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Optimization of α-Amylase Production by *Enterobacter cloacae* Strain D1 Isolated from Cassava Effluent-impacted Soil Using Response Surface Methodology

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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

Background: Production of amylase by *Enterobacter cloacae* D1 was optimized in this study using central composite design (CCD) of response surface methodology (RSM).
Methodology: Effects of five numeric factors (pH, temperature, inoculum concentration, peptone and yeast extract) on the production of amylase were examined. Amylase production was first screened using plate technique and amylase assay thereafter carried out using the dinitrosalicylic acid (DNSA) method. The CCD-RSM experimental set-up involved 30 runs with 5 levels of independent variables.
Results: The amylase-producing bacterium *Enterobacter cloacae* strain D1 was identified based on the phylogenetic tree analysis of its sequence. The sequence has been submitted to GenBank under the accession number: MZ477010. The isolate had 98% similarity to the GenBank match

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Enterobacter cloacae strain ATCC 13182. Optimum conditions that yielded maximum amylase (34.43 U/mL) were pH 5; temperature 40 ℃; inoculum concentration 3%; peptone 1.2% and yeast extract 0.5%.

Conclusion: This study has demonstrated efficient amylase production from *Enterobacter cloacae* strain D1 isolated from cassava effluent-impacted soil from Rumuosi, Port Harcourt, Rivers State. In addition, optimization of the critical factors of amylase production resulted in 3.4 fold increase in amylase activity. The enhancement of amylase production by the RSM techniques shows that amylase from this strain can be scaled-up for industrial application.

Keywords: α-Amylase; Response Surface Methodology (RSM); Enterobacter cloacae; optimization.

1. INTRODUCTION

Alpha-Amylases (E.C.3.2.1.1) represent a group of starch-degrading enzymes. They are routinely produced from plants, animal and microorganisms. However, microbial production of α-amylase has recently flourished due to the ease of associated downstream processes [1]. "From a biochemical perspective, alpha-amylases (E.C.3.2.1.1) catalyse the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose and maltotriose units" [2-4]. They are one of the most important industrial enzymes and have been applied in fermentation, pharmaceutical, detergent, textile, paper and food industries [5].

Many bacteria with capacity for α-amylases production have been reported [6]. Majority of α-amylases-producing bacteria belong to the genus *Bacillus* with the following species: *B. amyloliquefaciens, B. licheniformis,* and *B. stearothermophilus*, frequently reported [6]. However, besides species of *Bacillus*, other bacteria have been implicated; some of these bacteria include bacteria from stressed or extreme environment. These environments can specifically yield α-amylase with specific biotechnological characteristics [7,8]. For example, "the most common sources of thermostable α-amylase are Geobacillus bacterium isolated from Manikaran hot springs. The thermophilic alpha-amylases (*BLA*) have been shown to have more structural flexibility than mesophilic alpha-amylases (*BAA*)" [6]. Other bacteria have been also reported as efficient α-amylase producers and these include Nesterenkonia sp. and Pseudoalteromonas sp. However, starch-rich environments have emerged as major source of amylase-producing bacteria, with many studies reporting efficient amylase producers from such environment [9,10].

A major challenge confronting microbial α-amylase production is the simulation of appropriate nutritional and cultural conditions for the incubation of source microorganisms. Medium composition and physical conditions have been reported to critically influence the production of alpha-amylase. Some of these cultural and nutritional conditions include carbon sources [11,12], nitrogen sources [13], temperature (thermal stability) [14], pH [14-16], etc. In response to this obvious challenge, many biotechnological approaches have been devised to improve the production of α-amylase from microorganisms. This approaches manipulate the metabolic requirements of the producing microbe for enhanced production. A biotechnological approach commonly applied is optimization technique - optimization techniques leverage on the manipulability of bacterial metabolic factors and operating conditions for the production of desired products and metabolites [17].

