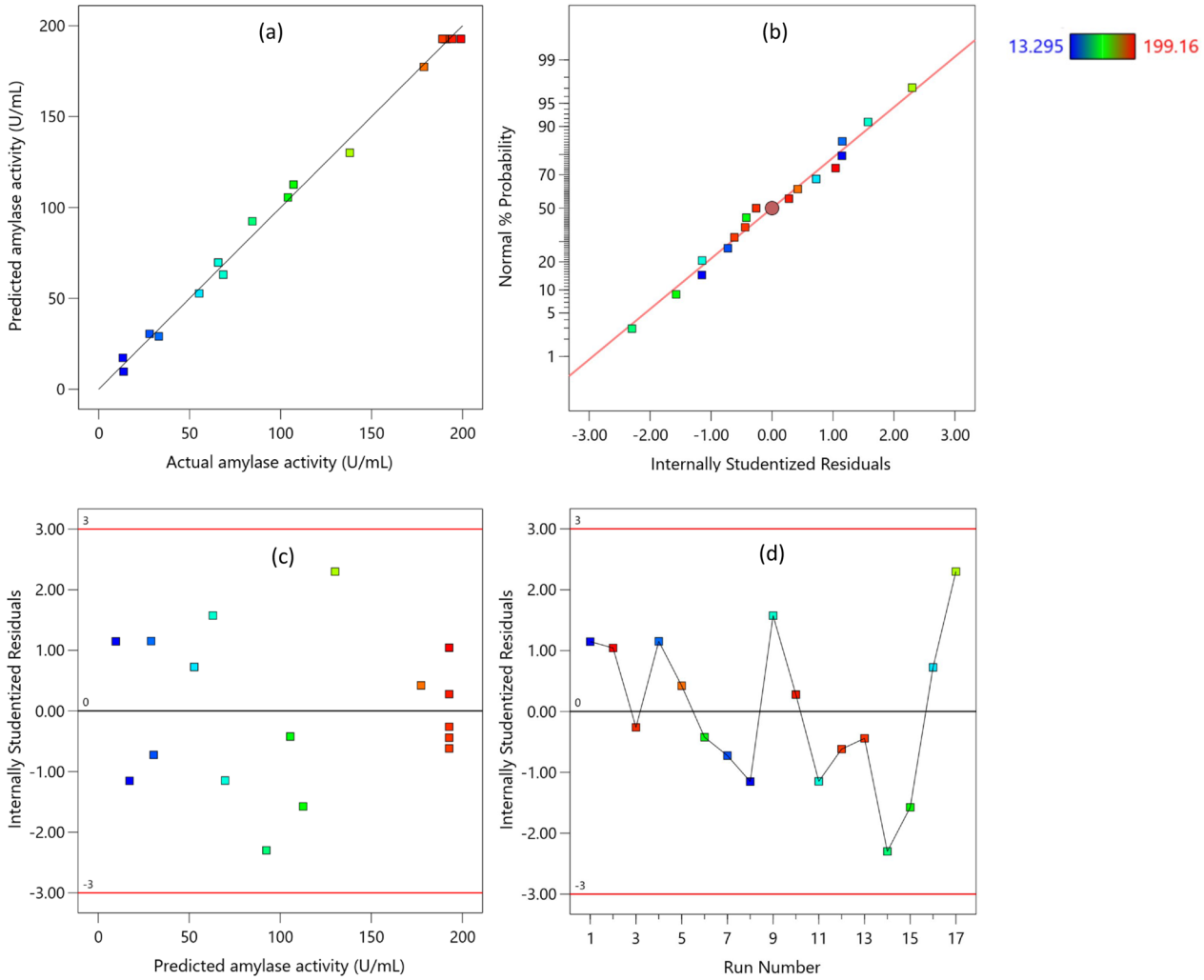

Fig. 6 Diagnostic plots for the model, **a** parity plot, **b** standard probability plot of residuals, **c** residual vs. predicted activity, and **d** residual vs. run number

$$Y = 192.72 + 2.61A + 8.52B + 27.39C + 9.22AB - 24.05AC + 15.08BC - 111.45A^2 - 48.87B^2 - 17.51C^2 \quad (2)$$

where Y is enzyme activity (U/mL), A is the pH, B is the incubation time (h), and C is the substrate concentration (%).

