Original Research Article

Screening, Isolation and Characterization of Amylase Producing Bacteria and optimization for Production of Amylase.

Abstract: The use of amylase enzyme has been extensive in different industrial sectors for quite a long time because of its various applications. In this study, soil from cassava processing site was screened for a bacteria with an amylase producing ability using starch iodine method and dinitrosalicyclic method. The biochemical and molecular characterization revealed the organism to be *Bacillus circulan* (CS2). Optimization of the enzyme production parameters was first carried out using the one factor at a time (OFAT) approach. The result obtained from OFAT study indicates that the following medium composition and culture conditions; medium pH 8, incubation temperature 45°C, incubation time 48 h, inoculum size 5%(w/v) of starch, lactose 1% (w/v), malt extract 1% (w/v), was suitable for the organism. Further optimization was done using Response Surface Methodology (RSM) based on Central

Composite Design (CCD). RSM model was found to have a predicted $R^2$and Adjusted R^2value of -0.0666 and 0.3980 respectively and can significantly ($p < 0.0500$) predicted the response variables. Within the model interaction, effect between the model variables medium pH (A) and Lactose (D) were found to be significant. Optimal conditions for Amylase production by *Bacillus circulans* are Temperature of 35°C pH of 10 Lactose 1(%) and Malt Extract 2(%) which gave an enzyme activity of 15.776U/mL and biomass of 1.648. *B.circulans* was partially purified using ammonium sulphate and was also seen to be stable at temperature of 80^0C and a pH of 6.

Keywords: Amylase; *Bacillus circulans*; OFAT; CCD; RSM; Ammonium sulphate precipitation; Enzymestability.

1 INTRODUCTION

Amylase constitutes a class of industrial enzymes, which represents approximately 30% of the world enzyme production (Van der Maarel et al., 2002). Amylase is an enzyme that hydrolyses starch into its monomeric compounds, the smallest being glucose. The glycosidic bonds that hold the monomers together are broken down by the enzyme. This is a very common and essential reaction that takes place within various living organisms in order to generate or store energy. Hence, amylase is a very prevalent enzyme produced biologically by various kinds of living beings. This includes plants, animals, humans and microorganisms.

There have been great advances in the use of amylase in industrial sectors as well. A large portion of the enzyme market share is owned by amylase (Gupta *et al*., 2003). A wide range of industries such as food industries, garments, textiles and beverage industries along with medicinal and clinical chemistry use amylase to manufacture their products. This requires a constant production of amylase enzyme. Extraction of this huge quantity of amylase directly from nature is not feasible and hence various methods are being constantly established to develop the mass production of commercial amylase (Dash *et al*.,2015).
Among the various types of amylase, the microbial amylase meets the industrial demands (Akcan *et al*., 2011). Quite a large variety of microorganisms have been identified and chosen as the source of amylase production because of the availability and simplicity of the ways in which they yield amylase. Fungal amylases are used worldwide along with different strains of bacteria. Each strain of bacteria requires specific growth conditions and nutrients to produce amylase. Soil is a primary source of these bacteria which can be isolated and commercially grown in large numbers to produce a vast amount of amylase. In order to provide this, industries use fermentation shake flasks to grow bacteria (Dash *et al*., 2015). In addition, the amylases that are extracted require optimum conditions to show greatest activity. This includes parameters such as temperature and pH. Considering the above importance, it is important to screen, isolate and characterize the amylase producing bacterial strains from soil sample of cassava processing site and also find out the optimum conditions for amylase activity through research before it can be used for industrialpurposes.

2. MATERIAL ANDMETHODS

2.1. Soil sample collection: Soil samples from cassava processing site was collected.

2.2. Isolation of amylase producing bacteria: The collected samples was subjected for enumeration for total bacterial population. About 10g each of the sample collected from the sampling site was aseptically transferred into individual conical flasks containing 90ml of normal saline. After thorough mixing, decimal dilutions of $10^{-}1$ to $10^{-}6$ was prepared by transferring 1ml of each sample to 9ml of the normal saline (0.85% NaCl). From each dilution, 0.1ml of the sample was aseptically transferred into already prepared nutrient agar plates. The inocula was spread properly by using a hockey stick (L-rod or spreader). The plates were incubated for 24h at 37°C. After incubation, number of colonies in each plate was counted. Average of the count obtained was calculated and total bacteria present per gram of sample calculated as:
No. of bacteria/g = No. of colonies x Dilution factor /Volume of the sample.

2.3. Screening of amylase producing bacteria: Each bacterial isolate was streaked onto starch agar plates and incubated for 24h at 37°C. After incubation, iodine solution (0.3% iodine and 1% KI) was poured unto the plates. Amylase positive bacterial strains was identified and recorded based on the clear zone formation around the bacterialgrowth.
2.4. Morphological and biochemical Characterization of bacteria: Screened amylase producing bacteria were morphologically characterized by Gram's staining method and characterized biochemically by catalase test, oxidase test (Filter paper method), indole Test, methylredtest,Voges-Proskauer(VP)test,citrateutilizationtest,gelatinhydrolysistest,urease

test.

2.5. Production of amylase and enzyme assay: The isolates showing clear zone around them was propagated in broth containing (Yeast extract - 5g, starch - 10g, MgSO4.7H2O - 0.5g, K2HPO4 - 1g, distilled water -1000ml and pH 7) shaking incubator at 150 rpm at 37°C for 48 hrs. After incubation, the resultant broth was centrifuged at 10,000 rpm for 15 minutes at 4°C.The supernatant was recovered and was used as source of crude enzyme.1ml of crude enzyme and 1ml of 1% soluble starch in Sodium phosphate buffer (pH 6.5) was added in a test tube. The test tubes were covered and incubated at 35°C for 30 minutes. Then 1ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 5 minutes. After cooling at room temperature, final volume was made to 5ml using distilled water. The absorbance was read at 540 nm by spectrophotometer.

2.6. Molecular Identification of bacterialstrain

The bacterial strain was further identified by sequencing the 16S rRNA gene of bacteria. This was done by the following methods: DNA extraction (Boiling method), DNA quantification, PahC gene amplification, 16S rRNA Amplification using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R:5'-CGGTTACCTTGTTACGACTT-3' primers, Sequencing and Phylogenetic Analysis.

2.7. Determination of optimum condition for the growth of the organism Using OFAT

The optimum temperature (30^0C – 45^0C), pH (5-8), carbon source (lactose, maltose and galactose) and nitrogen source (Yeast extract, Malt extract and Beef extract) and the right substrate concentration (1g-5g of starch) in which the organism can grow in was carried out using the one factor at a time methodology (OFAT). The medium parameter optimized was incorporated in the succeeding steps of optimization process.

2.8. Response Surface Methodology(RSM)

Response Surface Methodology was used to optimize the most significant variables screened by Central Composite Design, for the maximum yield of Amylase. It helps in understanding the interactions among variables. Optimization of medium using OFAT, involves changing one independent variable and keeping the other factors constant. This method is time consuming and incapable of detecting the true optimum. In fermentation process, the operational variables interact and influence each other's effect on the response. The four significant factors, pH, Temperature, lactose and malt extract were studied at five different levels (α, -1, 0, +1, and +α). The factors and their levels is represented in table 4.3. A set of 27 experiments were carried out based on the matrix built by the statistical software package Design-Expert. The experiment was explained by a second order polynomial equation. The model was statistically analyzed using analysis of variance (ANOVA), p- and F-values.

2.9. Partial Purification of Amylase Using AmmoniumSulphate.

A 48 hours fermented media was centrifuged at 15,000 rpm for 15 min. The supernatant and pellet were collected and proceeded for ammonium sulphate precipitation. Ammonium sulphate precipitation was performed in the concentration 2-8g/L of ammonium sulphate added in to different flasks containing 10ml of the crude extract with constant stirring for 30 min. The precipitate was separated by centrifugation at 15,000 rpm for 15 min. The enzyme activity of

supernatant and pellet was measured after each precipitation step. Pellet was dissolved in a minimum volume of 20mM sodium acetate buffer (pH 6.5). See figures 4.17 to 4.20 for results.

2.10. pH Stability

Crude extract from a 48 hours fermentation was obtained. The phosphate buffer is adjusted to different pH (3-11). 1 ml of the extract and 1ml of each of the different phosphate buffer with the various Ph were mixed in a test tube and incubated for 30mins and 1 hour at a temperature of 45^0C and then enzyme activity was carried out using the dinitrosalicyclic acid method.

2.11. Thermalstability

Crude extract from a 48 hours fermentation was obtained. The phosphate buffer of 6.5 was used. 0.5 ml of the extract and 0.5ml of the phosphate buffer were mixed in a test tube and incubated for 30mins and 1 hour at temperatures of 20^0C -90^0C and then enzyme activity was carried out using the Dinitrosalicyclic acid method.

III RESULTS AND DISCUSSION

3.1. Isolation and selection of amylase producing bacteria from soil

The soil collected for this study was from Elele in Etche local government area of Rivers State. After serial dilution and spread plating in nutrient agar plates, the bacteria acquired from $10^{-2}$and 10^{-4}dilution were selected. From the sample, 11 isolates were selected and starch iodine test carried out on them.

3.2: Colony Morphology

The colony characteristics of the bacteria was observed from a freshly streaked culture in a nutrient agar plate. Table 1 illustrates the colony morphology of isolate CS2.

CHARACTERISTICS	APPEARANCE
SHAPE	Circular
SIZE	Large
SURFACE	Dull
COLOUR	Cream
OPACITY	Opaque
ELEVATION	Flat
MARGIN	Entire

Table 1: Colony Morphology of Isolate CS2

3.3. Characterization of Isolate CS2.

Table 2 shows the biochemical characterization of isolate CS2.

It was observed that, a positive result was obtained for catalase test, motility,starchhydrolysis, citrate test, glucose utilization, Vogues-Proskauer. The results are presented in table 2.Wherea positive result is represented by + and are presented in table 2. Where a positive resultis represented by + and a negative result is represented by-.

Table 2: characterization of isolate CS2

BIOCHEMICAL TESTS	RESULTS
CITRATE UTILIZATION	+
MIU	Motility: +, Indole: -, Urease: -
STARCH HYDROLYSIS	+
VOGUES-PROSKAUER TEST	+
CATALASE TEST	+
GLUCOSE	+
LACTOSE	+
SUCROSE	+
NITRATE REDUCTION TEST	+
SPORE STAINING TEST	+
GELATINASE	+
TRIPLE SUGAR IRON (TSI)	Red slant, yellow butt, Gas and hydrogen sulphide production: -
METHYL RED TEST	-

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3.4.

Amplification and detection of 16S rRNA of the bacterial strain

After the extraction of the total genome and amplification of the 16S rRNA gene of the bacterial isolate CS2, it was observed through agarose gel electrophoresis that the size of the 16S rRNA gene for this bacterial isolate is just below 1650 base pairs. Plate 1 shows the agarose gel electrophoresis.

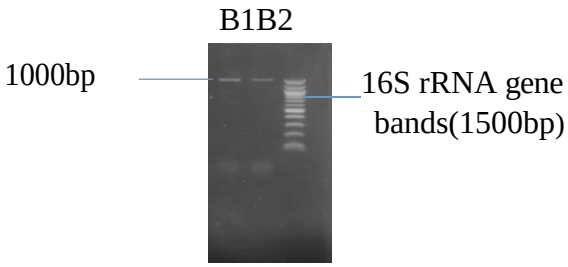
B1B2
1000bp
16S rRNA gene
bands(1500bp)

Plate 1: Agarose gel electrophoresis of the 16S rRNA gene of some selected bacterial isolates. Lanes B1 and B2 represent the 16SrRNA gene bands (1500bp), lane L represents the 100bp molecular ladder.

3.5: DNA Sequencing

Prior to sequencing, the purity and concentration of DNA sample was measured using NanoDrop 1000spectrophotometer.

The obtained 16s rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolate W1 showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Bacillus* sp and revealed a closely relatedness to *Bacillus circulans* (CS2).

