Original Research Article

Optimization of pectinase production from *Bacillus subtilis* PSE-8 using cassava peels as substrate in submerged fermentation through Response Surface Methodology (RSM)

Abstract
Pectinases are widely used in food processing, textile and other allied industries. Cassava peels generated from cassava processing outfits constitutes one of the major environmental pollutions in Nigeria and there is need to find alternative economic value of the waste instead of indiscriminately disposing them as currently done. This study reports the optimization of pectinase production from *Bacillus subtilis* PSE-8 using cassava peels as substrate in submerged fermentation through response surface methodology. The pectinase-producing bacterium was isolated from a dump site at a fruit market in Oyigbo, Rivers state. Pectinase production was screened on agar plate with pectin as sole carbon source and zone of clearance recorded. Optimization was performed using central composite design of RSM. Four independent variables (pH, temperature, incubation time, and cassava peel concentration) at five levels each were used in the design which generated a total of twenty-five runs. The responses monitored were pectinase production (U/mL) and biomass (mg/mL). The bacterial isolate was identified using cultural, biochemical, and molecular (16S rRNA gene) analyses. Out of 8 bacterial isolates screened for pectinase-producing potential, 5 isolates showed varying zone of inhibition. PSE-8 showed highest zone of inhibition of 9 mm when screened on Agar plate using pectin as sole carbon source. Data obtained from RSM indicated the highest pectinase production (117.5 U/mL) at pH 9, temperature 45 ^{o}C, cassava peel concentration 3 % w/v and incubation time of 3 days. The pectinase-producing bacterium was identified as *Bacillus subtilis* PSE-8 based on 16S rRNA gene sequence analysis. This study demonstrated that inexpensive cassava peel can be used as carbon source to produce pectinase by *Bacillus subtilis* PSE-8. Thus, will help to reduce the environmental pollution posed by the usual indiscriminate disposal of the waste as well as encourage reduction in the cost of pectinase production. RSM technique effectively showed a positive interaction between the different factors studied. The pectinase produced by this bacterium could be applied in various food processing and other allied industries.

Keywords**:** Pectinase, *Bacillus subtilis* PSE-8, submerged fermentation, RSM, cassava peel

1.0 Introduction

In response to “Goal 12” of the United Nations’ sustainable development goals which is to ‘ensure sustainable consumption and production patterns” set in 2015, there have been increase in the call for renewable energy resources as a sustainable approach to replacing the use of rather harsh chemicals in industries. The major sources of renewable feedstock come from agricultural and forest residues. Agricultural wastes and forest residues are mainly composed of cellulose, lignin, and pectin. Their use as feedstock for various industrial processes has been encouraged by their availability, cost effectiveness, eco-friendliness, and abundance. Yeast, mould and bacteria, can breakdown cellulose, pectin and the recalcitrant lignin, utilising their metabolites as

energy and carbon (Raghavendra et al., 2016). To achieve this breakdown, the microorganisms secrete different types of enzymes, depending on the substrate involved. Pectin and pectic substances, which refer to group of water-soluble carbohydrate substances found in the cell walls and intercellular tissues of some plants, are broken down pectinases or pectic enzymes.

Pectinases (EC 3.2.1.15) represent a group of enzymes that catalyse the breakdown of pectic substances via de-esterification and de-polymerization reactions Pedrolli *et al.*, 2009). They include long chains of galacturonic acid residues bounded with carboxyl group. They may be occasionally modified by addition of methyl groups yielding methoxyl group. Classification of pectinases is based on mode of action on the galacturonan part of the pectin molecule. About three (3) main classes of pectinases exist: polygalacturonase (PGase), pectate lyase (PLase), and pectin methyl esterase (PME) (Tariq and Latif, 2012). PGase and PLase catalyse the splitting of molecular chains of the respective polymers (Kashyap et al., 2001) whereas PME catalyse the hydrolysis of methyl ester group of galacturonate unit liberating methanol.