Optimization of medium parameters is a technique commonly employed to enhance the recovery of useful metabolites from production medium by conscious manipulation of fermentation parameters with resultant improved production of desired products [18]. Conventional optimization techniques consumes time and does not account for the interactions that exist among variable factors. Response surface methodology (RSM) as a statistical tool according to Othman et al. [19] and Rehman et al. [20], "can efficiently enhance enzyme production by screening larger number of significant factors and simultaneously evaluating the interaction between parameters thereby selecting the suitable conditions for the optimum response". "Therefore, RSM reduces the number of individual experiments desired for providing information on the interactions between different variables to define the most significant ones" [21]. This study therefore employed RSM in the optimization of α-amylase production by Enterobacter cloacae strain D1 isolated from cassava effluent- impacted Soil.

2. MATERIALS AND METHODS

2.1 Sample Collection and Processing

Cassava effluent-impacted soil samples were collected from Rumuosi, Port Harcourt, Rivers State, Nigeria. The soil was air-dried for 24 h, sieved to remove debris and thereafter used to isolate amylase-producing bacteria.

2.2 Isolation and Screening of Amylolytic Bacteria

Serial dilution was performed on the soil samples as described by Jalal et al. [22]. Nine (9) millilitres of normal saline (0.85% of NaCl w/v in distilled water) was dispensed into clean test tubes and the test tubes were sterilized in an autoclave at 121^{o}C (15 psi) for 15 min and then allowed to cool. A gram of dry soil sample was dissolved in the 9 ml sterile normal saline to make a stock solution. From this stock solution several (10^{-1} to 10^{-6}) dilutions were made. The serially diluted soil sample was spread plated on Nutrient Agar (HiMedia, India) and incubated at 35 ^{o}C for 48 h.

Discrete colonies after 48 h of incubation were sub-cultured on starch agar (HiMedia, India) and incubated at ambient temperature for 48h Bacteria that showed clearance zones on the agar plates were selected as amylase producers. These amylase producers were purified on nutrient agar plate and stored in an new agar slant containing the minimal medium.

2.3 Alpha Amylase Assay

2.3.1 Amylase production medium

A loopful of bacterial culture was transferred from starch-nutrient agar slants to starch- nutrient broth at pH 7 for cultivation and incubated in a shaker at 40 ^{o}C at 120 rpm for 24 h. Fermentation medium described by Vaidya and Rathore (2015) was used; the medium contained soluble starch (10 g/L) peptone (5 g/L), $(NH_4)_2$ SO4 (2 g/L), KH_2PO_4, (1 g/L), K_2HPO_4 , (2 g/L), $MgCl_2$, (0.01 g/L) at pH 7.The fermentation medium was inoculated with the bacterial culture (10% v/v) and incubated in shaker incubator set at 37 ^{o}C for 24 h. At the end of the fermentation period, the culture medium was centrifuged at 10,000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

2.3.2 Enzyme assay

Amylase activity was assayed as described by Vaidya and Rathore (2015) with some modifications. In brief, 1.5 ml of 1% starch in 2 ml, 0.1M phosphate buffer (pH 6.5) and 0.5 ml of diluted enzyme were incubated for 15 min at ambient temperature. The reaction was stopped by the addition of 1ml of DNS reagent and then placed in a boiling water bath for 10 min. Thereafter, the content was diluted with 8 ml of distilled water. The absorbance was measured at 540 nm against blank prepared as above without incubation. One unit of α-amylase activity was defined as the amount of enzyme that liberated 1µmole of reducing sugar (maltose equivalents) per minute under the assay conditions.

2.4 Optimization of Amylase Production by *Enterobacter cloacae* D1 Based on Central Composite Designs of RSM

The interactive effect of different variables on the production amylase by *Enterobacter cloacae* strain D1 was studied through response surface methodology based on the method previously described by Aloulou et al. [23]. The half fraction type of central composite design (CCD) was employed to study the effect of five (5) numeric factors (pH, temperature, inoculum concentration, peptone concentration and yeast extract concentration) on a response variable (amylase activity) with each factor set to 5 levels: plus and minus alpha (axial points), plus and minus 1 (factorial points) and the centre point. A set of 30 runs consisting of 26 non-centre points (2 axial and 24 fractional points) and 4 centre points were carried out. The ranges and levels of the components (independent variables) for the experiment are given in Table 1. Each factor in the design was studied on the five levels (– α, -1, 0, + 1 and + α) with zero as the central coded value. These levels were based on results obtained from preliminary experiments. The optimum values from the CCD were obtained by solving the regression equation and analysing the response surface contour plots. Analysis of variance (ANOVA) with 95% confidence was employed in determining factors or combination of factors with significant effects. ANOVA, determination of regression coefficients and the graph construction were carried out using Design -Expert® version 13.0. The experimentdesign behaviour can be described based on the following second-order polynomial equation (Eq. 1).