3.6. Effect of Different Parameters on Amylase Activity and Glucose Released usingOFAT.

3.6.1. Effect of starch concentration on amylase activity and glucosereleased

The effect of different concentrations of starch (1, 2, 3, 4 and 5 g/L) fermented by *B. circulans* strain CS2 is presented in Figures 3 to 5. Figure 3 describes the effect of different concentrations of starch fermented by *B. circulans* strain CS2 on amylase activity and glucose released after 24 h of fermentation. From the figure, 5 g/L of starch had the best effect on amylase production for the isolate.

Figure 4 describes the effect of different concentrations of starch fermented by *B. circulas* strain CS2 on amylase activity and glucose released after 48 h of fermentation. From the figure, 2 g/L and 5 g/L of starch had the best effect on amylase production for isolate *B. circulas* strain CS2, respectively.

Figure 5 describes the effect of different concentrations of starch fermented by *B. circulas* strain CS2 on amylase activity and glucose released after 72 h of fermentation. From the figure, 2 g/L of starch had the best effect on amylase production for the isolate.

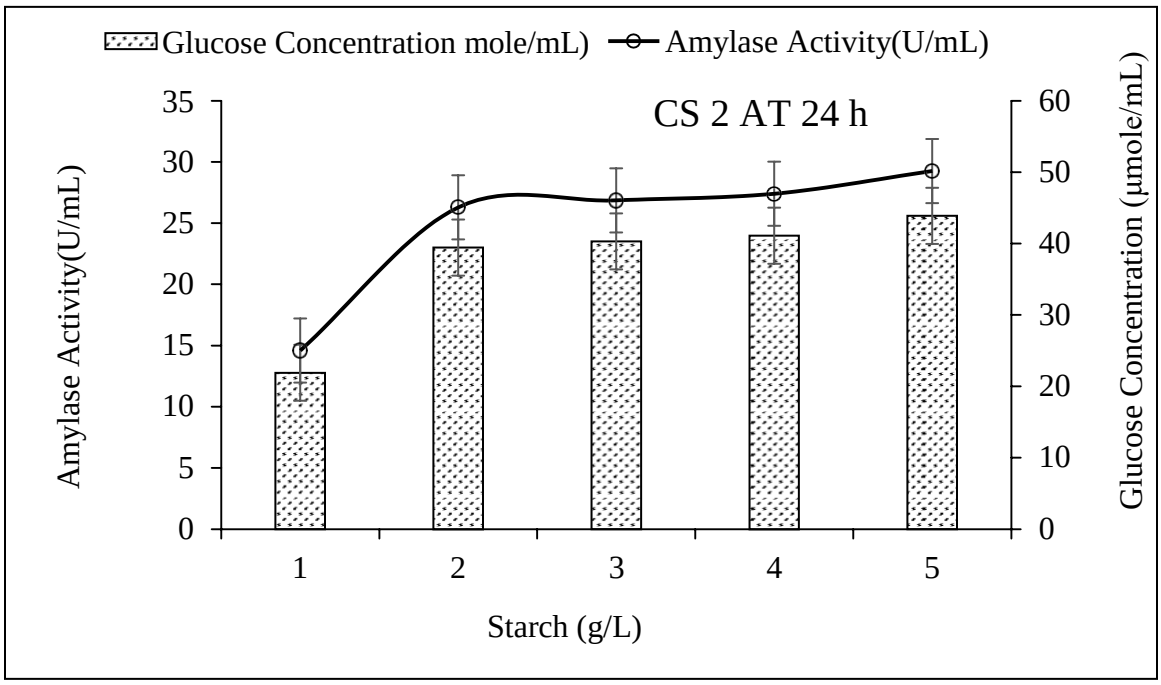

Figure 3: Effects of different starch concentration on amylase activity and glucose released by *B. circulans* strain CS2 after 24 h of fermentation.

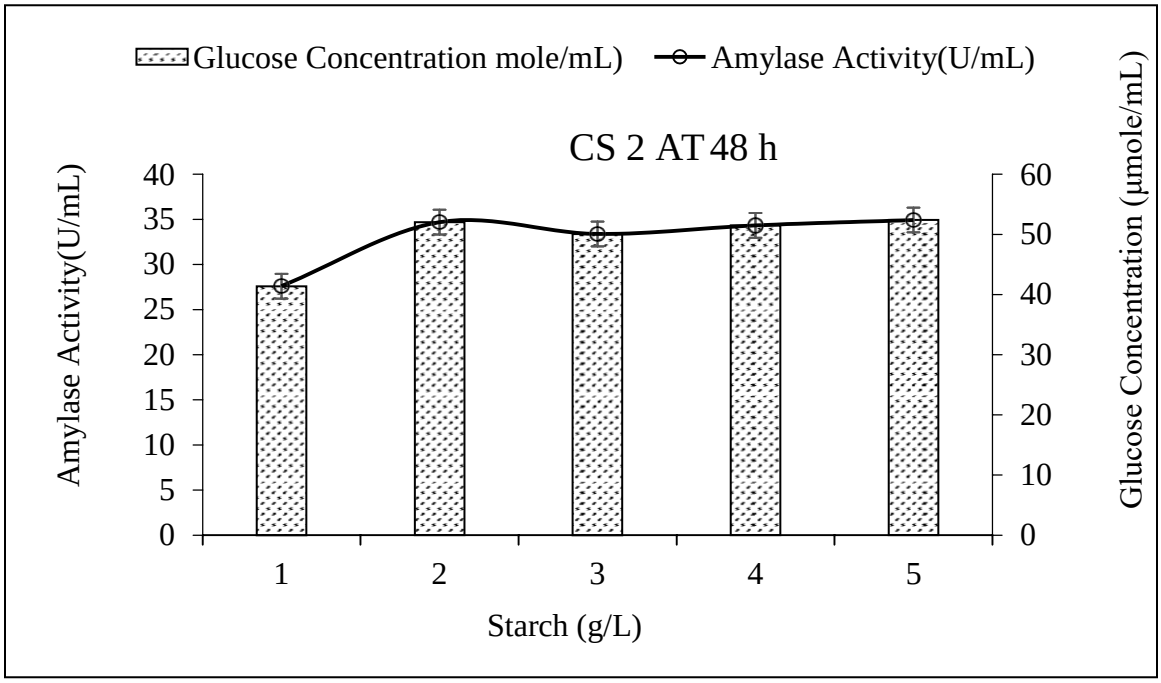

Figure 4: Effects of different starch concentration on amylase activity and glucose released by *B. circulans* CS2 after 48 h of fermentation.

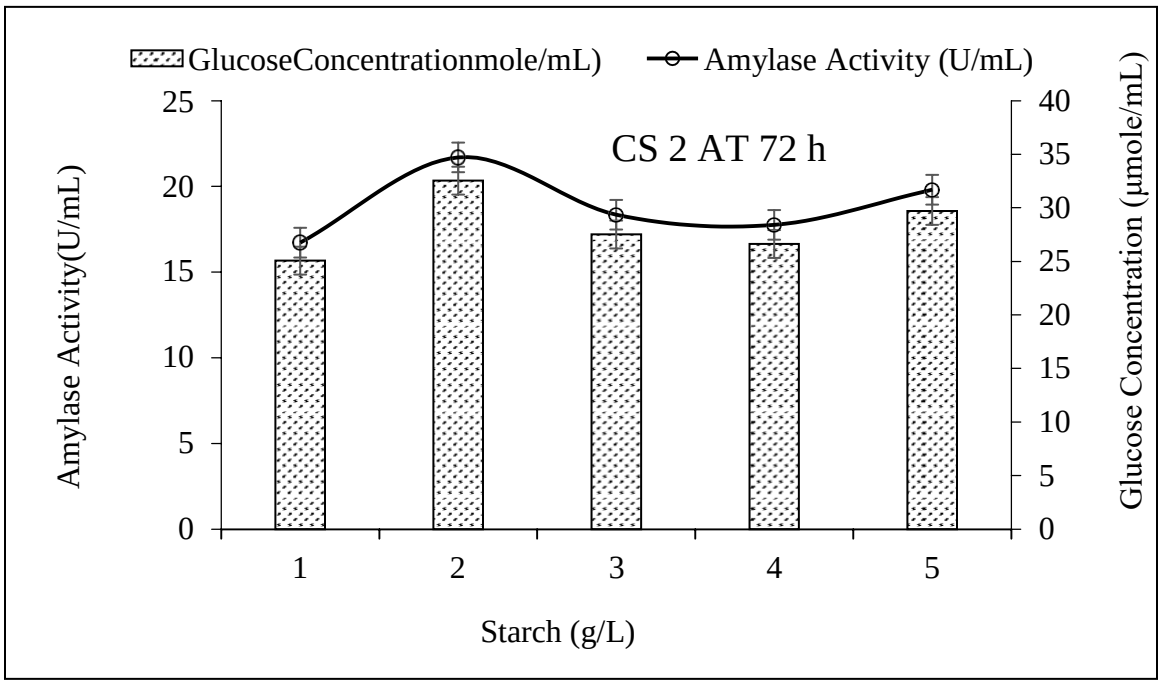

Figure 5: Effects of different starch concentration on amylase activity and glucose released by *B. circulans* CS2 after 72 h of fermentation.

3.6.2. Effect of Medium pH on Amylase Activity and GlucoseReleased

The effect of different pH (5, 6, 7, 8) fermented by *B. circulans* strain CS2 is presented inFigure 6. Figure 6 describes the effect of pH fermented by *B. circulans* strain CS2 on amylase activity and glucose released after 48 h of fermentation. From the figure, pH 8 had the best effect on amylase production for the isolate.

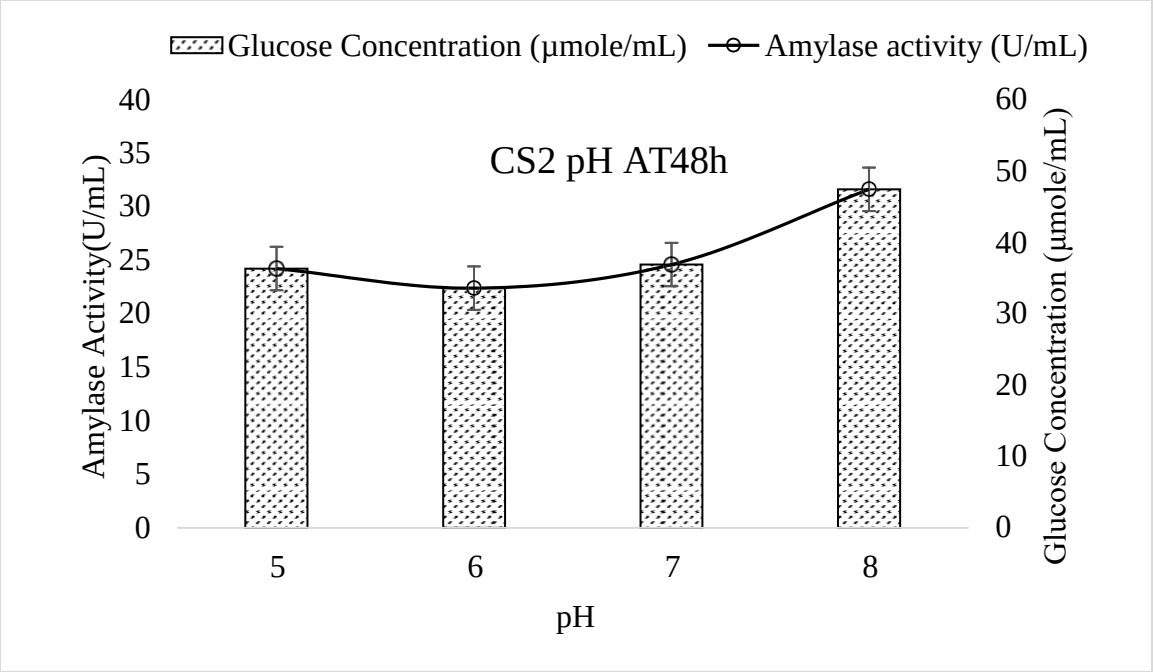

Figure 6: Effects of medium pH on amylase activity and glucose released *B. circulans* strain CS2 after 48 h of fermentation of 5 g/L of starch.