The increased interest on pectinases results from the many industrial applications of pectinases whose market share has been estimated to be 41.4 billion by 2020 (El Enshasy et al., 2018). Pectinases are used mainly in the food industries to produce fruit drinks, juices, and wines (Kashyap et al., 2001; Tapre and Jain, 2014). Acid pectinases are mainly used to extract, clarify, and remove pectin in fruit juices; they may be used to macerate vegetables to produce purees and pastes, and in making wine (Raghavendra et al., 2016; Kubra et al., 2018). Additionally, alkaline pectinases are used in the pre-treatment of wastewater obtained from the processing of vegetable foods with pectin residues. They have been employed in processing textile fibres (flax, hemp, and jute), tea and coffee fermentation, treating paper pulp, and extraction of vegetable oil (Kashyap *et al.*, 2001; Zhang et al., 2000; Kapoor et al., 2001; Hoondal et al., 2002).

Although there have been many studies on low-cost substrates for pectinase production, there is need to continue to search for more substrates. Additionally, there is need to optimise the bioprocess conditions necessary for pectinase production from this locally available substrate for improved yield. Nigeria is one of the major producers of cassava in the globe and cassava peels constitute one of the major environmental pollutions arising from agricultural products. These wastes most often are disposed at random without any regulation thereby posing a great nuisance to the environment. Hence, there is need to seek ways of converting these wastes to wealth and this forms the reason of assessing the suitability of cassava peels as a substrate in the production of pectinase which is widely applied in various industries. Therefore, this current study was aimed at screening pectinolytic bacteria a fruit market waste dump soil and applying RSM as a statistical tool to optimize the production conditions of the enzyme using cassava peels as substrate under in submerged fermentation (SmF).

2.0 Material and Methods

2.1 Collection of soil sample

Soil sample for the isolation of pectinolytic bacteria was obtained from a dumpsite at a fruit market in Oyigbo local government area, Rivers state, Nigeria. The soil samples were collected with a soil auger from several points at depth between 0 and 5 cm and then merged to make a composite sample. The composite sample was then transferred into a sterile plastic bag and transported to the laboratory for further analysis.

2.2 Isolation and Screening of Pectinase-Producing Bacteria

Prior to ten-fold serial dilutions, 9 mL of normal saline (0.85 % of NaCl w/v in distilled water) was first distributed into each clean 25 mL test tube and sterilized in an autoclave at 121^{o}C for 15 min at 15 psi and thereafter allowed to cool. To isolate the pectinase-producing bacteria, 1 g of the composite soil sample was dissolved in 10 mL of distilled water; this yielded the 10^{-1} dilution, from this, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were made. A volume of 0.1 mL of each of dilutions (10^{-3} to 10^{-5}) was spread onto a pectinase agar medium described by Mukesh *et al.* (2012) containing (in g/L of distilled water): $NaNO_3$ 1.0, KCl 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.5, yeast extract 0.5, citric pectin 10 and agar 20 with pH adjusted to 7.0. The plates were incubated at 35^{o}C and observed for bacterial growth. Growths with distinct morphology were selected, subcultured and stored in agar slants.

Pectinase-producing bacteria were screened by plating the isolates on pectin agar plates and incubated at 35°C for 48 h. Further screening was performed by adding Gram's iodine solution to the 48 h-culture plates and incubated for 5 min with intermittent gentle swirling of the plate. Thereafter, the plates were gently rinsed with distilled water and the observed for clear zones. Bacterial colonies with halo zone around them were considered as positive.

2.3 Identification of pectinase-producing bacterial isolate

2.3.1 Cultural, morphological and biochemical characterisation

The morphological and biochemical characteristics of the isolate was determined using Standard Microbiological Methods. The macroscopic properties such as colony size, elevation, shape, margin, colour, texture, surface was determined after incubation in Petri dish at 35^{o}C for 24 h. Microscopic and biochemical examination was carried on the isolate and some of the biochemical tests include oxidase, catalase, motility, methyl red/vogues Proskauer, indole, sugar fermentation tests as well as starch and gelatin hydrolysis. The isolate was tentatively identified with the aid of the ninth edition of Bergey's Manual of Determinative Bacteriology.