$$Y = \beta_0 + \sum_{i=1}^{K} \beta_i x_i + \sum_{i=1}^{K} \beta_{ii} x_i^2 + \sum_{i=1}^{K-1} \sum_{i<j=2}^{K} \beta_{ij} x_i x_j + e \quad (1)$$

Where:

Y represents dependent variable, x_i and x_j independent variables, β_0, β_i, β_{ii} and $\beta_{ij,}$, the model's regression coefficients and e, model's error.

Estimation competence of the process was tested by comparing actual response with predicted response as generated from RSM. ANOVA and R^2 statistic were used to evaluate any significant differences between various factors and the model's adequacy, which is best when close to 1. Lack-of-fit (a model's adequacy test tool), was employed to compare the pure error from measurement replications to the lack of fit from the performance of the model.

F-value (the ratio of the lack-of-fit mean square to the pure error mean square), was calculated to determine the significance of the lack-of-fit. Validation of the statistical model was based upon amylase production at Erlenmeyer flasks' level under the predicted conditions by the model. Sampling was carried out at desired intervals and amylase activity determined.

2.5 Molecular Identification of Isolates

DNA extraction, PCR amplification of the bacterial 16S rRNA gene and gel electrophoresis of the extracted DNA were carried out at Regional Centre for Biotechnology and Bioresources Research (South-South Zonal Centre for Excellence), Port Harcourt Rivers State, Nigeria. The PCR products were sent to Inqaba Biotech West Africa, Oyo State Nigeria where the Sanger Sequencing was carried out.

Genomic DNA was extracted directly from the isolate using Quick-DNA™ Fecal/Soil Microbe Microprep Kit (Inqaba, USA) following the manufacturer's instruction.

Polymerase chain reaction amplification of 16S rDNA gene was carried out using the primer set 27F (5'- AGA GTT TGA TCM TGG CTC AG-3')′, and 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3'). The method described by Yamada et al. [24] and Katsura et al. [25] were employed. Twenty microlitres (20 µl) reaction mixture containing 1X PCR buffer (Solis Biodyne, Estonia), 1.5 mM Magnesium chloride (Solis Biodyne, Estonia), 0.2 mM of each dNTP (Solis Biodyne, Estonia), 2 U Taq DNA Polymerase (Solis Biodyne, Estonia), 20 pMol of each primer and sterile water was used to make up the reaction mixture. PCR was carried out in an Eppendorf Nexus thermal cycler with the following cycling parameters: an initial denaturation step at 95°C for 5 min, followed by 30 consecutive cycles of denaturation at 95°C for 30 sec., annealing at 55°C for 45 sec., and extension at 72°C for 1 min. After this, a final extension at 72°C for 10 min was carried out.

The PCR reaction was followed by separation of PCR product on a 1.5 % agarose gel (Solis Biodyne, Estonia). The 100 bp DNA ladder (Solis Biodyne, Estonia) was used as DNA molecular weight marker. Electrophoresis was carried out at 80 V for 40 min, and the gel thereafter visualized under UV light after staining with EZ-Vision Blue Light DNA dye (VWR Life Science, USA.)

The sequences generated by the sequencer were visualized using Chromaslite for base calling. BioEdit was used for sequence editing, before performing a Basic Local Alignment Search Tool (BLAST) using NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Similar sequences were downloaded and aligned with ClustalW and phylogenetic tree was drawn with MEGA X software [26].

Table 1. Range and levels of experimental variables for amylase production by *Enterobacter cloacae* strain D1

Factors	Levels				
	-α	**-1**	**0**	**+1**	**+ α**
pH	3	5	7	9	11
Temperature (^oC)	25	30	35	40	45
Peptone (%)	1	3	5	7	9
Inoculum(%)	0.6	0.8	1	1.2	1.4
Yeast Extract (%)	0.2	0.3	0.4	0.5	0.6

3. RESULTS

3.1 Plate Hydrolysis and Amylase Screening Characteristics of Bacterial Isolates

The screening result of the 16 bacterial isolates is presented in Table 2 and Table 3. From data obtained, the maximum amylase activity (11.51 U/mL) was observed with isolate D1, which was later characterized as *Enterobacter cloacae* strain D1. Only 4 isolates showed amylase activity out of the 16 bacterial isolates. Isolate D1 was selected and utilized for further studies.