3.6.3. Effect of Incubation Temperature on Amylase Activity and GlucoseReleased

The effect of different incubation Temperatures in ^{0}C (30, 35, 40, 45) fermented by *B. circulans* strain CS2 is presented in Figure 7. Figure 7 describes the effect of different incubation Temperatures fermented by *B. circulans* strain CS2 on amylase activity and glucose released after 48 h of fermentation. From the figure, the Temperature of 45^{0}C had the best effect on amylase production for theisolate.

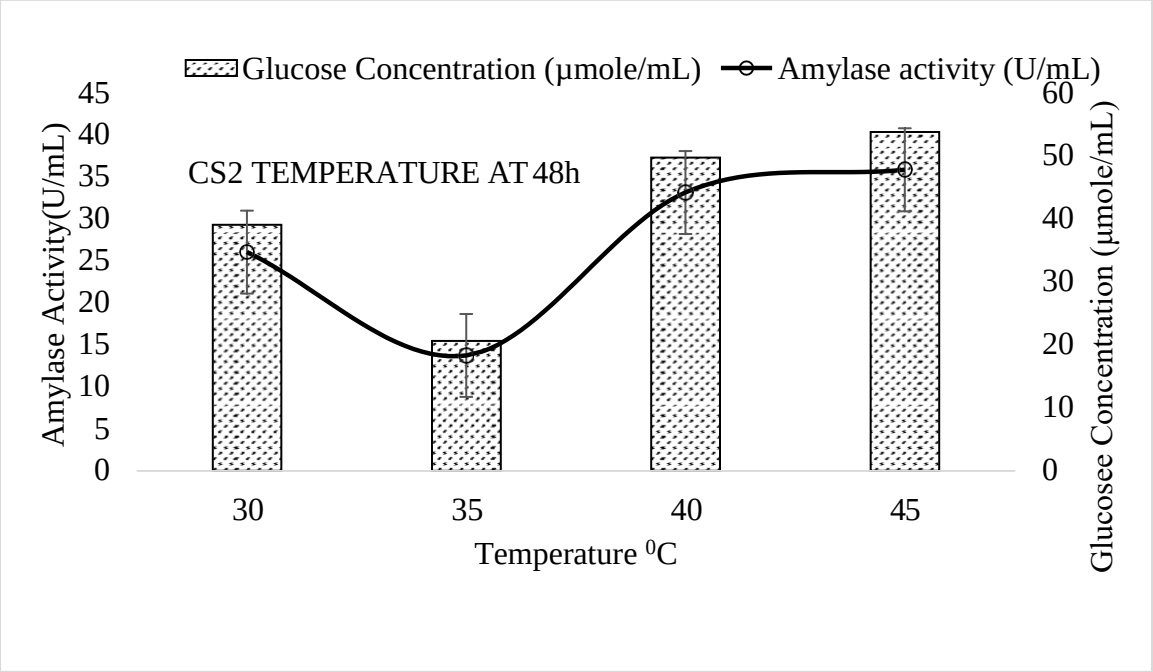

Figure 7: Effects of incubation temperature on amylase activity and glucose released by *B. circulans* strain CS2 after 48 h of fermentation of 5 g/L of starch.

3.6.4. Effect of Carbon Source on Amylase Activity and GlucoseReleased

The effect of different carbon sources fermented by *B. circulans* strain CS2 is presented in Figure 8. Figure 8 describes the effect of different carbon sources (lactose, maltose, galactose) fermented by *B. circulans* strain CS2 on amylase activity and glucose released after 48 h of fermentation. From the figure, lactose had the best effect on amylase production for theisolate.

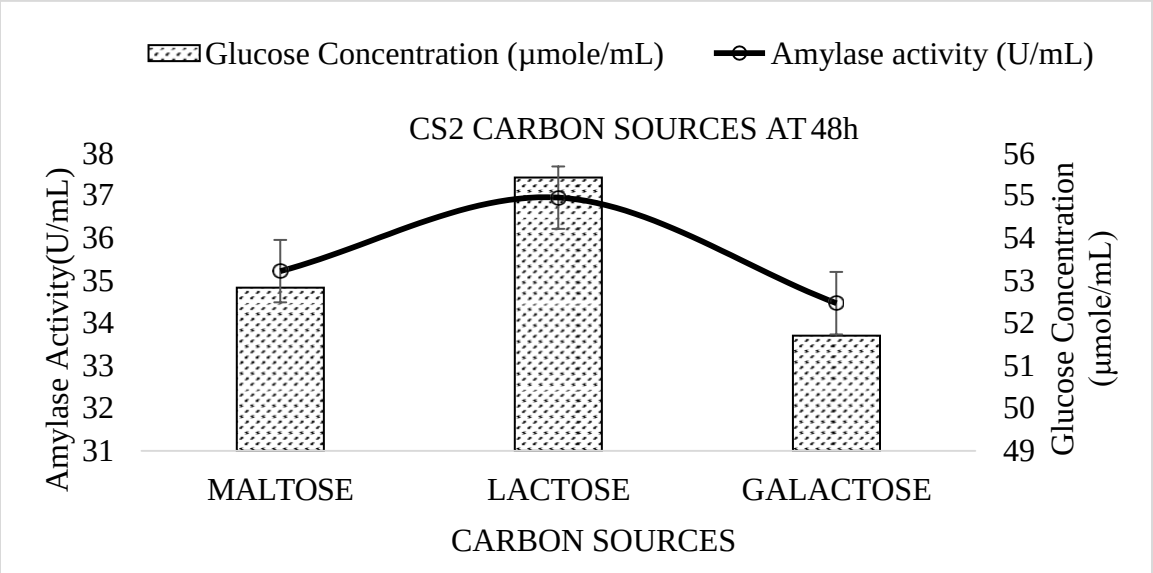

Figure 8: Effects of different Carbon Sources on amylase activity and glucose released by *B. circulans* strain CS2 after 48 h of fermentation of 5 g/L of starch.

3.6.5. Effect of Nitrogen Source on Amylase Activity and GlucoseReleased

The effect of different Nitrogen sources fermented by *B. circulans* strain CS2 is presented in Figure 9. Figure 9 describes the effect of different carbon sources (yeast extract, beef extract, malt extract) fermented by *B. circulans* strain CS2 on amylase activity and glucose released after 48 h of fermentation. From the figure, malt extract had the best effect on amylase production for the isolate.

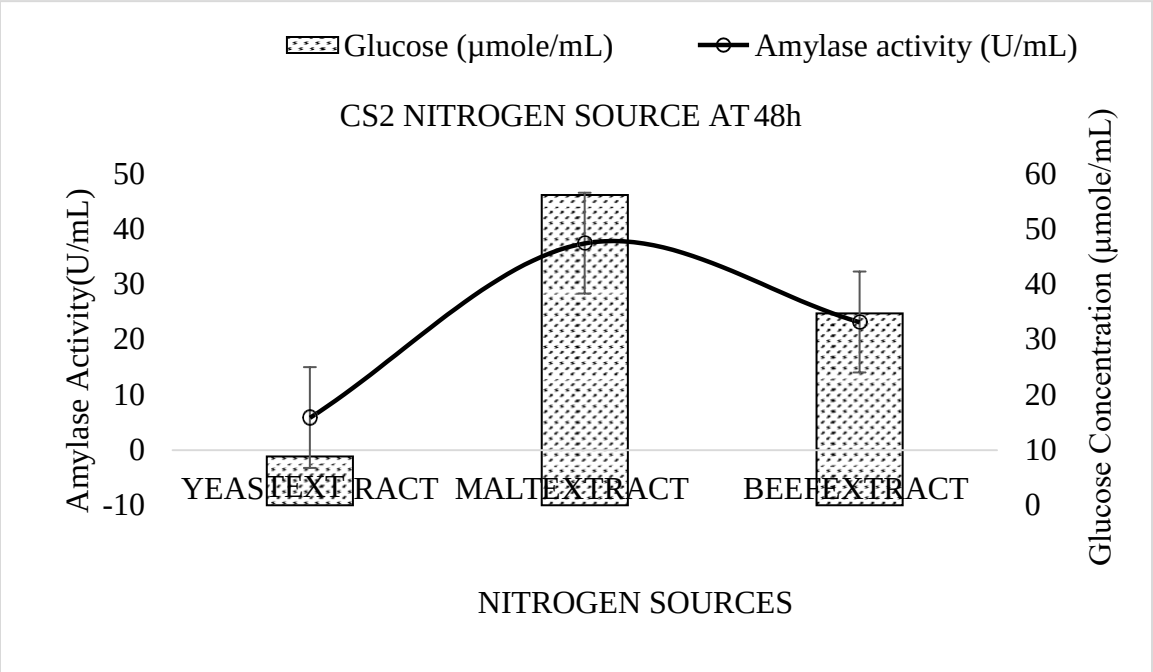

Figure 9: Effects of different Nitrogen Sources on amylase activity and glucose released by *B. circulans* strain CS2 after 48 h of fermentation of 5 g/L of starch.

3.6.6. pHStability

The pH Stability of *B. circulans* strain CS2 for 30mins and1 hour at a temperature of 45^0C is presented in Figure 10 to 11 respectively. From the figures, it is seen that the enzyme was stable at pH of 6 at 30mins and pH of 7 at 1 hour.

