**Production and Optimization of Pullulanase by *Stenotrophomonas maltophilia* under Submerged Fermentation using Response Surface Methodology (RSM)**


## Abstract:

Pullulanase (pullulan α- hydrolase EC 3.2.1.41) is starch debranching enzyme, that cleave the α-1,6, glucosidic linkage. This study investigated the screening, production, and optimization of pullulanase from bacterial isolates. Five different soil samples were collected from Choba (River State) and Oshodi (Lagos State). The total culturable heterotrophic bacterial counts ranged from $2.21x10^{6}$ to $3.2 \times 10^{6}$Cfu/ml. A total of eight bacterial isolates exhibited potential to degrade pullulan which produced a clear halo zone at 70% around the colonies. The isolate with the highest pullulanase degradation was further investigated for production of production using wheat bran as substrate under submerged fermentation (SmF) applying RSM. Identification and characterization of the isolate was done using both conventional and molecular techniques. This isolate was identified molecularly as *Stenotrophomonas maltophilia.* The 16S rRNA sequence was deposited in GenBank under accession number MH225448.1The optimum production of pullulanase obtained using One-factor-at-a-time (OFAT) was achieved with wheat bran (2.469U/ml) at 48h, pH 7 and temperature of 37ºC. Optimization using RSM revealed the following optimum conditions; temperature 36ºC, pH 7.5, incubation period of 36h and pullulan concentration of 1.5%. The result gathered from this study showed that the bacterial isolate can successfully utilize agro waste materials for production of pullulanase. This poses great potential in ameliorating the environmental pollution arising from their poor disposal as well as encouraging production of the enzyme at low cost. The data from this study also makes the isolate a good candidate for the production of pullulanase for industrial purposes.



**Keywords:** Pullulanase, Optimisation, *Stenotrophomonas maltophilia*, Submerged fermentation, Response Surface Methodology (RSM)


## Introduction

Pullulanase is one of the members of the family 13 glucosyl hydrolase, which is also called α-amylase family. Pullulanase has an interesting feature, where it can specifically attach branch points in pullulan, starch and dextrins.Pullulanase belongs to (pullulan α-glucano hydrolase; EC 3.2.1.41) an extracellular enzyme which debranches the extracellular yeast polysaccharide and pullulan. This was first discovered by [1] from mesophilic organism *Klebsiella pneumoniae* (formerly known as *Aerobactor aerogenes* or *Klebsiella aerogenes*). Pullulanase is responsible for the hydrolysis of pullulan and amylopectin to produce maltotriose [2]. These enzymes microbial pullulanase attracts more interest because of its specific action on α- 1, 6 linkages in pullulan, a linear α- glucan consisting basically of maltotriosyl units connected by 1, 6 α-bonds. There are three kinds of bond that cleave by enzyme called pullulanase (EC.3.2.1.41), isopullulanase (EC 3.2.1.57) and neopullulanase (3.2.1.35).The pullulanase are sub-divided into two categories based on substrate specificity: (i) Pullulanase type I, which specifically cleaves the α 1,6-glycosidic linkages in pullulan and branched oligosaccharides, forming maltotriose and linear oligosacharides, respectively and (ii) Pullulanase type II, or amylopullulanase, which hydrolyse both α 1,6-glycosidic bond and α 1,4- glucosidic bonds are capable of amylose degradation, yielding glucose, maltose, maltotoris, and other oligosaccharides. However, in the absence of a debranching enzyme capable of hydrolyzing α 1,6-glucosidic bond, amylopectine degradation is incomplete [3]. Some of the *Bacillus species* that produce pullulanase are *Bacillus acidopullulyticus*,

*Klebsiella planticola, Geobacillus stearothemophilus, Bacillus deramificans* and *Bacillus cereus* FDTA-I3 [4]. The development of fermentation during the latter part of the last century aimed specifically at the production strains made it possible to manufacture enzymes as purified well characterized preparations even on the large scale. This development allowed the production of enzymes into true industrial products and processes [5].

Pullulanase is of great significance due to its wide area of potential application. It is a very potent enzyme for degradation of starch to glucose and maltose. It has been reported that pullulanase enzyme is used on a large scale in glucose and maltose syrup industries. It is widely used in industries in the saccharification of starch. It converts starch into glucose and maltose which are used in the production of glucose syrup more efficiently [6]. Pullulanase are used in detergent industry as effective additives in dish washing and laundry detergents for the removal of starch under alkaline conditions [7] baking industry, used as dental plaque control agent [8] and for the production of cyclodextrins which in turn is used in the production of biotechnological products and low calorie beer. Cyclodextrin glucosyl transferase (CGTase) is used with α- amylase. But the α-1,6 glucosidic bond of amylopectin blocks the action of (CGTase). These bonds are broken by the pullulanase enzyme hence increasing the percentage yield of cyclodextrin production. In order to produce pullulanase, fermentation process is being carried out using submerged fermentation (SmF) for pullulanase production. The main advantage of using substrates is that nutrient rich in waste materials can be easily recycled in cheaper substrates. In the present study various found culture were isolated from different soil sample and screened for pullulanase production under submerged fermentation the best screened isolates were selected as reference culture, which was further utilized for optimization for the various process parameter for the enhanced production of pullulanase under submerged fermentation. The selected bacterial culture was further screened by submerged fermentation for pullulanase production and the best selected bacterial culture was further utilized for optimization process. Besides, the study using the Response surface methodology (RSM) techniques may also improve the pullulanase enzyme and the production.

Response surface methodology (RSM) is a statistical design used as a modelling and optimization technique for identifying effective factors and interaction among factors, which have significant influence on the final yield of product, in many bioprocesses [9][10]. Central composite design (CCD) of RSM helps in estimating the key factors out of large number of media components and fermentation conditions by performing low experimental runs [11]. This design helps to estimate coefficient of quadratic model and has been extensively used for bioprocess optimization. This study evaluated the isolation, production and optimization of cultural and nutritional parameters for the production of pullulanase from *Stenotrophomonas maltophilia* under submerged fermentation using statistical techniques (RSM).