3.2 Optimization of Amylase Production by *Enterobacter cloacae* D1 Using RSM – CCD

3.2.1 Composition of various experiments of the CCD for independent variables and responses

The composition of various experiments for the optimization of amylases production by Enterobacter cloacae D1 using CCD with independent variables (pH, temperature, inoculum concentration, peptone concentration and yeast extract concentration) and response variable (amylase activity) are presented in Table 4. The table reveals the actual and predicted values for amylase production by the isolate D1 D1.

3.2.2 Model fitting and ANOVA for the production of amylase by *Enterobacter cloacae* D1

Summary of ANOVA for response surface quadratic models for amylase production by *Enterobacter cloacae* D1 is given in Tables 5 and 6. Respective Model F and P values of 10.21 and 0.0006 imply that the model is significant. There is only a 0.06% chance that an F-value this large could occur due to noise. In this case E, AD, BD, BE, CE, B^2 are significant model terms. Lack of fit F and P values of 3.98 and 0.1421 imply that the Model was not significant in relative to the pure error. There is a 14.21% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good, as it implies that that the model can fit properly.

The Predicted R^2 of 0.0528 is not as close to the Adjusted R^2 of 0.8640 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with the model and/or data. There was adequate signal from the model as Adeq Precision, which measures the signal to noise ratio had a ratio of 15.567. Values greater than 4 are desirable thus, the model can be used to navigate the design space.

Table 2. Screening characteristics of amylase-producing bacteria isolated from cassava effluent-impacted soil

Isolate code	Zone of clearance on starch agar (cm)	Amylase activity (U/mL)
A1	-	-
A2	1.6	1.05
A3	-	-
A4	-	-
A5	-	0.8
A6	-	-
B1	1.1	0.85
B2	-	-
B4	0.38	0.6
B6	-	-
C1	-	-
C3	-	
C5	-	-
D1	9.24	11.51
D2	-	-
D3	-	-

Table 3. Morphological, biochemical, molecular and hydrolytic characteristics of the bacterial isolate

	Isolate code	**D1**
Biochemical	**Colony morphology**	**White, mucoid, large, with entire edge**
	Gram Stain	- (rod)
	Citrate	+
	Motility	+
	Oxidase	-
	Catalase	+
	Indole	-
	Urease	-
	Methyl Red	-
	Vogue Proskauer	+
	TSI	
	Slant	A
	Butt	A
	H_2S	-
	Gelatin hydrolysis	-
	Sugar Oxidation	
	Maltose	+
	Glucose	+
	Lactose	+
	Mannitol	+
	Sucrose	+
	Probable genus	*Enterobacter*
Molecular	**Accession number**	**MZ477010**
	GenBank Closest match	***Enterobacter cloacae* DS3**
	% identity	**98**
Starch hydrolysis and amylase actviity	Halo zone (cm^2)	9.24
	Maltose released (µmol/mL)	11.67
	Amylase activity (U/mL)	7.78

Table 4. Composition of various experiments of the CCD for independent variables and responses (actual and predicted) by *Enterobacter cloacae* D1

Run	**A:pH**	**B:Temp. (°C)**	**C:Inocul -um (%)**	**D: Peptone (%)**	**E: Yeast extract (%)**	**Amylase activity-FC (U/mL)**	
						Actual	**Predicted**
1	9	40	3	1.2	0.3	30.67	30.7
2	9	40	7	1.2	0.5	32.86	32.85
3	9	30	3	0.8	0.3	32.66	32.92
4	5	40	7	0.8	0.5	30.61	30.69
5	9	30	7	0.8	0.5	32.16	32.38
6	7	35	5	1	0.2	32.31	31.71
7	7	35	5	1	0.4	32.53	32.34
8	5	30	3	0.8	0.5	31.74	31.79
9	11	35	5	1	0.4	33.04	32.77
10	9	40	7	0.8	0.3	30.56	30.85
11	9	30	3	1.2	0.5	30.53	30.5
12	7	35	5	1	0.6	33.37	33.52
13	5	40	3	1.2	0.5	34.43	34.25
14	7	45	5	1	0.4	29.39	29.41
15	5	40	7	1.2	0.3	31.94	32.1