The statistical significance of the second-order quadratic model equation was evaluated by F-value, probability (p-value), and ANOVA of the response surface, as laid out in Table 5. The significance level of each coefficient was determined by their p-values ($p < 0.05$ is significant). The significance of the model was confirmed based on an F-value of 181.86 and a very low probability value for the response ($p < 0.0001$). The F-value value explained the experimental data distribution around the fitted model. There was a probability of only 0.01% that a large model F-value could occur due to noise. The p-values of the linear effects of variables B and C and the two-level interaction of all the variables (AB, AC, and BC), as well as all quadratic terms (A^2, B^2, and C^2), demonstrated their significance on the response (Table 5). The model term A (pH) was not significant in the present study, with a p-value = 0.3212 (Table 5). On the other hand, the lack of fit F-value of 5.12 indicates it is not significant compared to the pure error [65], which is suitable for the model to fit. The p-value is greater than 0.05 ($p = 0.0744$), which is desired because the aim is to have a model that fits the experimental data [24].

The fit statistics for the model are presented in Table 6. The coefficient of determination (R^2) was almost unity, depicting a good model fit [66]. This high value of R^2 (0.9957) showed the efficacy of the developed model in

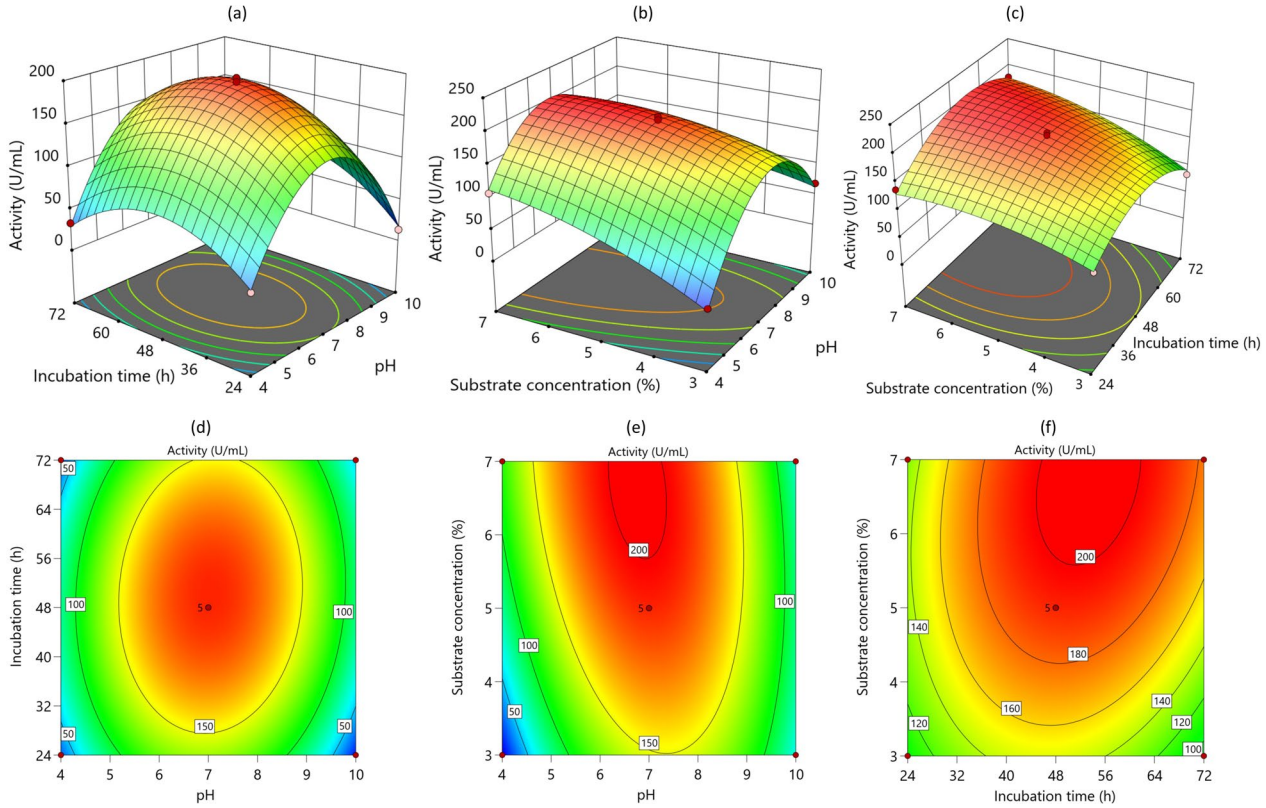

Fig. 7 Surface and contour plots of the parameter interactions on enzyme activity

satisfactorily describing the system behavior within the investigated range of operating parameters. The predicted R^2 of 0.9446 agreed with the adjusted R^2 of 0.9903 since the difference is < 0.2 [67]. In like manner, a low coefficient of variation (CV) of 6.34% implied a reliable and precise experiment since the value is < 10% [24]. Additionally, an adequate precision ratio of 34.541, which compared the range of the predicted values at the design points to the average prediction error, also indicates an adequate signal, confirming that the model is suitable for maneuvering the design sphere. This ratio must be greater than 4 for desirability [67].

Furthermore, the diagnostic plots for evaluating the model are shown in Fig. 6. The parity plot shows a good agreement between the predicted and actual data (Fig. 6a), where the data points were well distributed close to the trendline [68]. The normal probability plot against the internally studentized residuals in Fig. 6b shows that the data follows a straight line and not an S-shape, suggesting a normal distribution of the residuals [68]. The standard