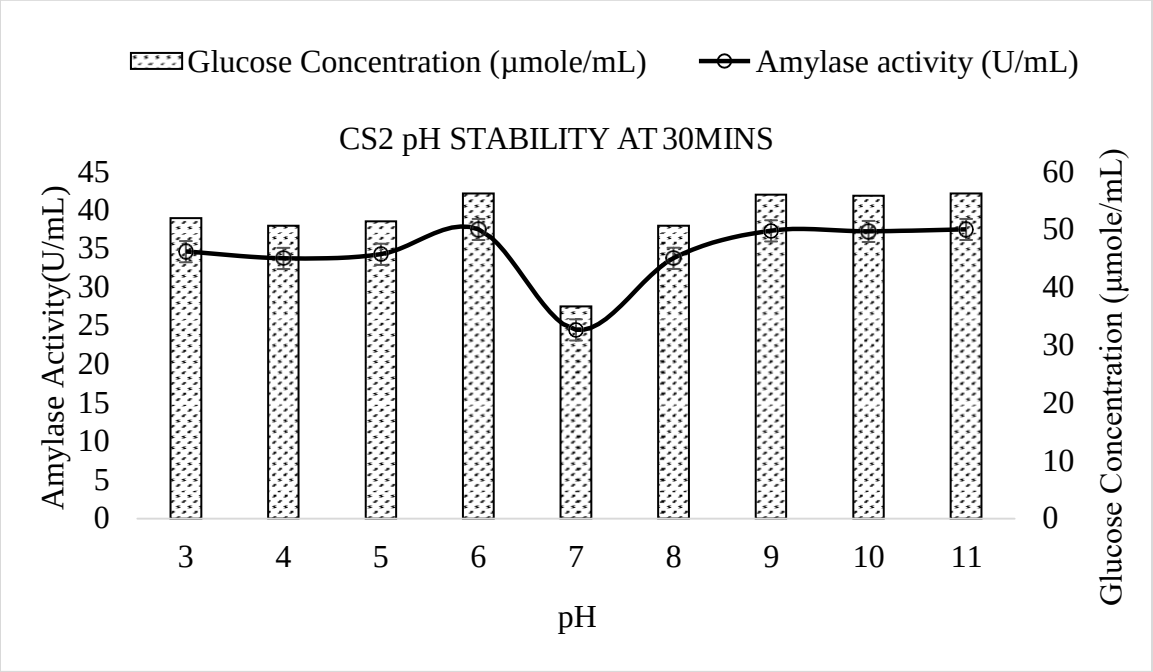

Figure 10: pH Stability for *Bacillus circulans* at 30mins

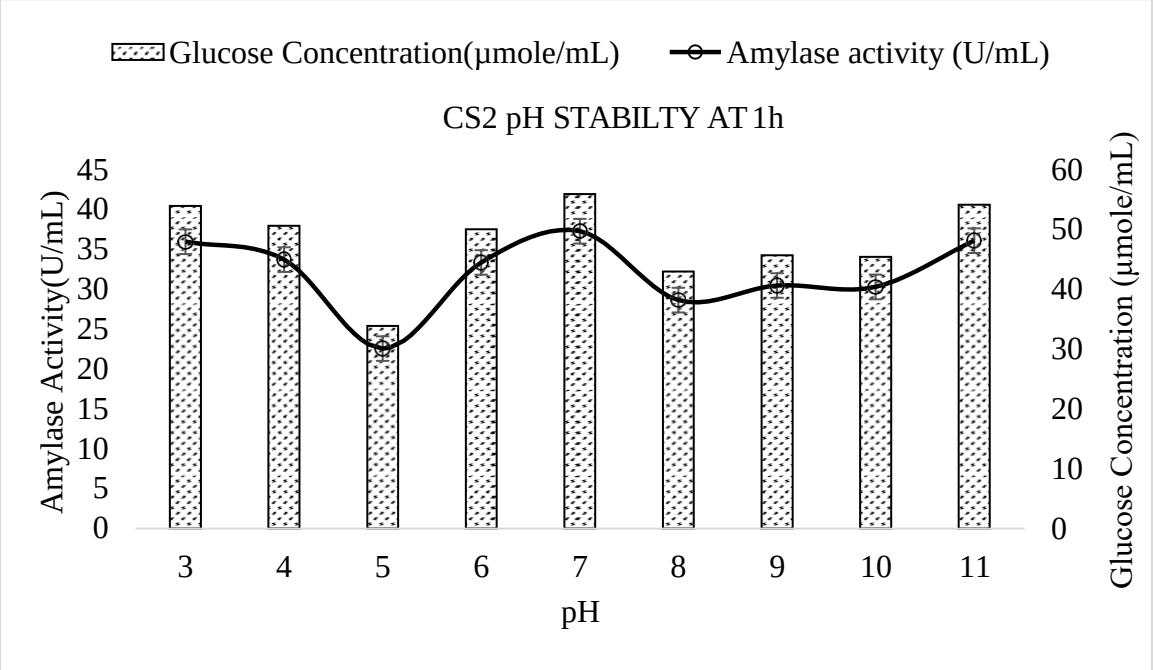

Figure 11: pH Stability for *Bacillus circulans* at 1 hour

3.6.7. Thermal Stability

The Thermal Stability of *B. circulans* strain CS2 for 30mins and 1hour is presented in Figure 12 to 13 respectively. From the figures it is seen that the enzyme was stable at a temperature of 80^0C at 30mins and 70^0C at 1 hour which means that the longer the time, there is tendency for the enzyme to bedenatured.

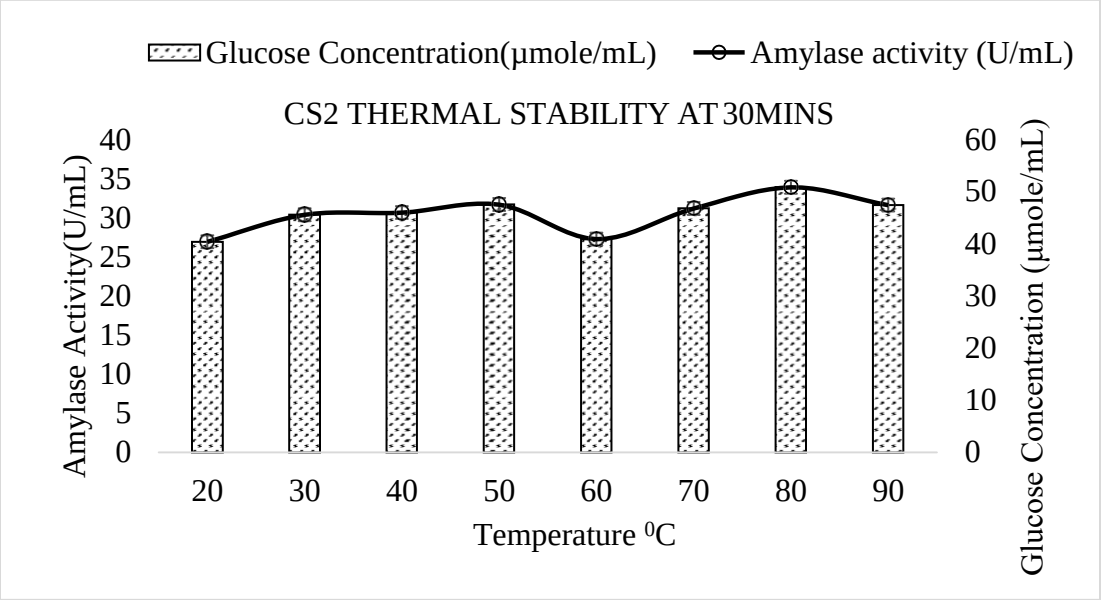

Figure 12: Thermal Stability for *Bacillus circulans* at 30mins.

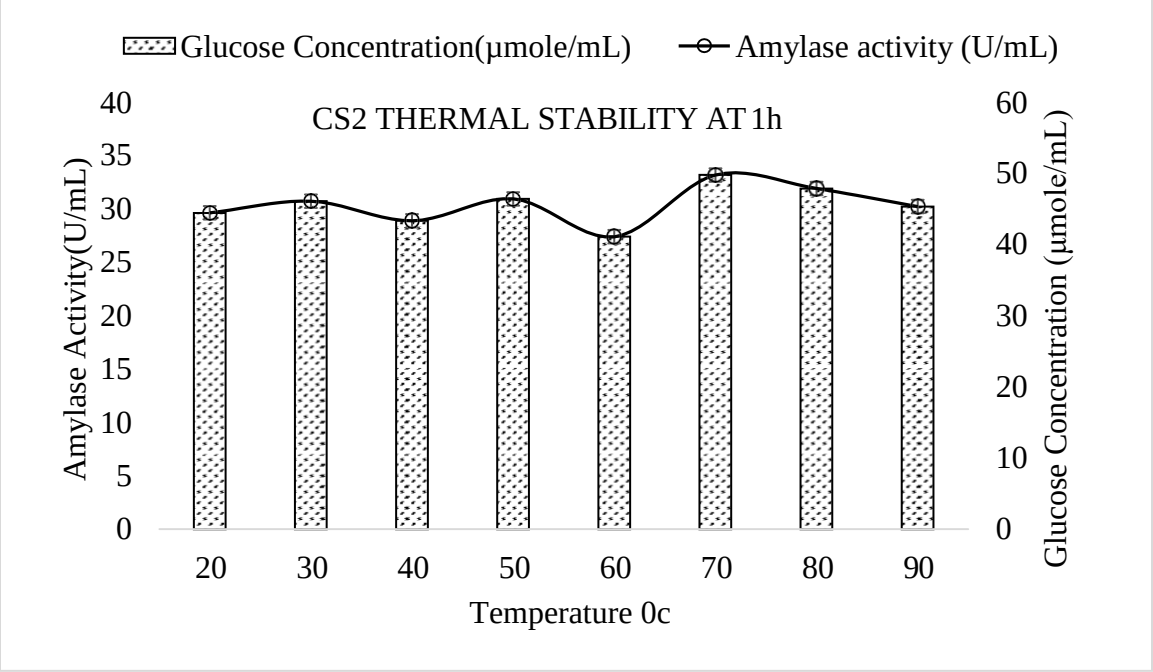

Figure 13: Thermal Stability for *Bacillus circulans* at 1 hour.

3.6.8. Effect of the Partial Purification ofAmylase

The effect of the partial purification of the Pellet and Supernatant of the amylase released is seen in figures 14 to 17. From the figures 14 to 15, it is seen 8g/L at 30mins and 6g/L at 1 hour worked best for the pellet while for figures 16 to 17, it is seen that 2g/L at 30mins and 4g/L at 1 hour worked best for the supernatant respectively.

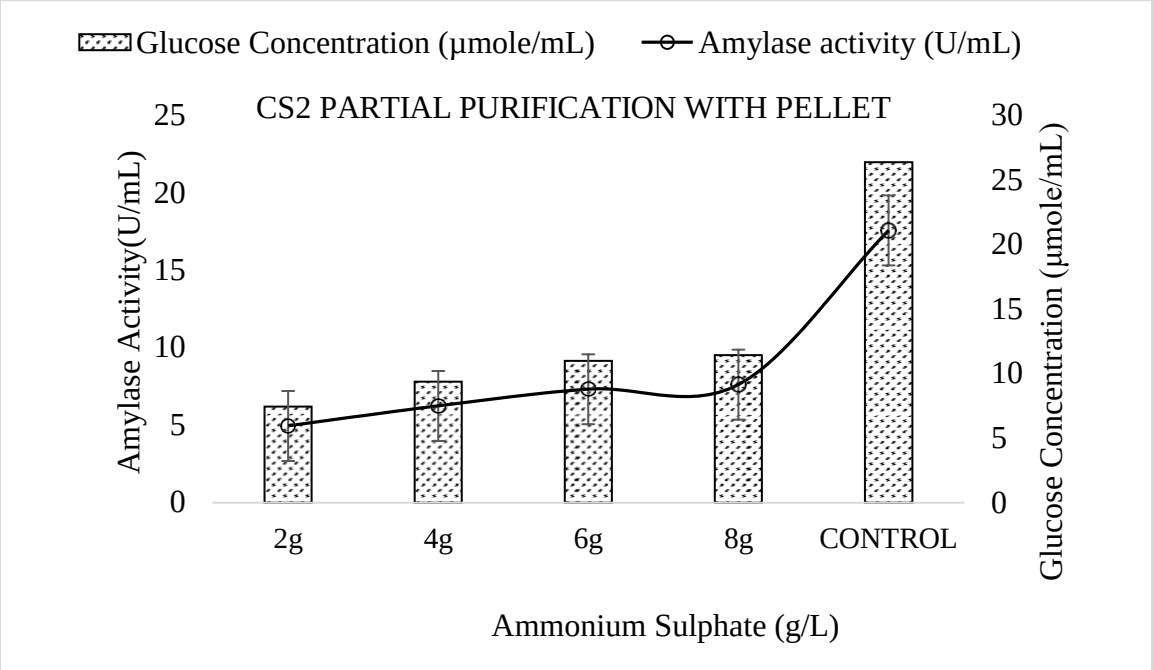

Figure 14: Partial Purification of pellet for *Bacillus circulans* at 30mins.

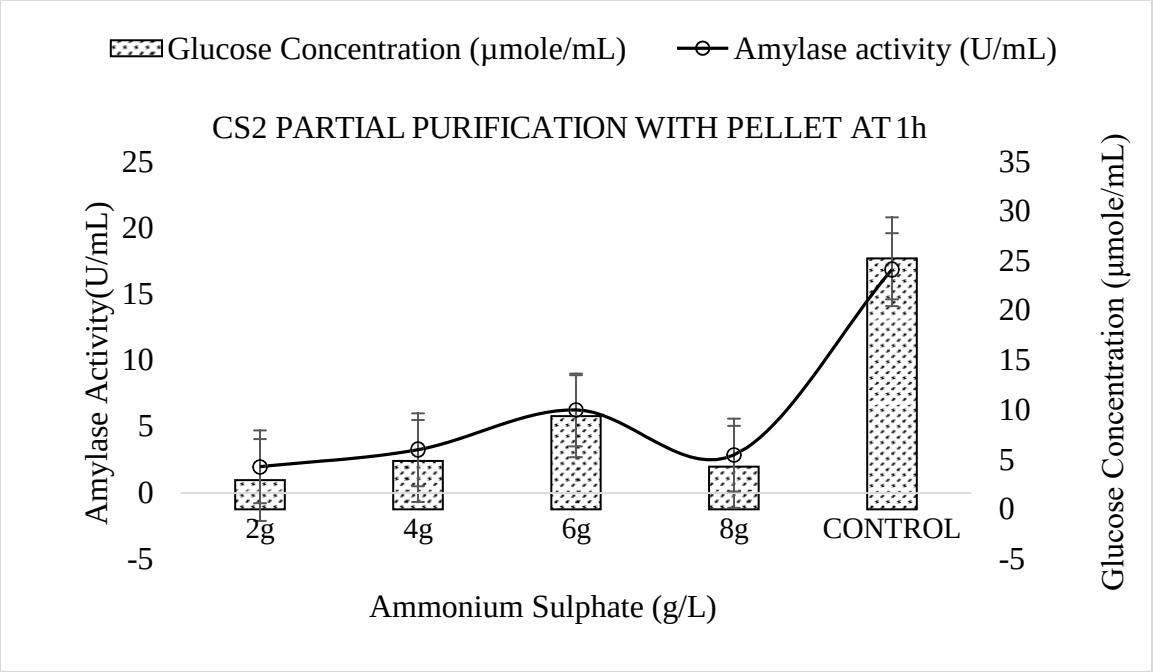

Figure 15: Partial Purification of pellet for *Bacillus circulans* at 1 hour.