Run	A:pH	B:Temp. (°C)	C:Inocul -um (%)	D: Peptone (%)	E: Yeast extract (%)	Amylase activity-FC (U/mL)	
						Actual	Predicted
16	7	35	9	1	0.4	33.32	32.79
17	5	30	7	0.8	0.3	32.26	32.65
18	7	35	5	1	0.4	32.13	32.34
19	7	35	1	1	0.4	31.95	32.02
20	9	30	7	1.2	0.3	31.23	31.53
21	9	40	3	0.8	0.5	32.71	32.66
22	5	30	7	1.2	0.5	31.64	31.73
23	7	35	5	1	0.4	31.9	32.34
24	5	40	3	0.8	0.3	27.96	28.07
25	7	25	5	1	0.4	30.41	29.94
26	5	30	3	1.2	0.3	30.66	30.79
27	7	35	5	1	0.4	32.36	32.34
28	7	35	5	0.6	0.4	31.93	31.49
29	7	35	5	1.4	0.4	32.12	32.1
30	3	35	5	1	0.4	32.37	32.19

Table 5. Equation of the parameters for amylase production as the function of pH, temperature, inoculum concentration, peptone concentration and Yeast extract concentration in coded factors

Source	Amylase – FS10-FY		
	F-value	p-value	
Model	10.21	0.0006	significant
A-pH	2.24	0.1684	
B-Temperature	1.87	0.2043	
C-Inoculum concentration	3.99	0.0769	
D-Peptone	2.51	0.1477	
E-Yeast extract	21.85	0.0012	
AB	0.6937	0.4264	
AC	0.5603	0.4732	
AD	22.01	0.0011	
AE	1.68	0.2270	
BC	0.6253	0.4494	
BD	45.67	< 0.0001	
BE	29.03	0.0004	
CD	0.2055	0.6610	
CE	10.61	0.0099	
DE	0.3869	0.5493	
A^2	0.1272	0.7296	
B^2	52.99	< 0.0001	
C^2	0.0276	0.8718	
D^2	2.23	0.1693	
E^2	0.5243	0.4874	
Lack of Fit	3.98	0.1421	not significant

Table 6. Summary of ANOVA for amylase production by *Enterobacter cloacae* D1

Responses	Parameters	Model	Lack of fit
Amylase (U/mL)	p-value	0.0006	0.1421
	F-value	10.21	3.98
	Coefficient of determination	0.9578	-
	Adjusted coefficient of determination	0.8640	-
	Predicted coefficient of determination	0.0528	-

3.2.3 Effect of reaction parameters on production of amylase by *Enterobacter cloacae* D1

Interaction effects of all the variables on amylase production were studied by plotting 3D curves for any two given independent variables, while keeping others at central level (Fig. 1).

3.2.3.1 Effect of pH on production of amylase by Enterobacter cloacae D1

Combined effects of pH-temperature, pH-inoculum concentration, pH-peptone concentration and pH-yeast extract concentration on amylase production by *Enterobacter cloacae* strain D1 are presented in Fig. 1. The pH range that had the maximum effect on amylase production was pH 5.0 to 8.0

3.2.3.2 Effect of temperature on production of amylase by Enterobacter cloacae strain D1

Combined effects of temperature-pH, temperature-inoculum concentration, temperature-peptone concentration and temperature-yeast extract concentration on amylase production by *Enterobacter cloacae* strain D1 are presented in Fig. 1. The temperature range that had the maximum effect on amylase production was 35 to 40^{o}C.

3.2.3.3 Effect of inoculum concentration on production of amylase by Enterobacter cloacae strain D1

Combined effects of inoculum concentration-pH, inoculum concentration-temperature, inoculum concentration-peptone and inoculum concentration-yeast extract on the production of amylase by *Enterobacter cloacae* strain D1 are presented in Fig. 1. The optimum inoculum concentration range that had the maximum effect on amylase production was 3 to 6%.