2.3.2 Molecular characterisation

DNA extraction, PCR amplification of the bacterial 16S rRNA PCR gene and gel electrophoresis of the isolates were carried out at the Molecular Biology Laboratory of Godfrey Okoye University, Enugu, Nigeria. The PCR products were sent to Iqaba Biotech, South Africa where the 16S rRNA sequencing was carried out.

2.3.3 Chromosomal DNA extraction

Genomic DNA extraction was carried out directly from the samples using a Qiagen QiaAMP DNA extraction kit according to manufacturer's instruction. The extracted DNA was kept at -20 °C until needed. The concentration and purity of the extracted DNA was estimated using a Nanodrop™ spectrophotometer. The absorbance was taken at 260 nm and 280 nm for each sample and the ratio of absorbance at 260 nm and 280 nm used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA while a ratio of ~2.0 is generally accepted as "pure" for RNA (Wilfinger et al., 1997).

2.3.4 PCR amplification of bacterial 16S rRNA gene

Amplification of the 16S rRNA gene was carried out using the primer set 27F- 5′- AGA GTT TGA TYM TGG CTC AG -3′, and 515R 5′- TTA CCG CGG CKG CTG GCA C-3′as previously described by Yamada et al. (2000) and Katsura et al. (2001).

2.3.5 Sequence analysis

The sequences generated by the sequencer were visualized using BioEdit, 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 6 software.

2.4 Collection, processing, communition, pretreatment of biomass

The cassava peels used in the study was obtained from a local market in Choba, Port Harcourt, Rivers state Nigeria. The cassava peel was soaked in water for 30 min; making for easy removal of the brown skin. Thereafter, the peel was washed and dried at atmospheric temperature for 3 days. The dry biomass was milled, filtered with a 60 Mesh sieve, and stored under dry conditions for further use. The cassava peel was pretreated first by soaking overnight and then by steam explosion method described by Ire et al. (2016).

2.5 Inoculum development

About a loopful of 24 h culture of the bacterial isolates, respectively, was inoculated into 100 mL of mineral salt broth (containing in g/L of distilled water: $NaNO_3$ 1.0, KCl 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.5, yeast extract 0.5, sucrose 10, and pH 7.0). The medium was sterilized in an autoclave at 15 psi (121^oC) for 15 min and incubated in a shaker incubator at 35^oC for 24 h. After 24 h, vegetative cells obtained, served as inoculum source for pectinase assay in submerged fermentation process.

2.6 Submerged fermentation (SmF)

Submerged fermentation (SmF) in 250 mL Erlenmeyer flasks using the basal medium described by Mukesh *et al.* (2012) containing (in g/L of distilled water): $NaNO_3$ 1.0, KCl 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.5, yeast extract 0.5, and pH adjusted to 7.0. Dry pretreated cassava peel (5 g) was added to the flask as the sole carbon source. Prior to inoculation with the pectinolytic bacterium 50 mL sterile medium was added to the each of the 250 mL Erlenmeyer flasks, cocked and sterilized in an autoclave at 121 ^oC (15 psi) for 15 min. Thereafter, 5% v/v inoculum of the pectinolytic bacterium (standardized with 0.5 McFarland standard, representing approximately 1.5 x 10^8 cells) was added to the flasks and incubated at 30 ^oC for 4 days under agitation condition (100 rpm). After four day, the cell-free broth was obtained from the medium and used as crude enzyme.

2.7 Extraction of crude enzyme and pectinase assay

Crude enzyme was obtained by filtering culture broth of the isolate with Whatman™ Qualitative Filter Paper. The clear supernatant served as crude enzyme source while the pellet was discarded. Pectinase activity of the crude enzyme extract was assayed by estimating the amount of reducing sugar liberated using 3, 5-dinitrosalicylic acid (DNS) method according to Miller (1959) with slight modification. A volume of 0.5 mL the crude enzyme was added to 1% citrus pectin in 0.1M acetate buffer (pH 7.0) and incubated at 40^oC for 10 min. About 1.5 mL of DNS reagent was added to the reaction mixture and the tube incubated in a boiling water bath for 5 min and colour development observed.