## Materials and methods

### Collection of samples

Five soil samples were collected from different locations at Choba (Rivers State) and Oshodi (Lagos State) using sterile plastic container. All the soil samples were collected from the surface (0-5cm depth) using the soil auger and transferred into the plastic containers. These plastic bags were maintained at $4^{o}C$ to ensure minimal biological activity. To provide homogenized soil samples, the soil was thoroughly mixed together.

### Isolation of bacteria

Soil suspension was prepared by mixing 1g of soil sample in 9ml of sterile distilled water (Saline). It was stirred and allowed to settle to ensure that the microorganisms to come in water phase. The suspension was serially diluted from $10^{-1}$ to $10^{-10}$. The diluted suspensions were picked from dilutions of $10^3$, $10^4$, $10^5$ and $10^8$ for the isolation of microorganisms. Nutrient agar and pullulan agar was weighed. 0.1ml of each dilution was spread on the agar medium plates in duplicates using pour plate method. The plates were sterilized at 121°C for 15min in an autoclave and incubated at 37°C for 24h. The colonies obtained from the plates were marked and numbered. They were further sub-cultured from their respective medium in order to obtain pure culture. Bacterial pure culture were maintained on nutrient agar slants and stored for further analysis.

**Identification and biochemical characterization of pullulanase isolates**

The morphological characteristics of the isolated bacterial strains or colonial properties such as colonial size, shape, colour, margin, elevation, and opacity were examined macroscopically. These isolates were identified based on phenotypic characteristics and biochemical test which include oxidase, catalase, motility, sugar fermentation, urease, citrate, methyl red/vogues proskauer, indole, triple sugar iron agar and casein hydrolysis according to the ninth edition of Bergey's Manual of Systematic Bacteriology [12].

**Molecular identification of bacterial isolates**

The isolated bacterial strains were further subjected to molecular identification using 16S ribosomal deoxyribonucleic acid (rDNA) for confirmation.

**DNA Extraction**

DNA extraction was carried out on the isolates using the Zymo Fungal/Bacteria DNA extraction kit according to manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a NANODROP (ND 1000) Spectrophotometer (Thermo Scientific, USA).

All the samples showed a DNA yield between 5ng - 25ng, and the extracted DNA were optimally pure showing A260/A280 between 1.60-1.80.

**PCR Amplification of the ITS gene**

Polymerase chain reaction was carried out to amplify the ITS gene of the bacteria using the primer pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG) and ITS-4 (5'-TCCTCCGCTTATTGATATGC). The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol *DNA* polymerase (Solis Biodyne); however, additional Taq DNA polymerase was incorporated into the reaction mixture to make a final concentration of 2.5 units of Taq DNA polymerase, Proofreading Enzyme, 2µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.

Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) for an initial denaturation of 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute at 58°C and 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a

1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder was used as DNA molecular weight standard.

**PCR Amplification of the 16SrRNA gene (27F and 1492R)**

Polymerase chain reaction was carried out to amplify the 16SrRNA gene of the bacteria using the primer pair 27F- 5′- AGAGTTTGATCCTGGCT CAG -3', and 1492R 5'- GGTTACCTTGTTACGACTT -3'. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM MgCl2, 200µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) for an initial denaturation of 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute at 61°C and 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker.

**Sequencing**

All PCR products were purified with Exo sap and sent to Epoch Life science (USA) for Sanger sequencing. The corresponding sequences were identified using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi

**Primary screening of bacterial isolates**

Eight bacterial isolates were screened for pullulanase production by culturing in pullulan agar. The medium consisted of (w/v). Pullulan powder (1%), yeast extract (0.5%), $MgSo_4 7H_2O$ (0.02%), $K_2HPO_4$ (0.03%), $(NH_4)_2SO_4$ (0.1%), $CaCl_2.2H_2O$ (0.02%), $MnCl_2.4H_2O$ (0.0001%), $FeSO_4 7H_20$ (0.001%), bacteriological agar (3.5%) and pH was adjusted to 7 and sterilized at 121°C for 15min. Pullulan as unique carbon source for screening of hydrolyzing pullulan strains [13]. Point inoculation was done at the centre of the plates and was incubated at 37°C at 48h. Several pullulanase producing bacterial colonies were identified after flooding the plates with gram's iodine solution [14]. The presence of transparent zone of hydrolysis around the colonies after the 48h of incubation indicates the presence of pullulanse producers. Where halo zone diameter H in (mm) and the colony diameter C in (mm) were measured. The degradation of efficiency of all the pullulanase producers were calculated using this formular (H-C)/C (%) [15]. The bacterial isolates which indicated the degradation efficiency is 33% and 70% were selected for further experiments [16].

**Submerged fermentation**

**Preparation of wheat bran (*Triticum aestivum*)**

Wheat bran was gotten from Oshodi market, Lagos State. The wheat bran was grinded in powdered and kept for fermentation analysis.

**Inoculum preparation for submerged fermentation**

The preserved culture on the nutrient agar medium slant was first inoculated on a fresh nutrient agar medium and incubated at 37°C for 24h to get the pure culture that was used for the production of enzyme. The activated isolates were removed and suspended in Tween 80 and 2ml of bacterial suspension was used for inoculation in Submerged Fermentation flask [17].