3.2.3.4 Effect of peptone concentration on production of amylase by Enterobacter cloacae strain D1

Combined effect of peptone–pH, peptone-temperature, peptone-inoculum concentration andpeptone-yeast extract on the production of amylase by *Enterobacter cloacae* strain D1 are presented in Fig. 1. The optimum peptone concentration range that had the maximum effect on amylase production was 1.1 to 1.2%.

3.2.3.5 Effect of yeast extract concentration on production of amylase by Enterobacter cloacae strain D1

Combined effects of yeast extract–pH, yeast extract-temperature, yeast extract-inoculum concentration, and yeast extract-Peptone and on the production of amylase by *Enterobacter cloacae* strain D1 are presented in Fig. 1. The yeast extract concentration range that had the maximum effect on amylase production was 0.45 to 0.5%.

3.2.4 Final equation in terms of coded factors

The final equation in terms of coded fctors is presented in Eq. 2:

$$+32.34+0.1450A-0.1325B+0.1933C+0.1533D+0.4525E+0.0988AB-0.0887AC-0.5562AD-0.1538AE-0.0937BC+0.8013BD+0.6387BE+0.0538CD-0.3863CE+0.0737DE+0.0328A^{2}-0.6685B^{2}+0.0153C^{2}-0.1372D^{2}+0.0665E^{2} \quad (2)$$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. (A: pH; B: Temperature; C: Inoculum concentration (%); D: Peptone (%); E: Yeast extract concentration (%)

3.3 Molecular Identification of Amylase-Producing *Enterobacter cloacae* Strain D1

The phylogenetic of the amylase-producing *Enterobacter cloacae* strain D1 usied in this study is presented in Fig. 2. The accesion numbers are given in parenthesis. The tree is rooted with the isolate and astericked.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown