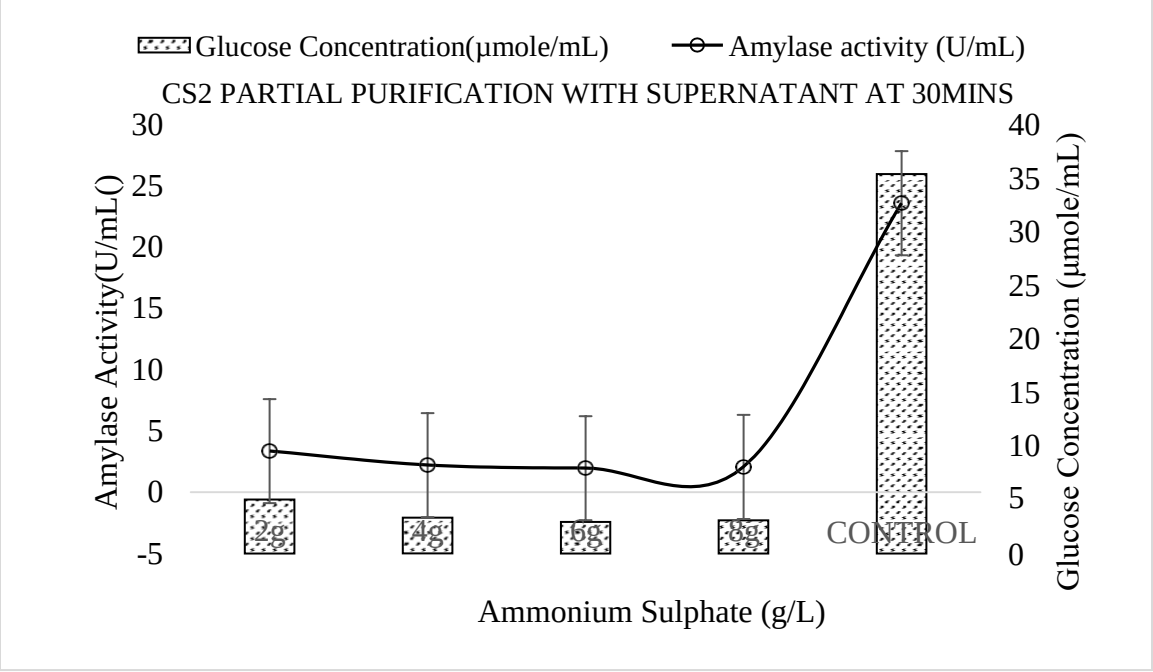

Figure 16: Partial Purification of supernatant for *Bacillus circulans* at 30mins.

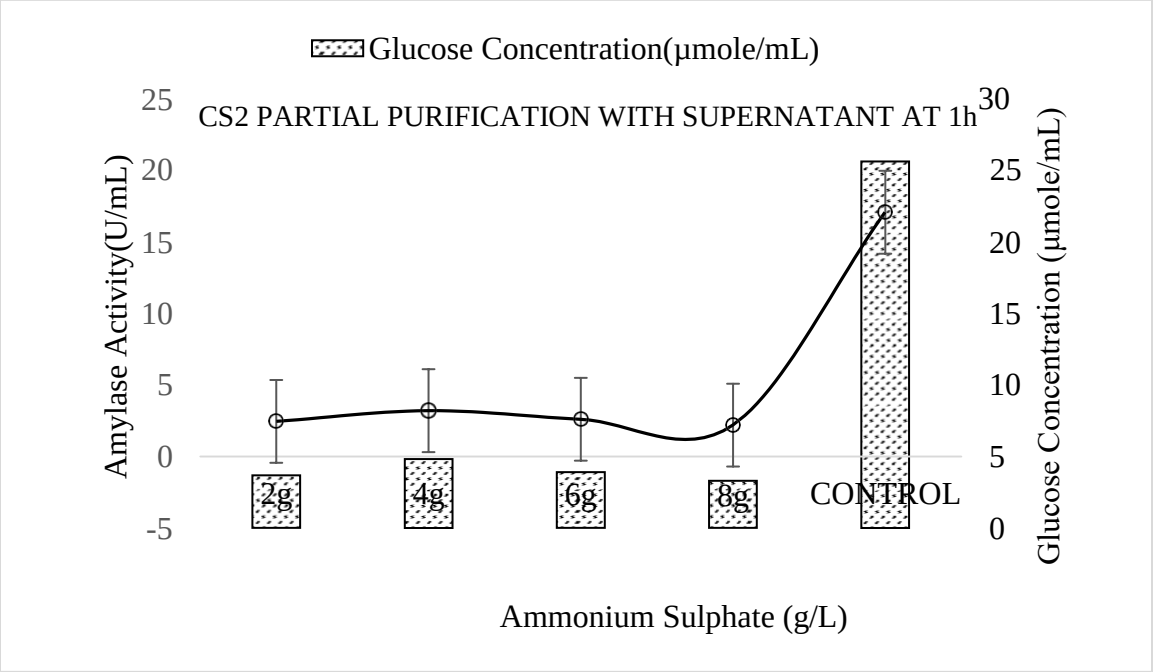

Figure 17: Partial Purification of supernatant for *Bacillus circulans* at 1 hour.

3.6.9. Response Surface Optimization of *Bacillus circulans* based onCCD

Table 3: Range and levels of experimental variables for amylase production by *Bacillus circulan* CS2

Factors	**Levels**				
	-α	-1	0	+1	+ α
pH	4	6	8	10	12
Temperature	20	30	40	50	60
Malt extract	0.25	0.5	1	2	4
Lactose	0.25	0.5	1	2	4

Table 4: Composition of various experiments of the CCD for independent variables and responses (actual and predicted) by *Bacillus circulans* CS2

Run	A: pH	B: Temp. (°C)	C: Malt extract(%)	D: Lactose (%)	Amylase activity (U/mL)		Biomass (A_{600})	
					Actual	Predicted	Actual	Predicted
1	10	35	1	1	12.06	14.69	0.1180	0.0949

2	6	35	1	1	17.09	14.61	0.2400	0.2352
3	10	55	2	2	15.81	14.01	0.2010	0.2884
4	8	45	1.5	2.5	8.10	12.01	0.1390	0.2160
5	10	35	1	2	8.07	5.48	0.1130	0.2417
6	10	35	2	2	7.62	10.34	0.1930	0.2830
7	10	55	1	2	5.14	4.90	0.2100	0.2974
8	8	65	1.5	1.5	12.68	16.29	0.1830	0.2319
9	6	55	2	1	14.54	12.84	0.1710	0.1669
10	8	45	1.5	1.5	12.77	14.09	0.3340	0.2417
11	6	35	1	2	19.02	19.07	0.2800	0.2197
12	6	55	1	2	21.02	16.61	0.2860	0.1972
13	8	45	2.5	1.5	12.04	15.27	0.1150	0.2694
14	8	45	0.5	1.5	5.39	12.91	0.2730	0.2414
15	6	35	2	1	5.70	7.86	0.1910	0.1765
16	6	55	2	2	18.59	17.88	0.2310	0.1742
17	8	45	1.5	0.5	17.62	16.17	0.2370	0.1937
18	4	45	1.5	1.5	16.07	15.99	0.1980	0.3612
19	6	35	2	2	19.20	16.09	0.2340	0.3097
20	10	35	2	1	15.65	15.78	0.3280	0.2465
21	8	25	1.5	1.5	12.52	11.89	0.2780	0.2497
22	8	45	1.5	1.5	17.00	14.09	0.2780	0.2417
23	10	55	2	1	20.77	22.63	0.1790	0.2357
24	6	55	1	1	16.14	15.34	0.1730	0.1604
25	8	45	1.5	1.5	12.78	14.09	0.2580	0.2409
26	10	55	1	1	18.47	17.29	0.2150	0.1210
27	12	45	1.5	1.5	18.54	12.19	0.7590	0.4787

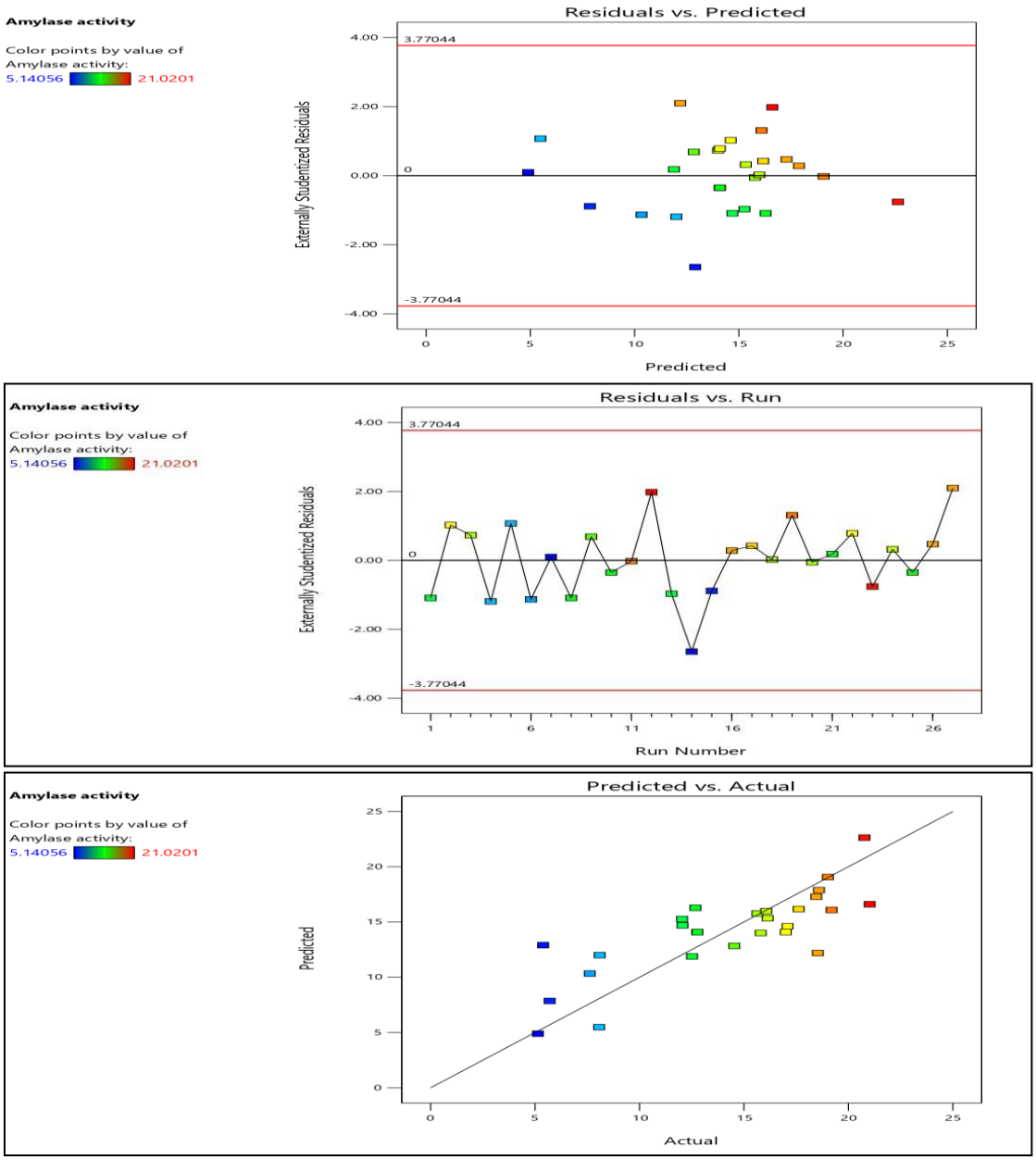

Figure 18: Plots of residual vs run, residual vs predicted and predicted vs actual values for amylase production by B. circulan CS2 under central composite design.