**Enzyme production**

Enzyme production was carried out under submerged fermentation (SmF). The fermentation medium consisted of (g/l) (1) pullulan, (5) yeast extract, 0.3 $K_2HPO_4$, (0.2) $MgSO_4.7H_2O$, (1) $(NH_4)_2SO_4$, (0.2) $CaCl_2.2H_2O$, (0.01) $FeSO_4.7H_2O$ and (0.001) $MnCl_2.4H_2O$ which is the same as the one used for screening except that it does not contain agar. The pH was adjusted to 7 [13]. Wheat bran (4.5g) was weighed into 150ml of each Erlenmeyer flask containing the fermentation medium and were autoclaved (DIXON) at 121°C for 15min allowed to cool and 2ml of the bacterial suspension was inoculated into the Erlenmeyer flask. The incubation was done at 37°C for 3days in a shaker incubator (New Brunswick Scientific Co INC). The flasks were taken out at the end of fermentation and 10ml of contents were extracted and centrifuged (Hettich EBA 8S) at 10,000rpm for 10min. The supernatant was used as a source crude pullulanase for further analysis [18].

**Pullulanase assay**

The method described by [16] was used to determine the pullulanase activity. Enzyme activity was measured in the reaction mixture consisted of 0.46ml of 1% pullulan in 0.5ml potassium phosphate buffer, pH 7 and concentration of the extracellular enzyme crude 0.5ml incubated at 40°C for 10min. The released sugars were assayed by the DNS method using spectrophotometer. After incubation the reaction was stopped by cooling the tube in an ice bath or water bath and the reducing sugar release by enzymatic hydrolysis of soluble starch and pullulan was determined by addition of 1ml dinitrosalicic acid (DNSA) reagent then incubated in boiling water for 100°C for 5min and the enzyme activity was measured using glucose standard at 540nm in spectrophotometer [13]. Sample blank was also used to correct the non enzymatic release of reducing sugars. One unit of pullulanase was defined as the amount of enzyme required to catalyse the liberation of reducing sugar equivalent to 1µmol of D-glucose per minutes under the assay conditions [19]. Protein concentration in the supernatant was determined by method described by [20] optical density was read at 600nm wavelength of the spectrophotometer

**Protein concentration determination**

Lowry method of quantitative estimation of protein was used. Solution A alkaline sodium carbonate solution (20g/l) in 4g sodium hydroxide, solution B copper sulphate sodium potassium tatarate solution 5g/l $CuSO_4.5H_2O$ in 10g/l NaKtatarate. The alkaline solution was prepared by mixing of solution A in 50ml and solution B in 1.0ml. 0.2ml of the enzyme extract was measure into tube, 0.8ml of distilled water was added to it. Thereafter, 5ml of alkaline copper reagent was added to the mixture properly and the mixture was allowed to stand for 10mins at room temperature. 0.5ml for Folin Cicaultua solution was added to all tubes and left for 30 minutes after which the optical density was read at 600nm wavelength of

the spectrophotometer against a blank prepared with 0.2ml buffer. A standard curve was constructed with each experiment using bovine serum albumin as a known protein. The amount of the soluble protein was calculated from the standard curve as mg protein per ml of test samples. The protein concentration was estimated using values extrapolated from the standard graph.

**Optimization of cultural condition for pullulanase production**

This study also involved in the optimization of different parameters governing pullulnase production. The effects of various temperatures, pH, incubation time, pullulan concentration on pullulnase production using the Response surface methodology (RSM) method, effects of various carbon sources and nitrogen sources using one factor at a time method (OFAT). The effect of these parameters was tested by taken the fermentation media at different temperatures, pH, incubation time, pullulan percentage. The fermentation media was withdrawn daily for pullulanase production till a decline time. The activity was measured daily using a UV spectrophotometer (T70 UV/VIS spectrophotometer PG Instrument LtD). The amount of protein present was assayed using bovine serum albumin (BSA) as standard. [18].

**Design of experiment for optimization pullulanase production**
**One-factor-at-a-time (OFAT)**

A preliminary investigation using One-factor-at-a-time (OFAT) method was applied to select the independent variables which influence pullulanase production by the selected bacterial strain. This was performed by evaluating one factor while the other variables were kept constant. The nutritional parameters such as carbon sources (glucose, maltose, xylose and pullulan) and nitrogen sources ($KNO_3$, $NH_4NO_3$ and yeast extract) were studied to select the best among them. The different carbon and nitrogen sources were substituted by equivalently to the original amounts in the basal medium.

**Effect of carbon sources on pullulanase production**

Carbon sources play a vital role in the production of pullulanase. Among different carbon sources used are glucose, maltose, xylose and pullulan. This experiment was done by using the one factor at a time (OFAT). Wheat bran (4.5g) was weighed into 150ml of Erlenmeyer flask containing fermentation medium as it was stated in enzyme production and were autoclaved at 121°C for 15minutes allowed to cool, 2ml of the bacterial suspension was added to the flask and distributed to each bijou bottles containing 0.5% of each carbon source at a time was incubated at 37°C for 4 days at pH 7, the broth were centrifuged at 10,000rpm at 10minutes. Supernatant were assayed for enzyme activity, protein concentration, and specific activity was measured.

**Effect of nitrogen sources on pullulanase production**

Among the two inorganic and organic nitrogen sources $KNO_3$, $NH_4NO_3$ and yeast extract. This experiment was done by using the one factor at a time (OFAT). 4.5g of wheat bran was weighed into 150ml of Erlenmeyer flask containing fermentation medium as it was stated in enzyme production and were autoclaved at 121°C for 15min allowed to cool, 2ml of the bacterial suspension was added to the flask and distributed to each bijou bottles containing 2% of each carbon source at a time was incubated at 37°C for 4 days at pH 7, the broth were centrifuged at 10,000rpm at 10min. Supernatant were assayed for enzyme activity, protein

concentration, and specific activity was measured**.** The amount of protein present was assayed using bovine serum albumin (BSA) as standard [18].

**Response surface methodology (RSM)**

Response surface methodology (RSM) is an experimental design strategy for selecting optimum conditions for a multivariable system, as it studies the integration among variables with less experimentation, time and cost. The central composite design (CCD) with 3 factors and 3 levels, including 6 replicates, all the centre point has used for a second order response surface.