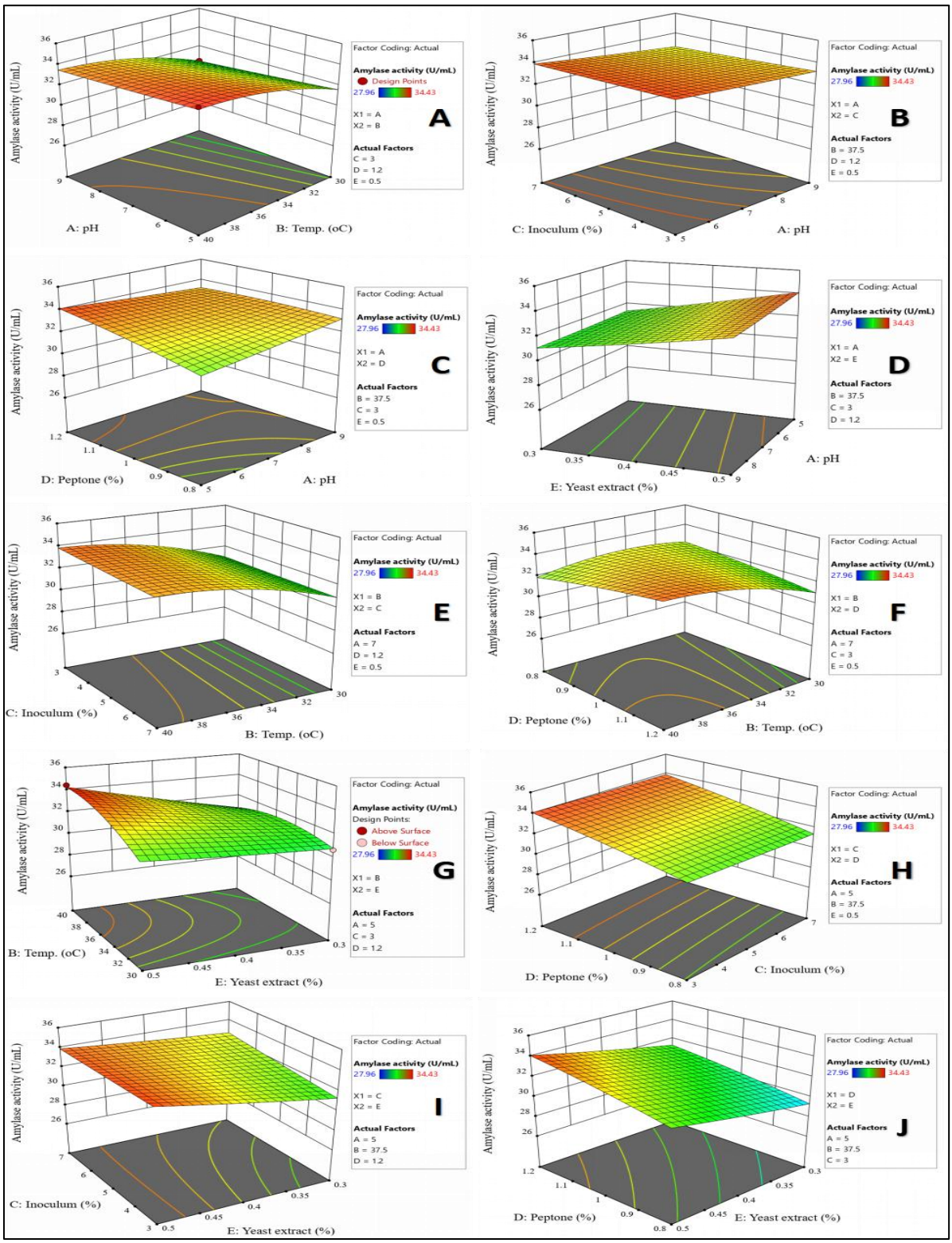

Fig. 1. Response surface (3D) for amylase production from *Enterobacter cloacae* strain D1 in batch fermentation as a function two given model variables

A: pH vs temperature; B: pH vs inoculum; C: pH vs peptone; D: pH vs yeast extract; E: Temperature vs inoculum; F: Temperature vs peptone; G: Temperature vs yeast extract; H: Inoculum vs peptone; I: Inoculum vs yeast extract; J: Peptone vs yeast extract

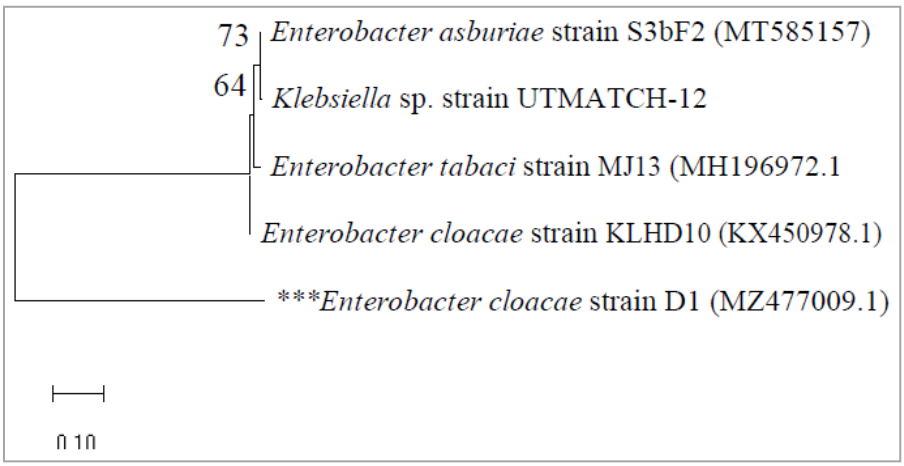

Fig. 2. Phylogenetic tree of the amylase-producing *Enterobacter cloacae* strain D1

next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. This analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 567 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [26].

4. DISCUSSION

This study was designed to optimize the production of α-amylase by *Enterobacter cloacae* strain D1 isolated from cassava effluent-impacted soil using response surface methodology. In this study, the amylase-producing bacteria was obtained from a cassava effluent impacted soil. Research has shown that starch-rich residues offer interesting alternative for the isolation of amylase-producing bacteria [9,10]. Amylase-producing bacteria can also be isolated from places such as cassava farms, soil, and processing flour factories [27]. The choice of cassava effluent for the isolation of amylase-producing bacteria was based on the fact that cassava effluent is rich in carbohydrate [28], especially in the forms of amylose and amylopectin [29] and these can encourage the growth of starch-hydrolysing bacteria. Due to the richness of cassava effluent in carbohydrates, many studies have explored the potentials of using cassava effluent or wastewater as source of amylase-producing bacteria. Results of these explorations have been rewarding with resultant amylases which are active at wide temperature and pH ranges [30-32].