deviation between the experimental and predicted values on a normal distribution indicates no significant violations of critical model assumptions (Fig. 6b). These assumptions include normal distribution and independence of errors, homogeneous error variances, and independence of residuals. Adherence to these assumptions is also observed in Fig. 6c and d. The pattern of the plot (Fig. 6c) must appear random rather than systemic [69], i.e., with an even spread, and the plots must fall within a horizontal band centered at zero. Based on this observation, and in Fig. 6d, with no evident outlier beyond -3 and 3 limits, it can be concluded that the model accurately described the process behavior within the investigated range of operating parameters for amylase enzyme production [70].

Interactive effects of growth condition parameters

The relationship between response and growth condition parameters was visualized by the three- and two-dimensional contour and surface plots (Fig. 7). There was significant variation in amylase production as the levels of independent variables changed. The maximum enzyme production was observed at the central values of these variables. The surface of Figs. 7a and d suggests there are well-defined optimal solutions. Maximum enzyme activity was obtained when both variables were at their chosen central values. A gradual reduction in the amylase activity was observed as the pH of the medium and incubation

Table 7 Comparative survey of enhanced amylase production by different optimization methods

Microbe	Optimisation method	Carbon source	Nitrogen source	Optimized parameters	Amylase activity (U/ml)	References
B. cereus	OFAT and BBD	Starch	Yeast extract	pH—7, time—48 h, SC—5%, T—45 °C	196.02	This Study
B. brevis	CCD	Cassava bagasse	Beef extract	pH—7, time—36 h, MHC—60%, T—60 °C	4667	Ray and Kar [75]
B. licheniformis	BBD	Starch	Yeast extract	NC—0.75%, $CaCl_2$—0.02%, SC—1%	384	Abdel-Fattah et al. [76]
Bacillus sp.	BBD	Starch	Yeast extract	pH—6, time—24 h, AR—130 rpm, T—35 °C	145.32	Khusro et al. [4]
B. subtilis	Non-statistical	Rice flour	Peptone + Tryptone	pH—8, time—24 h, SC—1.25%, T—37 °C	9	Dash et al. [77]
B. sonorensis	CCD	Starch	Ammonium sulphate	pH—9.5, time—72 h, SC—0.5%, T—50 °C	82.78	Vyas and Sharma [78]
B. licheniformis	OFAT and PBD	Starch	Yeast extract	pH—7, time—30 h, AR—160 rpm, T—40 °C	13.44	Abel-Nabey and Farag [79]
Bacillus sp.	2^4 factorial	Starch	Peptone	pH—6.2, NC—8.77 g/l, SC—17.58 g/l, T—37 °C		Tanyildizi et al. [80]
Bacillus sp.	PBD and CCD	Starch	Ammoniun sulphate	pH—8.33, NC—0.55%, SC—0.65%, T—50 °C	122	Singh et al. [81]
B. amyloliquifaciens	CCD	Starch	Yeast extract	pH—5, time—5 days, SC—0.60%, T—45 °C		Pathania et al. [48]