The central composite design (CCD) was developed as an imbedded factorial matrix with centre points and star points (replicate of axial point) around the centre point which allows estimation of the curvature. The response surface methodology was applied to understand the interaction of various variables and then used to find the optimum concentration of the significant independent variables (carbon source, nitrogen source, pH and metal ion) and the effects of their natural interactions on pullulanase production. The statistical analysis of the data was performed using Design Expert ver.10.0.7.0 (State-Ease, Inc. Minneapolis, USA) was used for RSM experimental design and data analysis. From the experimental data according to the design, a second-order polynomial regression model equation was derived to define the response in terms of the independent variables. The response surface graph obtained was used to explain the effect of variables individually and in combination, and to determine the optimum levels for maximum pullulanase production (Table 1).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1 A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{24} CD$$

Where Y: Predicted response

$\beta_0$: Intercept, A: pH, B: temperature, C: Incubation time, D: Pullulan percentage

$\beta_1$, $\beta_2$, $\beta_3$ and $\beta_4$ are the linear coefficients,

$\beta_{11}$, $\beta_{22}$, $\beta_{33}$ and $\beta_{44}$ are the squared coefficients,

$\beta_{13}$, $\beta_{14}$, $\beta_{24}$, and $\beta_{34}$ are the interaction coefficients,

$A^2$, $B^2$, $C^2$, $D^2$, AB, AC, AD, BC, BD and CD are the interactions between the variables as significant terms.

**Table 1: RSM design showing the experimental plan for optimization**

| Runs | pH | Temp($^o$C) | Incubation Time(h) | Pullulan(%) |
|---|---|---|---|---|
| 1 | 12.5 | 20 | 72 | 2.5 |
| 2 | 12.5 | 60 | 0 | 2.5 |
| 3 | 2.5 | 60 | 72 | 0.5 |
| 4 | 2.5 | 60 | 0 | 2.5 |
| 5 | 7.5 | 40 | 36 | 1.5 |
| 6 | 2.5 | 20 | 72 | 0.5 |
| 7 | 7.5 | 40 | 36 | 1.5 |
| 8 | 2.5 | 60 | 72 | 2.5 |
| 9 | 12.5 | 20 | 0 | 2.5 |

| | | | | |
|---|---|---|---|---|
| 10 | 2.5 | 20 | 72 | 2.5 |
| 11 | 12.5 | 60 | 72 | 2.5 |
| 12 | 2.5 | 60 | 0 | 0.5 |
| 13 | 7.5 | 40 | -36 | 1.5 |
| 14 | 12.5 | 60 | 0 | 0.5 |
| 15 | 7.5 | 40 | 36 | 3.5 |
| 16 | 12.5 | 60 | 72 | 0.5 |
| 17 | -2.5 | 40 | 36 | 1.5 |
| 18 | 7.5 | 80 | 36 | 1.5 |
| 19 | 7.5 | 40 | 36 | 1.5 |
| 20 | 7.5 | 0 | 36 | 1.5 |
| 21 | 7.5 | 40 | 108 | 1.5 |
| 22 | 2.5 | 20 | 0 | 0.5 |
| 23 | 12.5 | 20 | 0 | 0.5 |
| 24 | 17.5 | 40 | 36 | 1.5 |
| 25 | 2.5 | 20 | 0 | 2.5 |
| 26 | 7.5 | 40 | 36 | 1.5 |
| 27 | 7.5 | 40 | 36 | 1.5 |
| 28 | 12.5 | 20 | 72 | 0.5 |
| 29 | 7.5 | 40 | 36 | 1.5 |
| 30 | 7.5 | 40 | 36 | -0.5 |

## Results and Discussion

### Isolation, screening and identification of bacterial isolates

The unknown bacteria were observed for the colonial characteristics result are described by analysing the colonial characteristics on nutrient agar plates, the colonies of desired enzyme producing strain were found as translucent colonies of 3-5 and 1-2mm size, shape oval and entire. The pullulanase producing strains were gram negative bacterial organisms. When viewed under microscope with x40. The isolate FFW 10 was found to be *Stenotrophomonas maltophilia* strain on the basis of 16SrRNA gene sequence. The ribosomal RNA gene sequence has been submitted to the GenBank and accession number MH225448.1.Table 2 [2] reported pullulanase production by *Bacillus* sp. [18] and [16] have identified pullulanase producing bacterial isolates based on their microscopic and biochemical parameters.

**Table 2: Biochemical characterizationof the bacterial isolate**

| Basic characteristic | *Stenotrophomonas maltophilia* |
|---|---|
| Catalase | + |
| Oxidase | + |
| Gram stain | G- |
| Casein | + |
| Shape | Rod |
| Methyl red (M-R) | - |
| Urease | - |

| | |
|---|---|
| Vogasproskuer (V-P) | - |
| Citrate | - |
| Spore shape | + |
| Motility | + |
| $H_2S$ Production | - |
| Glucose | +G |
| Sucrose | +G |
| Lactose | +G |
| Galactose | +G |
| Fructose | +G |

Note: + = positive, - = negative, G = gas, G- = gram negative and G+ = gram positive

The isolate FFW 10 was found to be *Stenotrophomonas maltophilia* strain on the basis of 16SrRNA gene sequence. The ribosomal RNA gene sequence has been submitted to the GenBank and accession number MH225448.1.