Sixteen (16) bacteria were isolated in the study and screened for amylase production, out of these, *Enterobacter cloacae* strain D1was selected because of its high amylase activity (7.78 U/mL) when compared with others. *Enterobacter cloacae* has been reported as an efficient amylase producer [33,10]. Adomi [34] reported that two efficient amylase producers, namely *Citrobacter* sp. and *Enterobacter* sp. were isolated from cassava effluent; the maximum amylase activity they obtained was 1.2 U/mL. This value they obtained was lower than the maximum amylase activity recorded in this preent study before optimization (7.78 U/mL) and after optimization (34.43 U/mL). Other studies have reported the production of efficient amylase from other species of *Enterobacter* (Enterobacter hormaechei SR3). Arekemase et al. [35] optimized amylase production from amylolytic bacteria isolated from cassava peel dumpsite using submerged fermentation. Their study recorded maximum amylase production of 2.718±0.001 U/mL after optimization.

“In order to improve the production of amylase by the selected bacterial strain, cultural and nutritional condions for the production of amylase by the isolate were optimized using central composite design (CCD) of response surface methodology (RSM). Optimization methods are able to screen larger number of significant factors and evaluate their interactions with one another, thus selecting suitable conditions for the optimum response” [19]. “Such approaches lead to the enhancement of enzymes production for various applications” [18]. “Several biochemical

and biotechnological processes have effectively employed RSM in modeling and optimization studies for determining the effectiveness of factors and the relations between different physiological variables that affect enzyme production" [36,37].

"CCD model of RSM was employed in the optimization of amylase production in this present study. The CCD model represents an integral component of RSM. The biggest advantage of this type of optimization model is that it is more accurate and does not require a three-level factorial experiment for building a second-order quadratic model" [38]. In this study, the interaction effects of the varriables (pH, temperature, inoculum centration, peptone and yeast extract concentrations) on amylase production were investigated through 3D curves for any two given independent variables, while keeping others at central level. From the model obtained for the various interactions, the interaction between pH and peptone concentration, temperature and peptone, temperature and yeast extract, and inoculum concentration and yeast extract were all significant at p-values of 0.0011, <0.0001, 0.0004 and 0.0099, respectively. The amount of significant model terms is an indication that the CCD-RSM technique was efficient in the optimization of amylase. Moreover, the increae in amylase activity by the isolate from 7.78 U/mL to 34.43 U/mL, representing 3.4-fold increase is a confirmation of the the importance of the independent variables employed in the optimization study. Studies have shown that pH, temperature, inoculum centration, peptone and yeast extract concentrations all affect the efficiency of amylase production. Simair et al. [39] showed that yeast extract and beef extract as nitrogen sources enhanced amylase activity more than any other nitrogen source. They also showed that temperature between 40 and 55^{o}C favoured the production as well as pH of 8. For inoculum concnetrations, they found that optimum amylase activity was obtained with inoculum concnetrations of 10%. The result obtained in this present study is consistent with their report as the optimum pH, temperature, yeast extract and inoculum concnetrations that favoured amylase production were 5-8, 35-45^{o}C, 0.45-0.5 and 3-6%, respectively [40].

5. CONCLUSION

This study, optimization of α-amylase production by *Enterobacter cloacae* strain D1 isolated from cassava effluent-impacted soil using response surface methodology, has demonstrated that cassava effluent is a veritable source of amylase-producing bacteria. In addition, it demonstrated that pH, temperature, inoculum centration, peptone and yeast extract concentrations are critical factors whose interactive effect can significantly affect the production/yield of amylase under assay conditions. The study showed that by manipulating these factors up 3.4-fold increase in amylase was produced, buttressed by the increase from 7.78 U/mL to 34.43 U/mL in amylase activity achieved before and after optimization technique, respectively using CCD-RSM. The optimum conditions for the maximum amylase activity were pH 5; temperature 40℃; inoculum concentration 3%; peptone 1.2% and yeast extract 0.5%. Finally, considering the many applications of amylase this study has thus demonstrated that efficient amylase producing bacteria can be isolated from cassava effluent impacted soil.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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