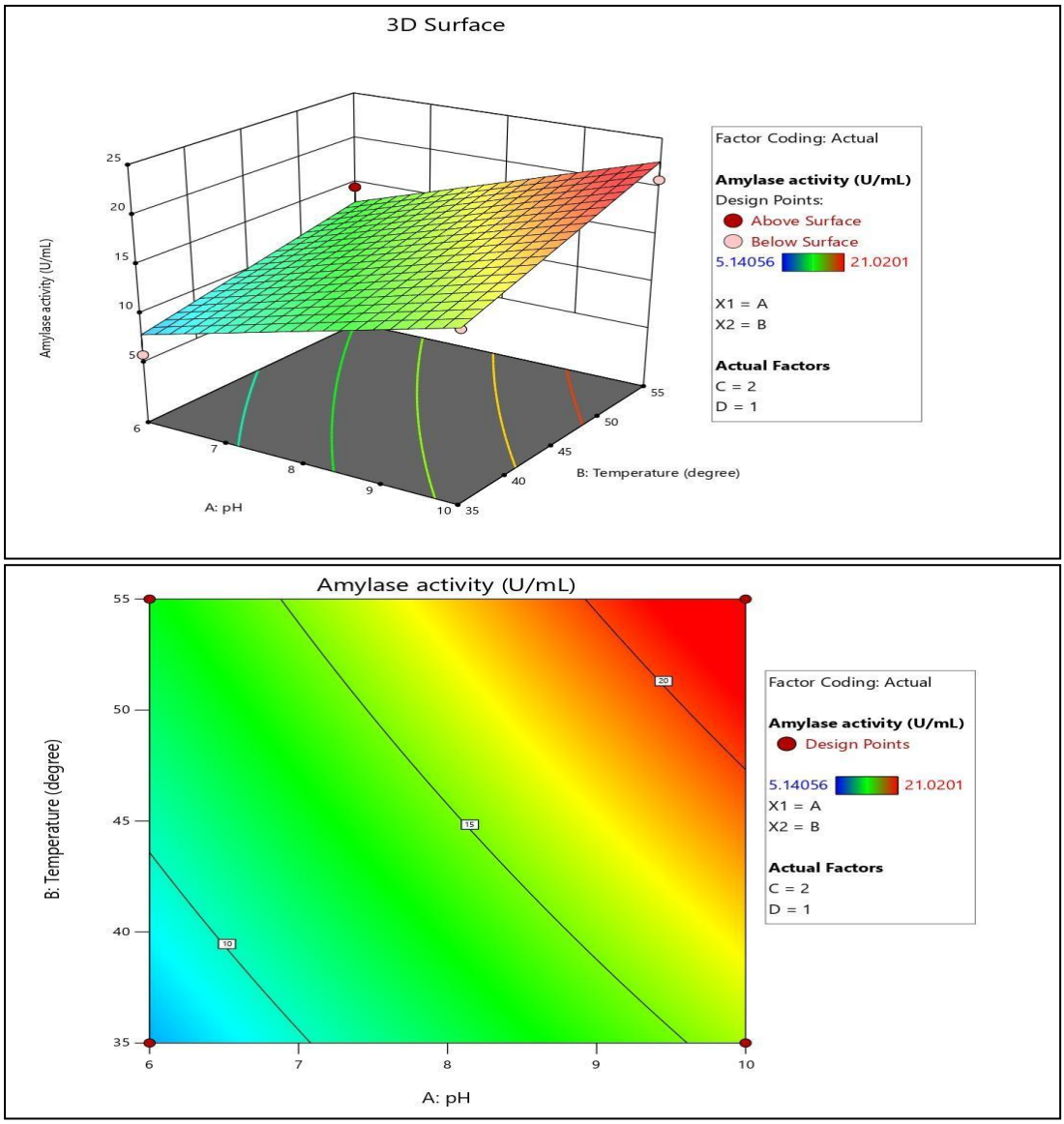

Figure 19: Response surface (3D) and contour plots for amylase production *B. circulans* CS2 in batch fermentation as a function of pH and temperature (°C).

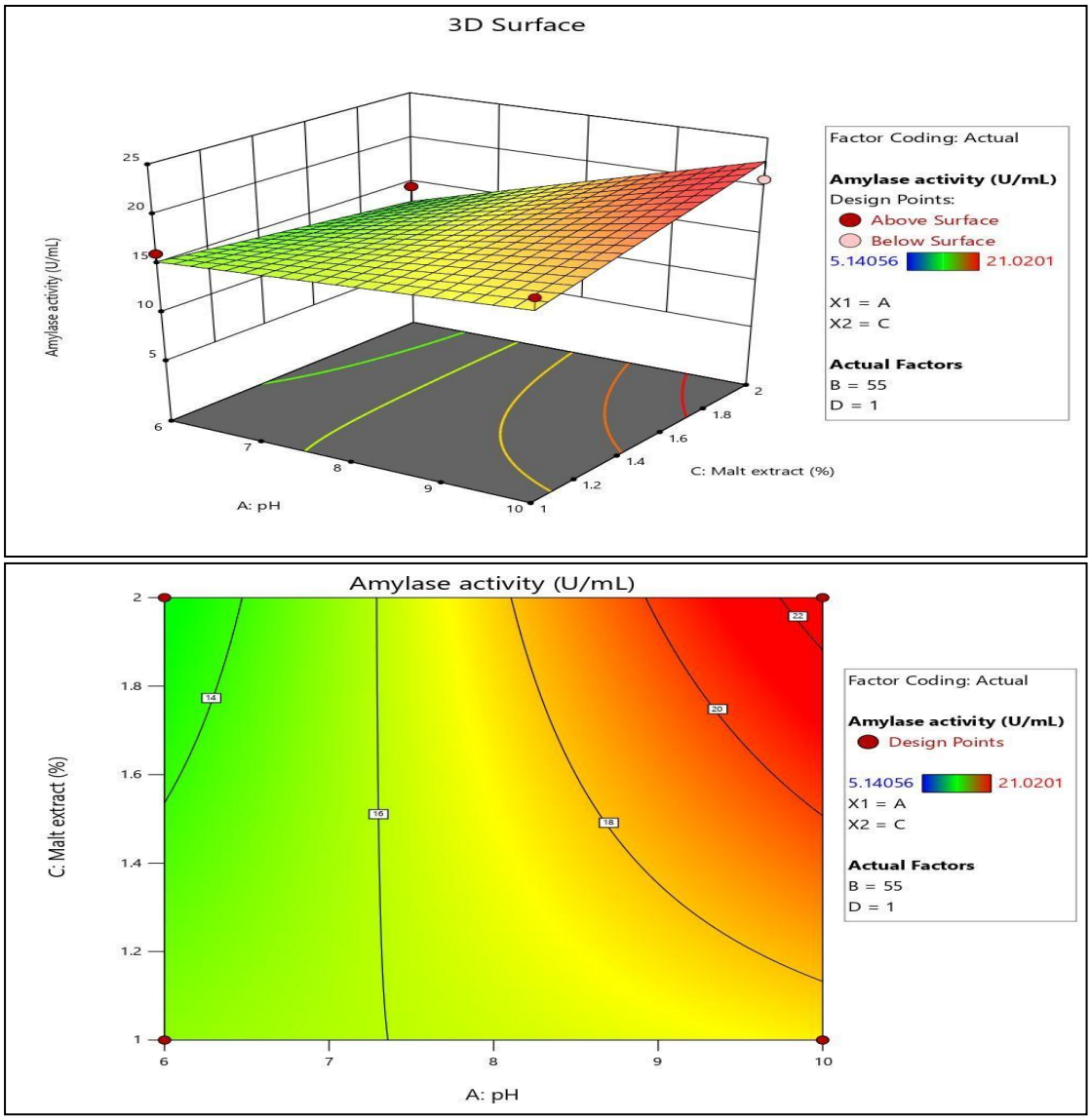

Figure 20: Response surface (3D) and contour plots for amylase production *B. circulans* CS2 in batch fermentation as a function of pH and malt extract (%).

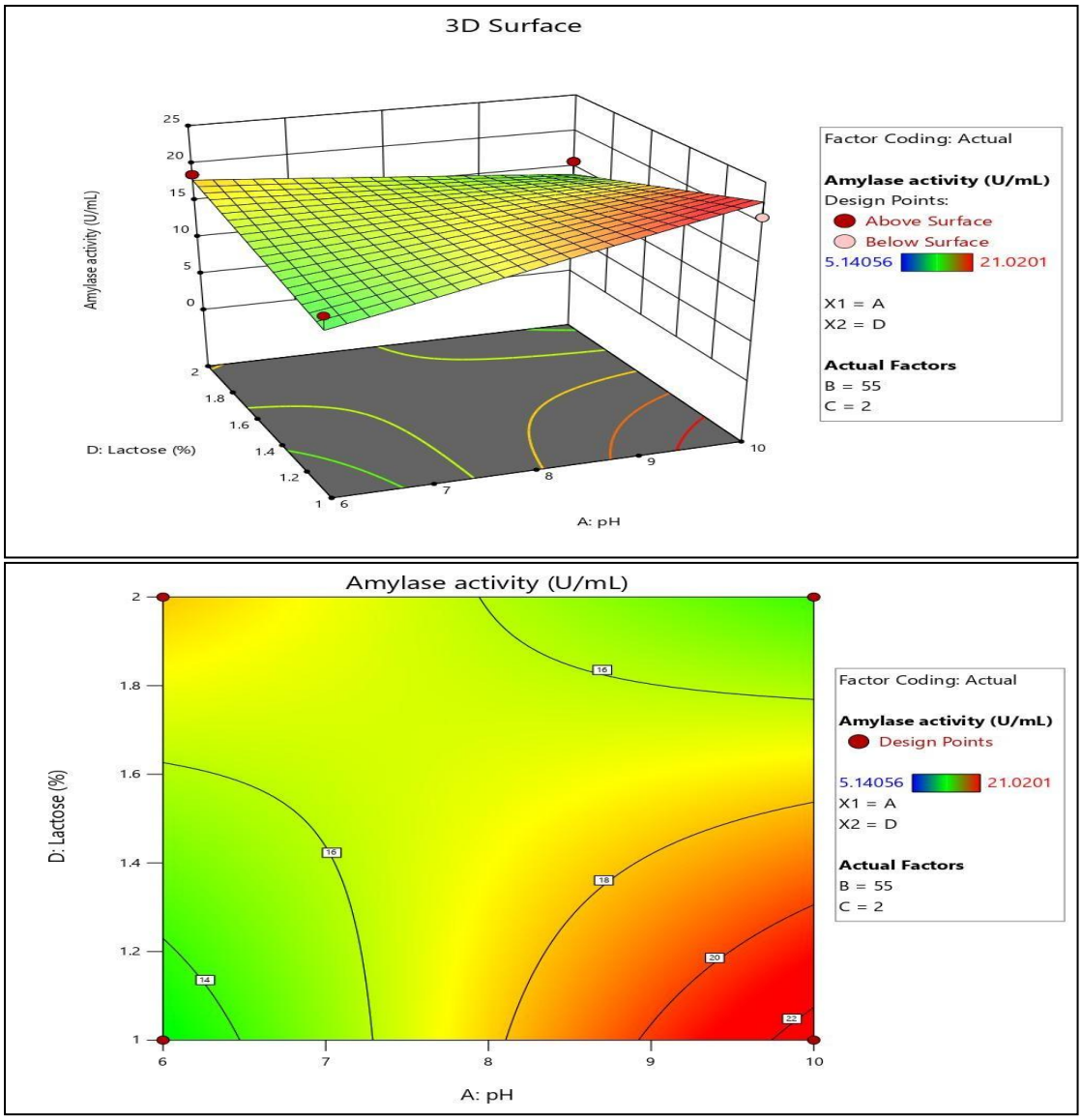

Figure 21: Response surface (3D) and contour plots for amylase production *B. circulan* CS2 in batch fermentation as a function of pH and lactose (%).