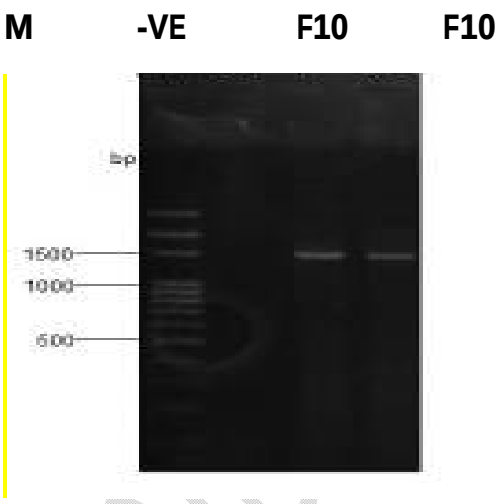


**Image 1: PCR amplification image of *Stenotrophomonas maltophilia***

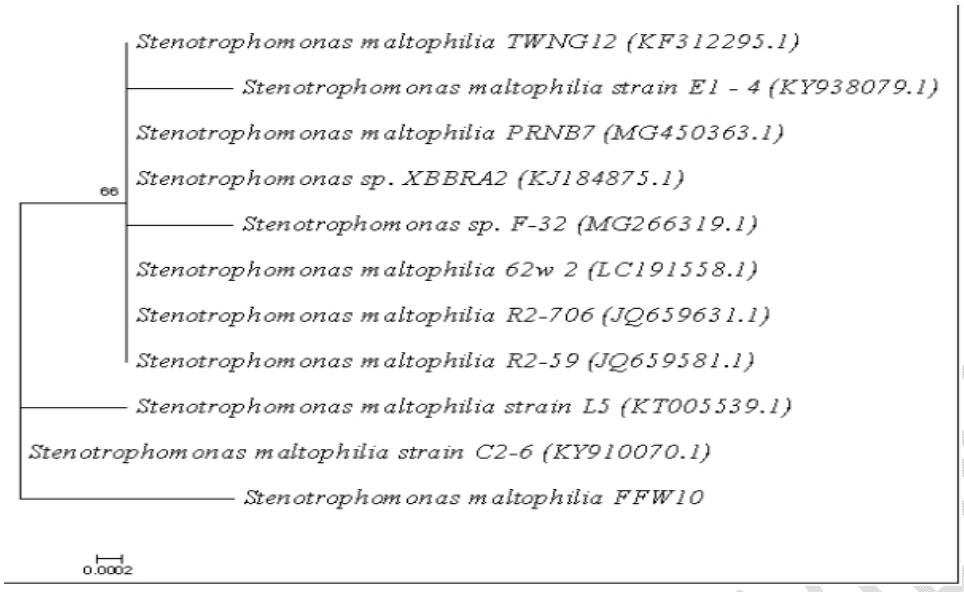


**Image 2: Estimated phylogenetic relationship of isolate FFW10 used in this study with closest GenBank isolates. The tree was rooted by designating strain FFW10 as the out-group.**

**Screening of pullulanase producing bacterial isolates**:

Table 3 showed the screening results of the 8 bacterial isolates which were able to produce pullulanase with different diameter of pullulan hydrolysis zones. The ratio of pullulan hydrolysis zones of the bacterial isolates ranged between 20-70%. The highest percentage degradation efficiency was recorded with isolate FFW 10 (70%), followed by FFW 8 (33%) while isolate RFW 7 had the least degradation efficiency of 20%. Among the 8 bacterial strains screened, only isolate FFW 8 and FFW 10 exhibited the ability to degrade pullulan. Hence, isolate FFW 10 was selected for further studies. [16] reported correlation between colony diameter (zone of clearance) and pullulanase production from the bacterial isolates were efficiency above 50% were selected and subjected to secondary screening [18] reported that the colony which showed a clear hollow zone around isolate after addition of KI solution on pullulan agar plate was regarded as pullulanase producer. The screening of these bacterial isolates on pullulan agar showed that the isolates FFW10 were able to produce pullulanase with diameter of pullulan hydrolysis zones at 37 ºC incubation

**Table 3: Screening for the potentials of pullulanase producing bacterial isolates obtained from soil samples collected from different locations in Rivers State and Lagos States.**

| S/No | Sample code | Halo zone H(mm) | Colony size C (mm) | Degradation efficiency(H-C/C) % |
|---|---|---|---|---|
| 1 | FFW 10 | 34 | 20 | 70 |
| 2 | FFW 8 | 20 | 15 | 33 |
| 3 | OFW 1 | 22 | 18 | 22 |
| 4 | RFW 7 | 24 | 20 | 20 |

| | | | | |
|---|---|---|---|---|
| 5 | OFW 3 | 26 | 20 | 30 |
| 6 | OBW 5 | 24 | 18 | 31 |
| 7 | FFW 11a | 22 | 17 | 29 |
| 8 | FFW 11b | 22 | 18 | 22 |

Figure 1 shows the preliminary study (with OFAT) on pullulanase production in submerged fermentation (SmF) using wheat bran and potato starch as substrates. The result revealed that both substrates encouraged pullulanase production by the bacterial strain although wheat bran proved to be better than potato starch in all fermentation periods under study. The highest pullulanase production (2.50 U/ml) was achieved after 48 h of incubation as against when wheat bran was used as 1.52 U/ml recorded with potato starch. However, optimum pullulanase production was obtained after 48 h of fermentation for both substrates. Wheat bran was selected as substrate for further analysis. Submerged fermentation was explored and efforts were made to utilize natural substrates that contained starch as the substrates for pullulanase production at incubation time at 48h, pH 7 and temperature at 37°C. The data showed that wheat bran played a positive role in the production of pullulanase by *Stenotrophomonas maltophilia*. Similar results were reported from *Bacillus* sp. in the production of pullulanase [13]. Other worker showed a high level of pullulanase production by *Bacillus halodurans* after 5 days incubation [18]. [13] reported temperature 40°C, pH 7 and incubation time 48h. [16] also reported incubation tine 48h, pH 7.5 and temperature at 37°C for the pullulanase production.