To end the reaction 1 mL of Rochelle's salt was added to the test tubes and optical density reading taken at 540 nm using a spectrophotometer.
For bacterial biomass measurement, 3 mL of the culture broth was added into a cuvette and optical reading taken at 600 nm. The amount of enzyme required to liberate 1 µM of reducing sugar at 40 °C per minute was defined as one unit of pectinase activity and calculated as:

$$\text{Pectinase activity} = \frac{\text{Glucose librated} * \text{dilution factor}}{\text{incubation time x vol. of crude enzyme} * 2} \qquad \text{Eq. 1}$$

where, glucose liberated is given in µmole and Volume of enzyme sample = 1 mL

2.8 Experimental design for Response Surface Methodology and statistical analysis

In this study, four (4) independent variables (pH, temperature, incubation time, and cassava peels) at five (5) levels (Tables 1), screened through twenty five (25) different experimental runs, with the insignificant ones being eliminated to obtain a smaller and more fitting collection of factors were performed. From Table 1 minimum and maximum ranges investigated variables in cultivation medium were pH (5 to 9), temperature (25 to 65 ^{o}C), incubation time (1 to 5%), and cassava peel (1 to 5%). Design Expert version 11 was used in analysing and interpreting experimental data obtained with CCD. The system's behaviour is explained by second-order polynomial equation given in Eq. 2.

$$Y = \beta_0 + \sum_{i=1}^{K} \beta_i x_i + \sum_{i=1}^{K} \beta_{ii} x_i^2 + \sum_{i=1}^{K-1} \sum_{i<jj=2}^{K} \beta_{ij} x_i x_j + e \qquad \text{Eq. 2}$$

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 as model's error.

To test the estimation competence of the process, caparison between the actual responses and the predicted responses generated from RSM were drawn. ANOVA and R^2 statistic aided evaluation of significant differences between various factors and the model's adequacy, which is best when close to 1. Validation of the statistical model was based upon pectinase production and biomasses production at Erlenmeyer flasks' level under the predicted conditions by the model. Sampling was carried out at desired intervals and pectinase production and biomasses production determined.

Table 1: Range and levels of experimental variables

	Level of factors				
Factors	-α	-1	0	+1	+α
Initial pH	5	6	7	8	9
Temperature (deg. C)	25	35	45	55	65
Incubation time (day)	1	2	3	4	5
Cassava peels (%)	1	2	3	4	5

3.0 Results

Table 2 shows the isolation and screening of bacterial isolates for pectinase production. The result demonstrated that out of eight (8) bacterial isolates screened only 4 isolates (PSE-1, PSE-2, PSE-3, PSE-5 and PSE-8) showed potential to secrete pectinase. Isolate PSE-8 had the highest zone of clearance (9.0±0.025 mm), followed by isolate PSE-5 (7.0±0.013 mm) while the least was isolate PSE-2 (4.0±0.020). Isolate PSE-8 which showed the highest zone of clearance on pectin agar plates was selected and used for further studies.

Table 2: Isolation and screening of bacterial isolates for pectinase secretion.

Bacteria Isolate	Zone of Clearance (mm)
PSE-1	5.0±0.028
PSE-2	4. 0±0.020
PSE-3	6. 0±0.015
PSE-4	0
PSE-5	7.0±0.013
PSE-6	0
PSE-7	0
PSE-8	9. 0±0.025

The cultural and morphological characteristics of the selected PSE-8 isolate is displayed in Table 3. The findings showed that the isolate (PSE-8) was a Gram + rod with irregular shape, wavy margin, raised, creamy, mucoid, veined and large in large in size.

Table 3: Morphological characteristics of the pectinase-producing bacterial isolate on Nutrient Agar plate

Colony feature/Gram's reaction	Characteristics
Gram stain	+ (rods)
Colony size	Large
Shape	Irregular
Margin	Wavy
Elevation	Raised
Colour	Creamy
Texture	Mucoid
Surface	Veined

Table 4 depicts the biochemical characteristic of the pectinase-producing bacterial isolate (PSE-8). The outcome of this study indicated that isolate PSE-8 was endospore positive, citrate positive, motile, oxidase negative, catalase positive, indole negative, urease negative, and methyl red negative positive among other properties. It was able to ferment some sugars with the production of acid and gas although it could not ferment lactose and mannitol. The bacterial isolate (PSE-8) was tentatively identified as *Bacillus* spp based on the cultural, morphological and biochemical characteristics.