CCD central composite design, *PBD* Plackett–Burman design, *SC* substrate concentration, *MHC* moisture holding capacity, *T* temperature, *NC* nitrogen concentration, *AR* agitation rate

time were further increased. Maximum amylase activity was observed at a pH of 7 and incubation time of 48 h (Figs. 7 a and d).

Media pH is one of the most critical factors affecting microbial enzyme production in fermentation processes by generating morphological change in the microbe and enzyme secretion and the product [71]. This is apparent in the surface plot (Fig. 7b), showing the main and interactive effect of pH and substrate concentration. The main impact of the increment in pH followed a similar trend to the one observed in the previous plot (Figs. 7 a and d). The surface plot shows that an increment in pH caused an increase in amylase activity up to a certain maximum (pH of 7), beyond which further increment resulted in a decrease in amylase activity. However, the maximum response was achieved at the highest substrate concentration (Figs. 7 b and e). The contour plot revealed the maximum enzyme activity at the highest substrate concentration and incubation time of 48 h (Fig. 7e).

Figures 7c and f show the surface and contour plots between the incubation time and substrate concentration. At high substrate concentration, the effect of the incubation time reached an optimum at 48 h. The contour plot revealed the maximum enzyme activity at the highest substrate concentration and incubation time of 48 h (Fig. 7f). The interactions observed in this study have been reported in the literature [72–74].

Model optimization and validation test

The model was used to predict the conditions for the maximum amylase activity by the *B. cereus.* The optimal submerged fermentation medium conditions predicted were pH of 7, substrate concentration of 5% (w/v) of soluble starch, and incubation time of 48 h while keeping the incubation temperature at 45 °C with an amylase activity of 192.72 U/mL. The conditions were used to conduct the triplicate sets of shake flask experiments in the laboratory with an average amylase activity of 196.02 U/ml. This confirmed the efficacy of the model to adequately describe the microbial enzyme production process and give accurate predictions of the response. A comparison of the results of different optimization methods for enhanced amylase production is presented in Table 7. The amylase activity observed in the study was significantly higher than most of the literature values (Table 7).

Conclusions

Microorganisms that can produce amylolytic enzymes are ubiquitous. This present study has successfully isolated and identified as *B. cereus* from the Ikogosi warm spring water. This bacterium demonstrated the potential for amylase enzyme production owing to its ability to withstand high-temperature conditions and wide pH operability. OFAT established optimal physical parameter conditions as the starch concentration of 5% w/v, inoculum volume of 2% v/v, pH of 8, temperature of 45 °C, and 48 h of incubation, leading to amylase activity of 172.6 U/mL by the isolated *B. cereus*. A second-order mathematical model was developed for the process of amylase production with high accuracy, reflected in high R^2 (0.9957). The study also suggests that the amount of starch concentration, incubation period, and pH significantly affected the amylase production via submerged fermentation. This optimization method adopted in this study was used to successfully maximize amylase production up to 1.2-fold (i.e., 196.02 U/mL) under the submerged fermentation medium condition of pH of 7, substrate concentration of 5% w/v of soluble starch for 48 h and at temperature of 45 °C.

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Author contributions
OAA: Investigation, Methodology, Writing—Original draft preparation; BOS: Conceptualization and Supervision; JAO: Methodology; EB: Supervision, Methodology, Writing- Reviewing and Editing.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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