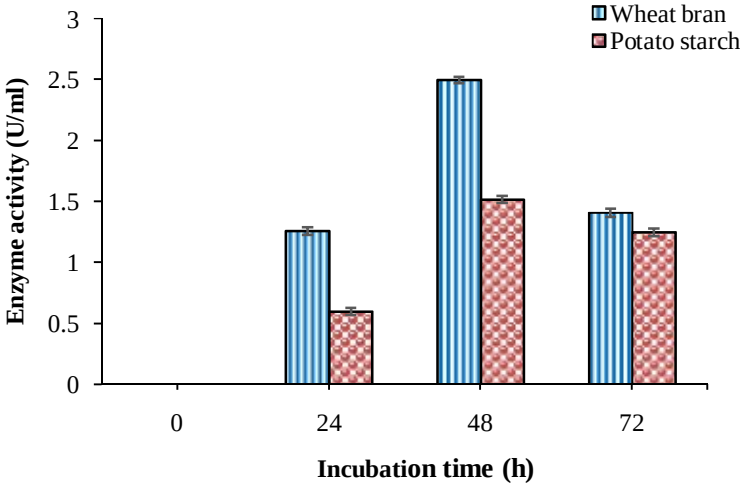


**Fig. 1: Preliminary study on pullulanase production in submerged fermentation (SmF) using wheat bran and potato starch as substrates**

### Effect of Carbon Source on pullulanase production

The Figure 2 showed the effect of different carbon sources on growth and pullulanase production by the isolate was determined by growing the bacterial isolates in SmF medium in which any of the following carbon sources: glucose, maltose, xylose and pullulan were used as a sole carbon source. The maximum pullulanase production was shown when pullulan was

used as sole carbon source at incubation period at 48 h, followed by maltose at the same incubation time on isolate A and B. and the least carbon source occurred on glucose and xylose. In the various carbon sources tested, pullulan was found to be the best carbon source for the substrates

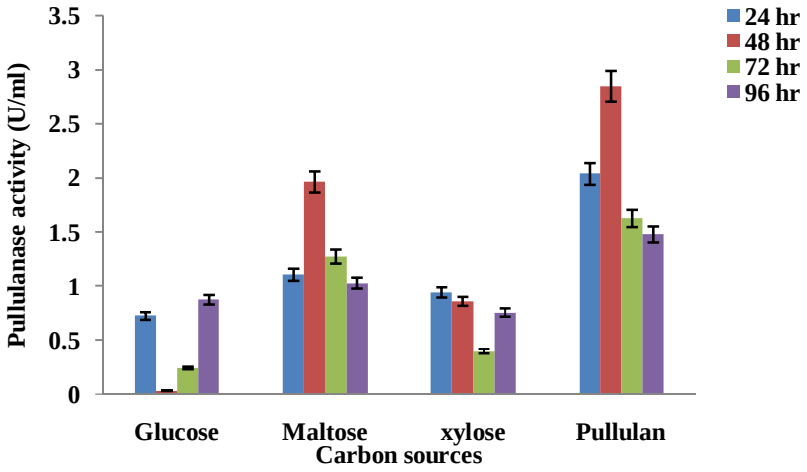


Figure 2: Effect of various carbon sources on pullulanase production by *Stenotrophomonas maltophilia*



**Effect of nitrogen sources on pullulanase production**

Figure 3 showed the effect of organic nitrogen source (yeast extract) and inorganic nitrogen sources ($NH_4NO_3$ and $KNO_3$) on pullulanase production by *S. maltophilia*. The maximum pullulanase production *Stenotrophomonas maltophilia* (2.87U/ml) was observed with yeast extract for the isolate at the same incubation time.

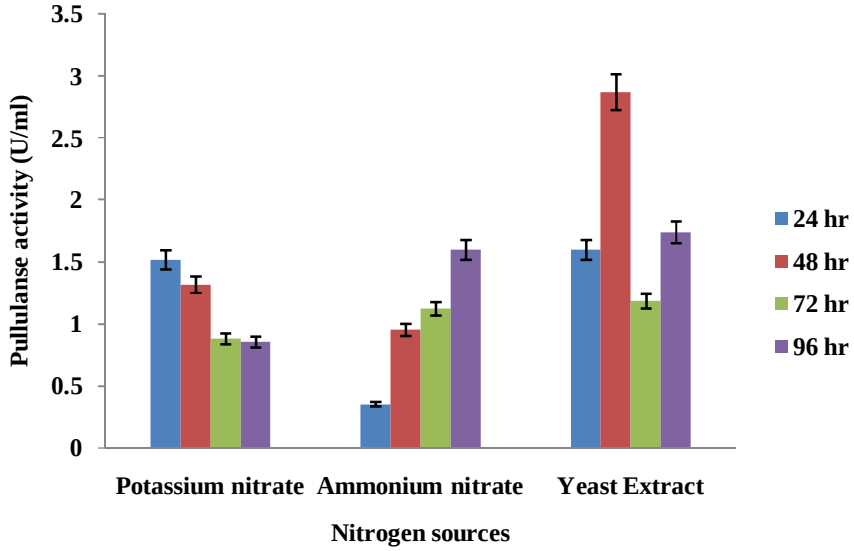

Figure 3: Effect of various nitrogen sources on pullulanase production by *Stenotrophomonas maltophilia*

## Optimization studies

Optimization processes were done with quadratic models of the central composite design (CCD) of response surface methodology, using Stat-Ease Design Expert 10.0 software. Pullulanase produced by *Stenotrophomonas maltophilia* were optimized respectively using the quadratic models of the CCD. The factors considered as independent variables were Temperature, pH, pullulan percentage and incubation time. The results of Central Composite Design showed the significance on the production of pullulanase as the various optimum condition were noted; temperature 36ºC (A), pH 7.5 (B), incubation period 36h (C) and pullulan percentage production 1.5% (D) by *Stenotrophomonas maltophilia*(Table 4). The interaction of the various variables (A, B, C, D), respectively in this study were found to be significant. [21] reported that the central composite design of response surface methodology was applied to understand the interactions between the process parameters for pullulan production by *Aureobasidium pullulans* MTCC 2195 using a second order quadratic model.