Table 4: Biochemical characteristics of the pectinase-producing bacterial isolate

Isolate code	PSE-8
Endospore	+
Citrate	+
Motility	+
Oxidase	-
Catalase	+
Indole	-
Urease	-
MR	-
VP	+
TSI	
Slant	K
Butt	A
H_2S	-
Starch hydrolysis	+
Gelatin hydrolysis	+
Sugar Fermentation	
Maltose	+/A
Glucose	+/A
Lactose	-
Mannitol	-
Sucrose	+/A
Probable genus	*Bacillus spp*

+ = positive; - = negative; K = alkaline; A = acid; MR = methyl red; VP = Vogues Proskauer; TSI = triple sugar iron.

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

Table 5 presents composition of various central composite design (CCD) experiments for independent variables (pH, temperature, incubation time and cassava peel concentration) and responses (pectinase production [U/mL] and biomass [mg/mL]). Additionally, actual and predicted values for both responses are presented also. The results obtained from this study revealed agreement between the actual values and the predicted values in Table 2. The highest pectinase yield (117.5 U/mL) was observed at pH of 9, temperature of 45^{o}C, incubation time of 3 days and cassava peel concentration of 3%. While the least pectinase production (77.63 U/mL) occurred at pH of 7, temperature 25^{o}C, incubation time of 3 days and cassava peel concentration of 3%. Highest Biomass yield (1.11 mg/mL) was obtained at temperature of 65^{0}C, incubation time of 3 days, cassava peel of 3 % (w/v) and pH of 3 while least biomass yield (0.17 mg/mL) was recorded at pH of 6, temperature of 35 ^{0}C, time of 2 days and cassava peel concentration of 4 % (w/v).

Table 5: Composition of various experiments of the CCD for independent variables and responses by *Bacillus subtilis*

Run	Factor				Pectinase (U/mL)		Biomass (mg/mL)	
	A	B	C	D	Actual	Predicted	Actual	Predicted
1	7	65	3	3	80.54	84.18	1.111	0.7161
2	8	35	4	4	98.74	96.08	0.781	0.9180

3	8	55	2	2	93.97	94.60	0.318	0.4607
4	6	35	4	4	78.47	81.67	0.461	0.5192
5	8	35	2	2	94.43	94.12	0.461	0.4087
6	7	55	2	4	116.7	111.15	0.95	1.19
7	7	45	3	1	84.89	88.94	0.54	0.2533
8	6	55	2	4	82.19	87.51	0.318	0.3772
9	6	35	2	4	83.85	80.35	0.17	0.0542
10	8	55	4	2	99.95	97.91	0.185	0.3685
11	7	45	5	3	94.45	97.32	0.331	0.3676
12	7	45	1	3	93.84	92.69	0.3	-0.0052
13	8	55	4	4	102	106.16	1.262	1.22
14	7	45	3	5	98.98	96.64	0.359	0.3771
15	7	45	3	3	102.1	102.10	0.214	0.2140
16	7	25	3	3	77.63	75.70	0.59	0.7163
17	5	45	3	3	84.39	86.95	1.24	1.10
18	6	55	2	2	82.17	79.29	0.219	0.4797
19	6	55	4	4	94.9	89.67	0.347	0.4670
20	6	35	4	2	90.51	90.52	1.292	1.12
21	8	35	2	4	94.67	101.90	0.402	0.5082
22	8	35	4	2	98.08	96.59	0.55	0.6917
23	6	35	2	2	81.24	80.90	0.543	0.7839
24	6	55	4	2	93.15	89.75	0.348	0.4427
25	9	45	3	3	117.5	116.66	1.609	1.48

A-pH; B-temperature (^{o}C); C-incubation time (days); D-cassava peel concentration

3.2: Model fitting and analysis of variance (ANOVA) for pectinase production by Bacillus subtilis PSE-8 using CCD

Summary of ANOVA for RSM quadratic polynomial models of pectinase production and biomass by *Bacillus subtilis* PSE-8 is presented in Tables 6. The model was significant with a Model F-value of 6.44. There is 0.27% chance that the large F-value could be attributed to noise. A P-value that is less than 0.0500 showed a significant model term. For this model, A and B^2 were the significant model terms. Instances of many insignificant model terms (not counting those required to support hierarchy), may require model reduction. "Adequate Precision" measures the signal to noise ratio. Ratios that are above 4 may be desirable; the ratio of 10.210 obtained for this model, shows an adequate signal. Thus, the design space can be effectively navigated with the model.