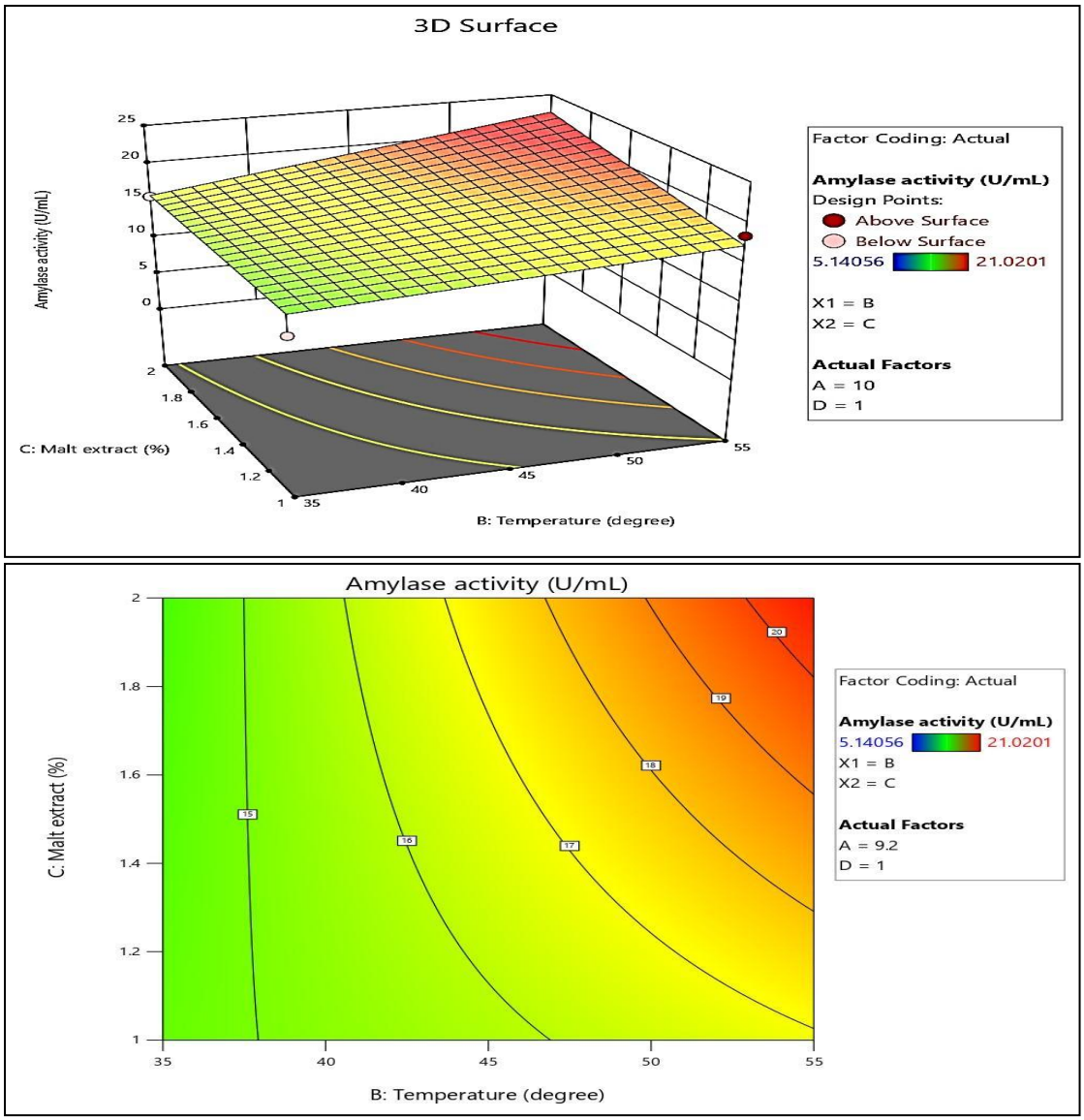

Figure 22: Response surface (3D) and contour plots for amylase production *B. circulans* CS2 in batch fermentation as a function of malt extract and temperature (^{o}C).

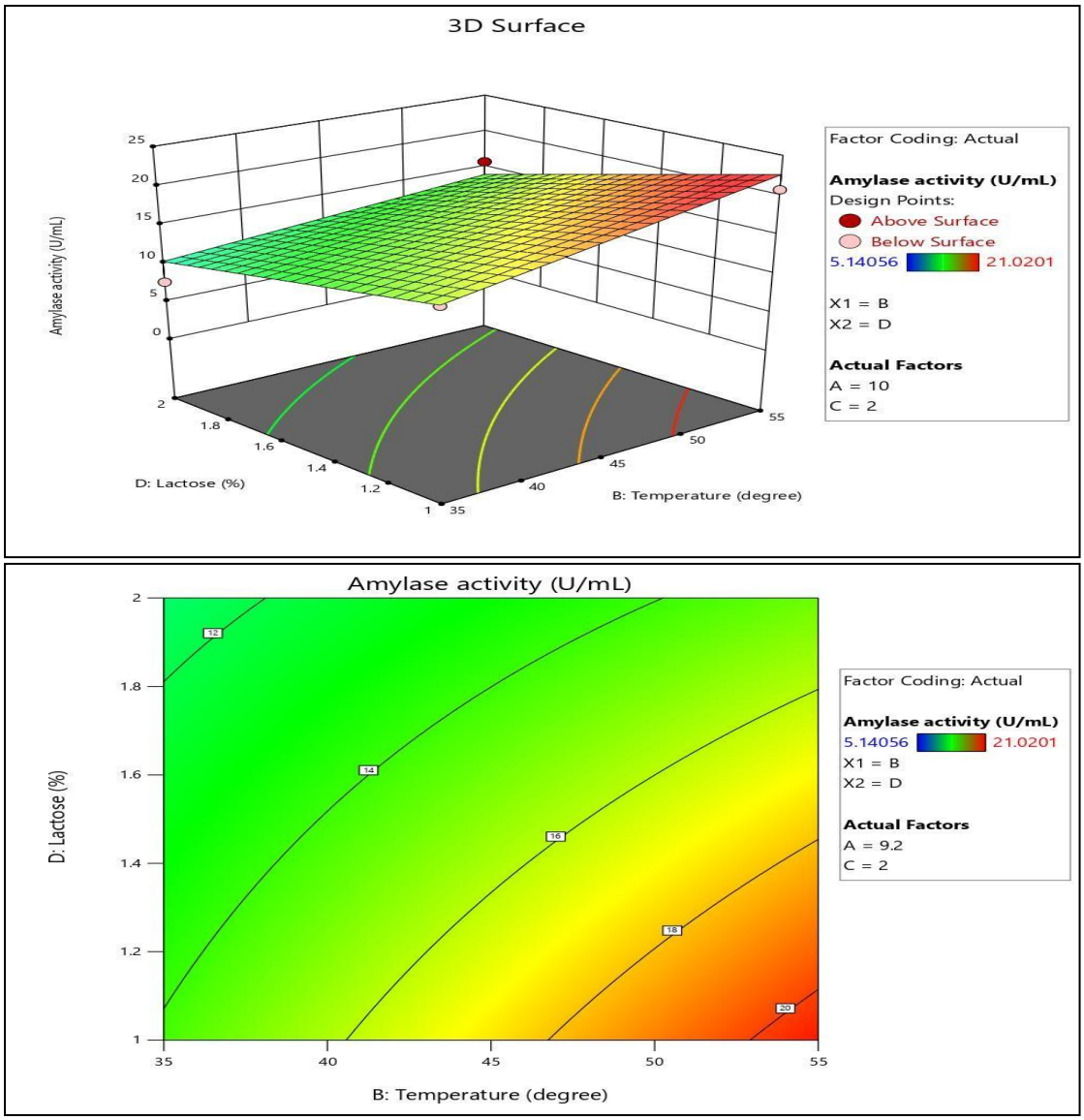

Figure 23: Response surface (3D) and contour plots for amylase production *B. circulans* CS2 in batch fermentation as a function of temperature (^{o}C) and lactose (%).

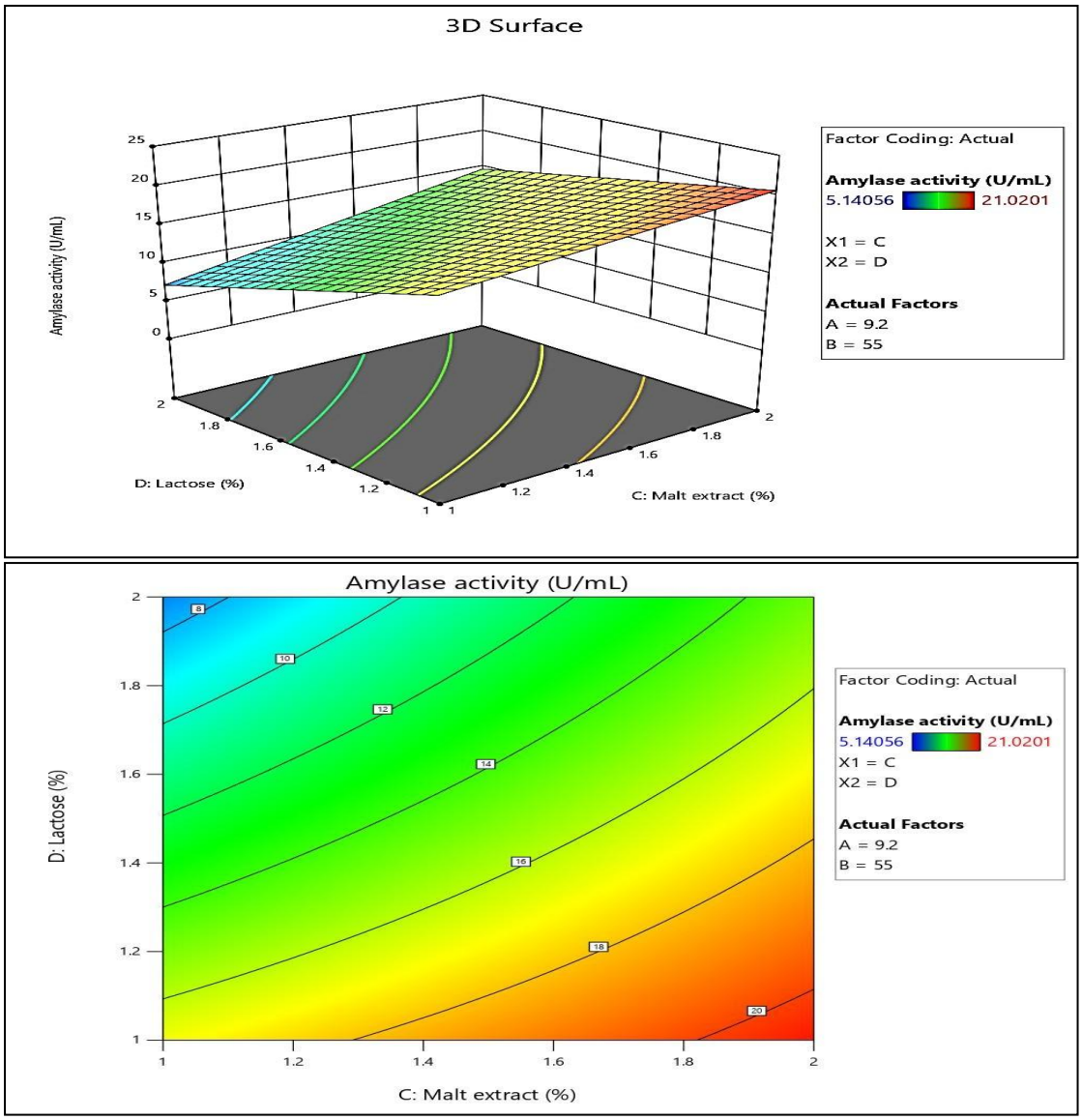

Figure 24: Response surface (3D) and contour plots for amylase production *B. circulans* CS2 in batch fermentation as a function of malt extract (%) and lactose (%).