**Table 4: RSM design the actual and predicted values of the pullulanase produced by *Stenotrophomonas maltophilia***

| Runs | pH | Temperature(ºC) | IncubationTime(h) | Pullulan(%) | Actual Pullulanase(U/ml) | Predicted Pullulanase(U/ml) |
|---|---|---|---|---|---|---|
| 1 | 12.5 | 20 | 72 | 2.5 | 1.75 | 2.02 |
| 2 | 12.5 | 60 | 0 | 2.5 | 1.01 | 1.54 |
| 3 | 2.5 | 60 | 72 | 0.5 | 1.19 | 1.36 |
| 4 | 2.5 | 60 | 0 | 2.5 | 1.32 | 1.65 |
| 5 | 7.5 | 40 | 36 | 1.5 | 5 | 4.78 |
| 6 | 2.5 | 20 | 72 | 0.5 | 1.21 | 1.15 |
| 7 | 7.5 | 40 | 36 | 1.5 | 3.1 | 4.78 |
| 8 | 2.5 | 60 | 72 | 2.5 | 1.78 | 2.06 |
| 9 | 12.5 | 20 | 0 | 2.5 | 1.11 | 1.43 |
| 10 | 2.5 | 20 | 72 | 2.5 | 2.1 | 2.24 |
| 11 | 12.5 | 60 | 72 | 2.5 | 2.5 | 2.24 |
| 12 | 2.5 | 60 | 0 | 0.5 | 1 | 1.19 |
| 13 | 7.5 | 40 | -36 | 1.5 | 2.12 | 1.20 |
| 14 | 12.5 | 60 | 0 | 0.5 | 1.3 | 1.65 |
| 15 | 7.5 | 40 | 36 | 3.5 | 2.71 | 2.31 |
| 16 | 12.5 | 60 | 72 | 0.5 | 1.78 | 2.11 |
| 17 | -2.5 | 40 | 36 | 1.5 | 2 | 1.51 |
| 18 | 7.5 | 80 | 36 | 1.5 | 2.2 | 1.72 |
| 19 | 7.5 | 40 | 36 | 1.5 | 5.2 | 4.78 |
| 20 | 7.5 | 0 | 36 | 1.5 | 1.88 | 1.40 |
| 21 | 7.5 | 40 | 108 | 1.5 | 2 | 1.96 |
| 22 | 2.5 | 20 | 0 | 0.5 | 0.34 | 1.09 |
| 23 | 12.5 | 20 | 0 | 0.5 | 0.97 | 1.15 |
| 24 | 17.5 | 40 | 36 | 1.5 | 2.21 | 1.75 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 25 | 2.5 | 20 | 0 | 2.5 | 1.81 | 1.95 |
| 26 | 7.5 | 40 | 36 | 1.5 | 5.3 | 4.78 |
| 27 | 7.5 | 40 | 36 | 1.5 | 5.1 | 4.78 |
| 28 | 12.5 | 20 | 72 | 0.5 | 1.33 | 1.49 |
| 29 | 7.5 | 40 | 36 | 1.5 | 5 | 4.78 |
| 30 | 7.5 | 40 | 36 | -0.5 | 1.87 | 1.32 |

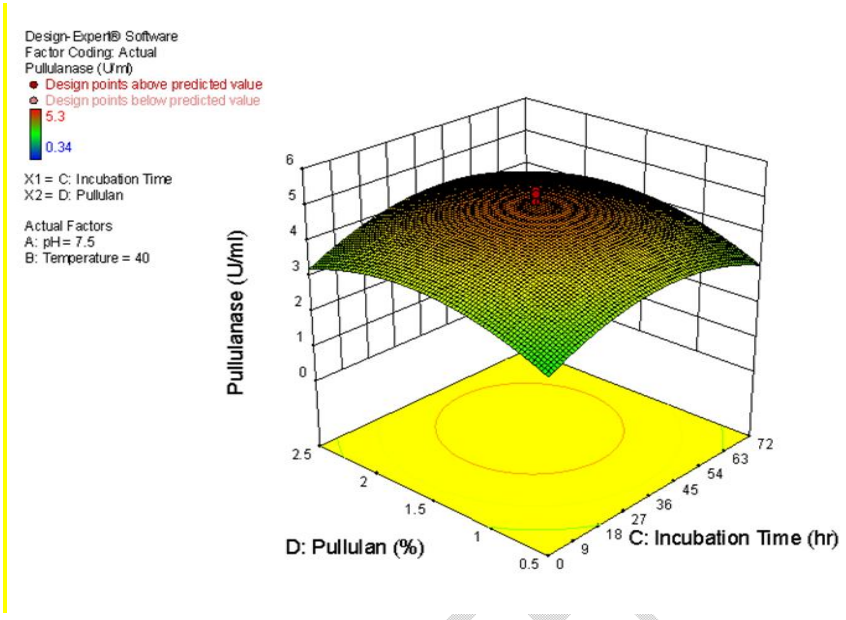


**Figure 4: RSM for 3Dimensional representation of pullulanase concentration (U/ml) produced by *Stenotrophomonas maltophilia* under varied pullulan (%) and incubation time, and pH 7.5 and Temperature 40$^0$C**

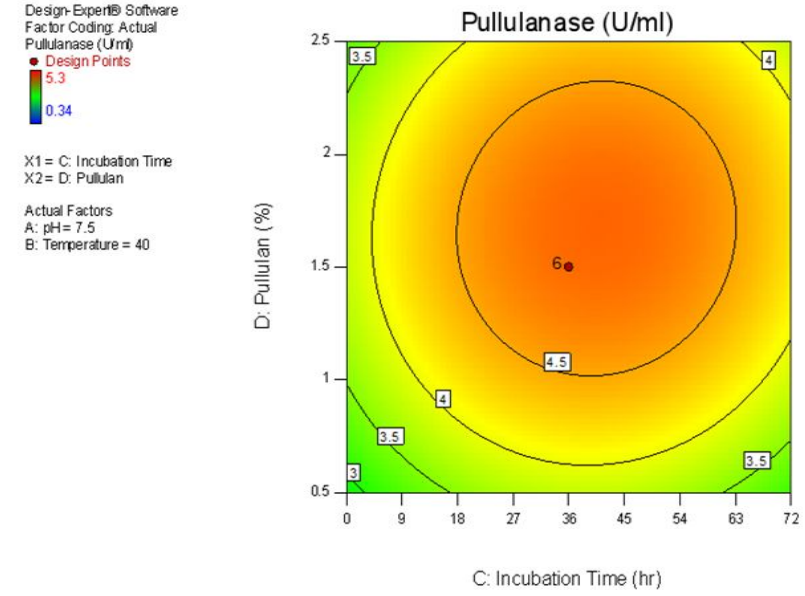

**Figure 5: Contour representation of pullulanase concentration (U/ml) produced by *Stenotrophomonas maltophilia* under varied pullulan (%) and incubation time, and pH 7.5 and temperature 40$^0$C**