Table 6: ANOVA for the production of pectinase by *Bacillus subtilis* PSE-8

Responses		**Model**
Pectinase production	P-value	0.0027
	F-value	6.44
	Coefficient of determination	0.9001
	Adjusted coefficient of determination	0.7602
	Predicted coefficient of	-

	determination	
	Adequate precision	10.21

Table 7: Equation for the production of pectinase and biomass by *Bacillus subtilis* PSE-8 as function of pH, temperature, incubation time and cassava peel concentration presented in terms of coded factors (P≤0.05).

Factor	**Parameters**			
	Pectinase (U/mL)	**Standard error**	**Biomass (mg/mL)**	**Standard Error**
Intercept	102.10	5.18	0.2140	0.2769
A-Ph	7.43	1.06	0.0950	0.0565
B-Temperature	2.12	1.06	-0.0000	0.0565
C-Incubation time	1.16	1.06	0.0932	0.0565
D-Cassava peel	1.93	1.06	0.0310	0.0565
AB	0.5225	1.29	0.0891	0.0692
AC	-1.79	1.29	-0.0138	0.0692
AD	2.08	1.29	0.2073	0.0692
BC	0.2100	1.29	-0.0938	0.0692
BD	2.19	1.29	0.1568	0.0692
CD	-2.07	1.29	0.0317	0.0692
A^2	-0.0744	1.54	0.2691	0.0824
B^2	-5.54	1.54	0.1256	0.0824
C^2	-1.77	1.54	-0.0082	0.0824
D^2	-2.33	1.54	0.0253	0.0824

3.3 Final equation in terms of factors

Table 8 shows the equation in terms of coded factors for pectinase and biomass productions by *Bacillus subtilis* PSE-8. This can be used to make predictions about the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. Hence, A (pH) had the most impact in pectinase production while D^2 had the least.

Table 8: Final equation in terms of coded factors for pectinase production and biomass production

Coded factors	**Pectinase activity**	**Biomass**
	= +102.10	= +0.2140
*A	+7.43	+0.0950
*B	+2.12	-0.0000
*C	+1.16	+0.0932

*D	+1.93	+0.0310
*AB	+0.5225	+0.0891
*AC	-1.79	-0.0138
*AD	+2.08	+0.2073
*BC	+0.2100	-0.0938
*BD	+2.19	+0.1568
*CD	-2.07	+0.0317
*A^2	-0.0744	+0.2691
*B^2	-5.54	+0.1256
*C^2	-1.77	-0.0082
*D^2	-2.33	+0.0253

3.4 Effect of reaction factors on pectinase productions by Bacillus subtilis PSE-8

Figures 1a-1f show interaction effects of the independent variables on the production of pectinase. Each figure shows interactions between any two given independent variables, while keeping another variable at its central (0). It was observed that moderate temperature, inoculation time and cassava peels concentration and high pH yielded the highest pectinase response (Fig. 1a-c), whereas moderate incubation time and temperature; moderate cassava peels concentration and temperature yielded the highest pectinase response (Fig. 1d and e), while moderate cassava peels concentration and high incubation time yielded the highest pectinase response (Fig.1f).