Table 5: Description of constraints for the optimization of amylase production by *B. circulans* CS2 under CCD design

Name	Goal	Lower Limit	Upper Limit
A:pH	is in range	6	10
B:Temperature	is in range	35	55

C:Malt extract	is in range	1	2
D:Lactose	is in range	1	2
Amylase activity	maximize	5.14056	21.0201
Biomass	maximize	0.119	1.821

4 DISCUSSION

Enzymes are extensively used in the industries due to their high specificity and catalytic characteristics and the amylase enzyme is not left out. Amylase is being used mostly in the textile, food paper and mostly in the detergent industry in Nigeria. Also the pharmaceutical and chemical industries, regularly use amylase for their product yield. (El-fallal et al., 2012). The lack of a commercial producing amylase industry has led to the importation of this enzyme and this costs billions to import into the country, and most times the organism doesn't thrive well here due to the climatic differences. This leads to the use of harmful chemicals as alternatives for amylase (Dash et al., 2015). For these reasons, it is essential to develop a process to generate amylase commercially.

The main aim of this study was to screen for and isolate a high amylase producing strain from the soil and characterize the strain by morphology, biochemical and genotypic method. The study also included optimizing for the best growth conditions to yield maximum amylase such as temperature, pH, and Carbon and Nitrogen source.

Since bacteria is more abundant in the soil, it was chosen as the bacterial source for the isolation. The soil collected for this study was from Elele in Etche local government area of Rivers State. 5 isolates out of 11 were observed to be amylase producers during the primary screening of the bacterial strains. This was achieved by the use of starch iodine test method and a zone of clearance was used to identify the positive strains. The clear zones produced, were due to the absence of starch hydrolyzed by the amylase enzyme extracted by the bacteria. The reason behind this low no of isolates gotten could be due to the extensive dilution of the sample during the sample processing. 2 isolates (CS1 and CS2) with largest ratio of clear zones were chosen.

Enzyme assay was carried out in other to determine the amount of amylase produced by the selected isolate using Dinitrosalicyclic acid method. Since it is widely used for the determination of the amount of reducing Sugar produced, it is seen as an indication of enzyme activity (El-fallal et al., 2012). Amylase produced reducing sugar by breaking down starch into glucose, thus a greater concentration of glucose indicates greater level of starch break down and hence a higher amylase activity. Isolate CS2 showed an activity of 15.776U/mL which was higher than that of CS1, thus it was chosen as the bacteria to continue thestudy.

Further step included the characterization of the bacterial strain. This is quite important because it leads to the development and optimization of the media according to the organism and can be improved if there is prior Knowledge of the bacteria genetic makeup. It is important to know how it is structured, because it helps one to know how it affects humans (katzung., 2014).

From this presumptive tests, it was seen that this organism is of the *Bacillus* sp. Any bacterial isolates having clear zone is a potential amylase producer of extracellular enzymes or proteins (Azad et al., 2012: Hasan et al., 2017: Joshi., 2011). Characterization on the genetic level was carried out using the 16S rRNA gene sequencing. The gene a part of the prokaryotic DNA and has been the most common housekeeping genetic marker in order to study the phylogeny and taxonomy of a bacterial strain. This is because (i) it is present in almost all bacteria and might often exists as a multigene family, or operons; (ii) over time, there has been no changes seen in the function of the 16S rRNA gene and hence any change in the sequence indicates a more accurate measure of time (evolution); (iii) the size of the gene is large enough to be used for informative purposes (1500 bp). Using the 16S rRNA sequence in characterizing microorganisms is more dependable and sensitive than culture-dependent techniques alone and the results obtained in this research is consistent with other related studies by (Wang et al., 2011: Dash et al., 2015: P. Deb et al., 2013: U. Dey et al., 2016: Kandarp et al.,2020).

The determination of the optimal characteristics for amylase production by *B. circulans* was first carried out using the one factor at a time method (OFAT). The results can be seen in figure 3 to 11 above. The data collected from the one factor at a time, was used to design the Response Surface Methodology (RSM). The enzyme was seen to be produced at the pH range of 8-10 and temperature of 45^0C using OFAT with the optimum temperature and pH at 35^0C and 10 respectively using the RSM and has been reported in literatures too. Several authors have reported that the majority of the bacterial amylases have optimum temperature range of 30-100^0C (Dibu et al., 2011). (Joshi., 2011) reported that the optimum pH and temperature for amylase production from *Bacillus* sp was 10 and 80^0C, (Hasan et al., 2017) reported pH and temperature to be 7 and 50^0C, (Dibu et al., 2011) reported pH and temperature of 6 and 50^0C, (Kandarp et al., 2020) reported a pH and Temperature of 5 and 35^0C, (Biplab et al., 2015) reported pH and temperature of 7 and 37^0C respectively. pH range from 7-10 has the been reported as the optimum for enzyme production by *Bacillus* sp. This is in line with the result obtained from this study.

Ammonium sulfate precipitation is a useful technique as an initial step in protein purification because it enables quick, bulk precipitation of cellular proteins (Aikawa et al., 2013). It is also often employed during the later stages of purification to concentrate protein from dilute solution following procedures such as gel filtration. The drawback of this method is that oftentimes different substances can precipitate along with the protein, and other purification techniques must be performed, such as ion chromatography or size-exclusion chromatography. In this research, the enzyme was purified by using ammonium sulfate alone with no chromatographic technique. 2-8g/L (i.e 2-8%) of ammonium sulphate used, gave an enzyme activity of 3.36U/mL for the supernatant at 30mins and for the pellet, and enzyme activity of 7.51U/mL was obtained. According to (Kandarp et al., 2020), amylase was purified by ammonium sulphate precipitation andcolumnchromatographyandreportedanamylaseactivityof258.31U/mLinthepelletand 130.19 U/mL in the supernatant for 0-30% ammonium sulphate used, 293.38U/mL in the pellet and 103.89 U/mL in the supernatant for 50% ammonium sulphate used, 378.68 U/mL in the pellet and 36.94 U/mL in the supernatant for 80% ammonium sulphate used. The low enzyme activity given by *B.circulans* could have been as a result of the percentage of ammonium sulphate used for its purification as can be seen according to (kandarp et al., 2020) result obtained.

Under the toughest operating conditions and for long durations, a good industrial catalyst is meant to be stable. When the crude amylase was treated at different pH as shown in figure 10 to 11, it showed a slightly acidic pH of 6 and a neutral pH of 7 for optimal activity. Similar preferred conditions have been found for amylase activity in previous studies (Demirkan., 2011). More than 60% residual activity was obtained from this range. (Joshi.,2011), reported thermal stability at 80^0C and pH stability at 8. (Yasser et al., 2012) reported thermal stability within the range of 50-60^0C and a wide range of pH stability between 6 and 11. Thermal stability of the enzyme was seen (figure 12 to 13) to be 80^0C at 30mins and 70^0C at 1 hour. This goes to show that the longer the period of incubation and high temperature, can denature the enzyme thus the reduction in enzyme activity at 1hour. However due to the high thermal stability of this enzyme, it can be used in temperature sensitive techniques like bioethanol production, and also in the detergent factory. The enzyme produced by *B.circulans* can be said to be a thermal stable alkaline amylase enzyme. This is in line with the report given by(Joshi.,2011).

RSM is a known statistical tool for optimizing parameters of fermentation. The enzyme activity obtained for *B. circulans* using RSM was 15.776U/mL. Similar works has demonstrated the efficiency of RSM in optimization of fermentation variables. (Sanjay et al., 2020) reported an enzyme activity of 4.16U/mL. (Ameer et al., 2017) reported an enzyme activity of 145.32U/Ml using RSM respectively.

In the testing for the effect of Carbon and Nitrogen sources suitable for *B.circulans* using the one factor at a time, lactose and Malt Extract were observed to be utilized more as carbon and nitrogen sources respectively. Applying these parameters in to the RSM design helped in giving a high yield of the enzyme. Thus a carbon and nitrogen source are important factors for amylase production. In a study by (Sanjay et al., 2020), starch and Yeast Extract were used as carbon and nitrogen sources respectively. In the research by (Joshi., 2011) Starch and Peptone were used, (Kandarp et al., 2020) reported the use of lactose and sodiumnitrate.

The composition of various experiments of the CCD for independent variables (pH, temperature, lactose and malt extract ratio) and response (Amylase concentration, %) are presented in Table 3. Table 4. shows the actual and predicted values for Amylase production of *B.circulans*.

Summary of ANOVA for response surface quadratic models for amylase production

.For amylase production by isolate *Bacillus circulan,* it showed a **Model F-value** of 2.72 which implied that the model is significant, meaning that there is only a 3.62% chance that an F-value this large could occur due to noise**.** P-**values** less than 0.0500 indicate model terms are significant. In this case AD was seen as a significant model term with a P- value of 0.0021 (<0.0500).Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve one's model. The **Lack of Fit F-value** of 2.53 implies the Lack of Fit is not significant relative to the pure error. There is a 31.94% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit.

Figure 18, shows the plot of residual vs run, residual vs predicted, and predicted vs actual for amylase production by *B. circulans* and its alignment to the glucose curve.

Figure 19 shows the Response Surface (3D) and contour plots of amylase production of *B. circulans* in batch fermentation as a function of temperature at 55^0C and pH of 10 indicating the optimum yield of amylase using the combination of thisparameters.

Figure 20 shows the Response Surface (3D) and contour plots of amylase production of *B. circulans* in batch fermentation as a function of malt extract at 1(%) and pH of 10 indicating the optimum yield of amylase using the combination of thisparameters.

Figure 21 shows the Response Surface (3D) and contour plots of amylase production of *B. circulans* in batch fermentation as a function of lactose at 2 (%) and pH of 10 indicating the optimum yield of amylase using the combination of this parameters. This showed to be the most significant factor necessary for amylase production by *B. circulans.*

Figure 22 shows the Response Surface (3D) and contour plots of amylase production of *B. circulans* in batch fermentation as a function of temperature at 55^0C and malt extract of 1.4 (%) indicating the optimum yield of amylase using the combination of thisparameters.

Figure 23 shows the Response Surface (3D) and contour plots of amylase production of *B. circulans* in batch fermentation as a function of temperature at 55^0C and lactose at 1(%) indicating the optimum yield of amylase using the combination of thisparameters.

Figure 24 shows the Response Surface (3D) and contour plots of amylase production of *B. circulans* in batch fermentation as a function of lactose at 1 (%) malt extract at 2(%) indicating the optimum yield of amylase using the combination of this parameters. Enzyme production by *B.circulans* is poorly documented thus the research provided the optimum parameters forenzyme production from *Bacillus.circulans.*

CONCLUCION

Using the two techniques; qualitative and quantitative screening, *Bacillus circulans* have shown to be a good Amylase producing organism based on the high zone of clearance from the starch iodine test. The optimal characteristics for enzyme production from *Bacillus circulans* are pH of 10, Temperature of 35^0C, Malt Extract 2(%) and lactose (1%). The enzyme produced by *B. circulans* has a thermal stability at temperature ranges from 70^0C to 80^0C and pH stability from 6 to 7.

RECOMMENDATION

Further work should be done to demonstrate the effect of tis enzyme produced on degradation of raw starch for starch hydrolysis. Other purification process like the use of column chromatographic methods can be carried out on the enzyme produced. The effect of metal ions ontheenzymecanalsobecarriedout.Furtherworkneedstobedonesoastoseehowthis

enzyme can be packaged and distributed.

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