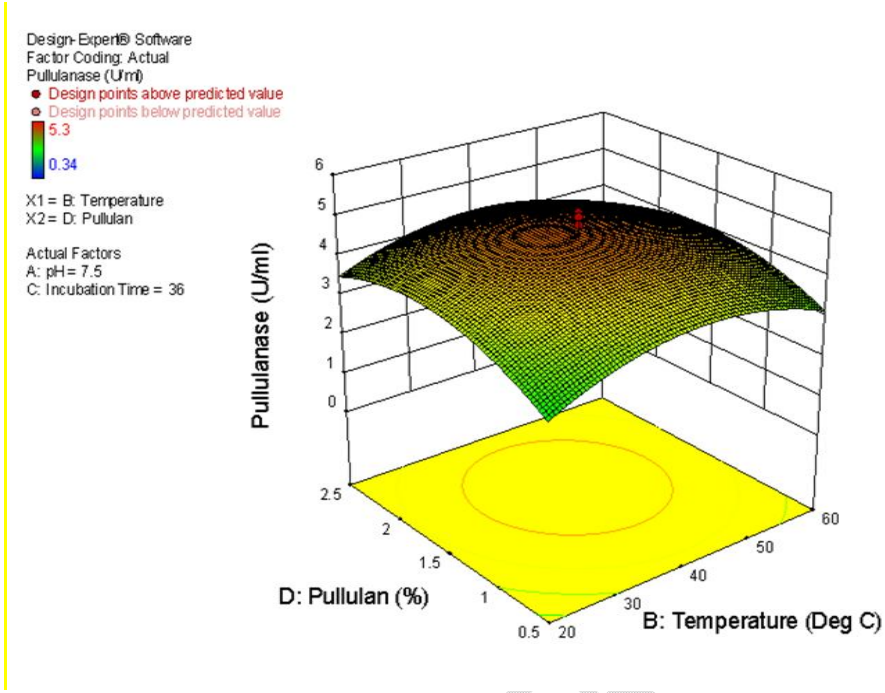


**Figure 6: RSM for 3Dimensional representation of pullulanase concentration (U/ml) produced by *Stenotrophomonas maltophilia* under varied pullulan (%) and temperature ($^0$C), and pH 7.5 and incubation time 36h**

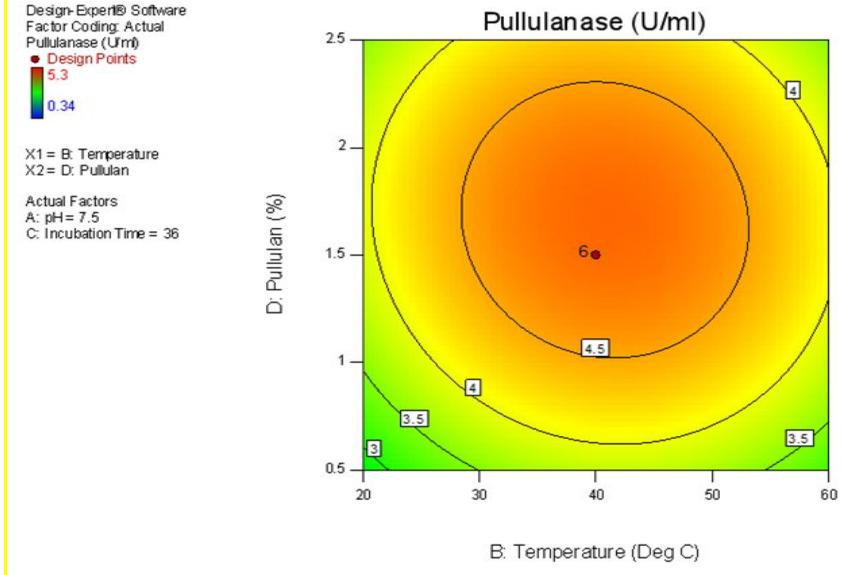


**Figure 7: Contour representation of pullulanase concentration (U/ml) produced by *Stenotrophomonas maltophilia*under varied pullulan (%) and temperature ($^0$C), and actual pH 7.5 and incubation time 36h**

## Conclusion

The cultural conditions and composition of media for optimal production of pullulanase by *Stenotrophomonas maltophilia* have been developed in this study. The outcome of this study indicated that optimum production of pullulanase by the bacterium was at 40$^{o}$C, pH 7 and incubation time of 48 h. Various carbon sources were investigated as media component using OFAT and pullulan 2.844 U/ml was observed to be the best for pullulanase production by *S. maltophilia*, while maltose, xylose and glucose were the least/lowest. Optimal enzyme production (2.87 U/ml) was with organic yeast extract as nitrogen source. The Central Composite Design of RSM was employed to optimize the fermentation medium for pullulanase production. The results of RSM showed that optimum pullulanase production was achieved at: temperature 36ºC, pH 7.5, incubation period of 36 h and pullulan percentage concentration of 1.5% using *Stenotrophomonas maltophilia*. It can be concluded that, *Stenotrophomonas maltophilia* can be an excellent producer of pullulanase which could find applications in various industry.

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