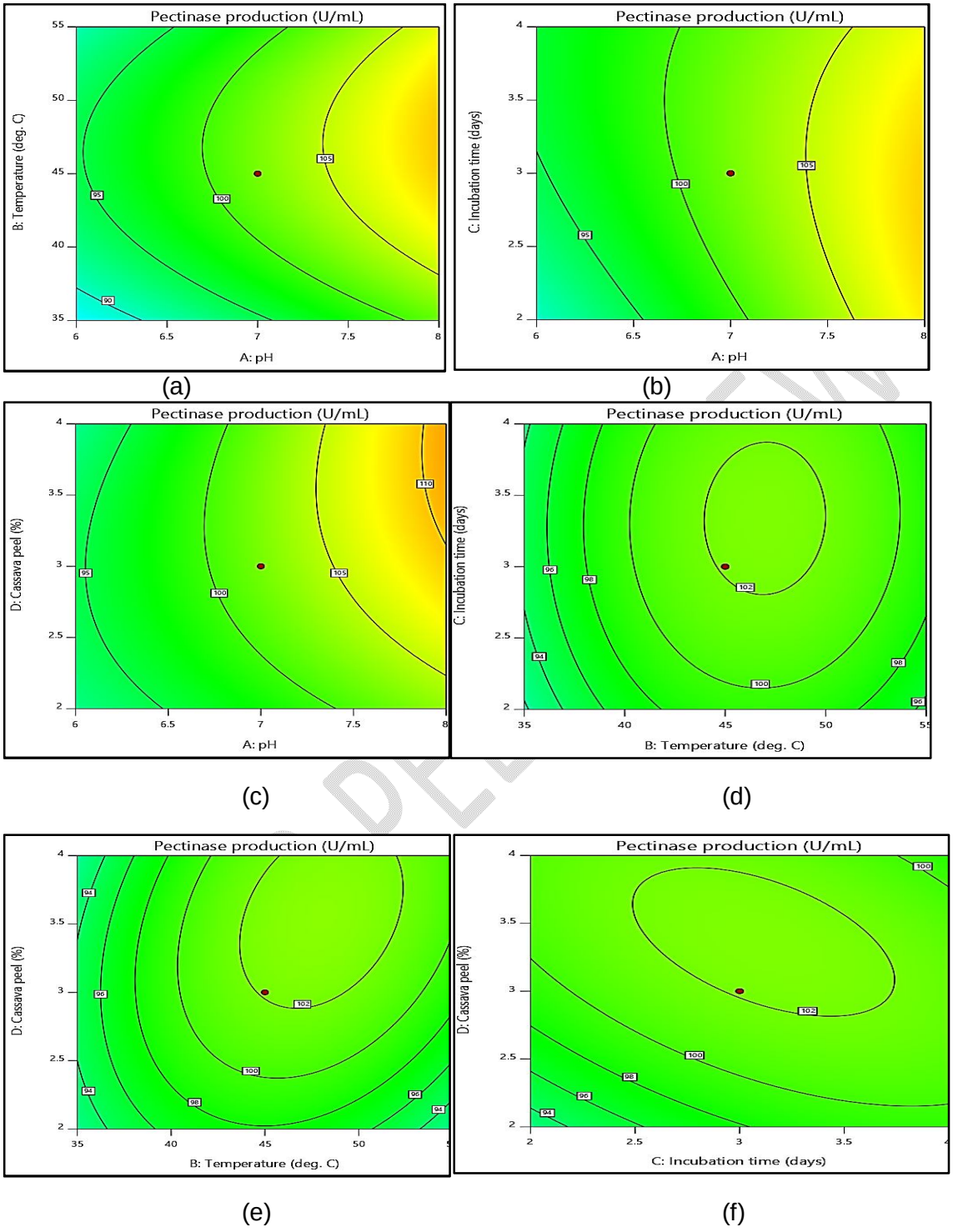

Figure 1: Response surface plots for pectinase production (U/mL) from *Bacillus subtilis* PSE-8 through submerged fermentation as a function of (a) pH and temperature (oC) (b) pH and incubation time (c) pH and cassava peel concentration (d) temperature and incubation time (e) temperature and cassava peel concentration (f) cassava peel concentration and incubation time

3.5 Identification of the bacterial isolate

BLAST search result revealed the ten closest matches in GenBank as *Bacillus subtilis* strains with *Bacillus subtilis* subsp. subtilis 6051-HGW which revealed a 99% identity whereas others showed 98% (Fig.2). Therefore, based on the phylogenetic relation the bacterium was identified as *Bacillus subtilis* strain PSE-8.

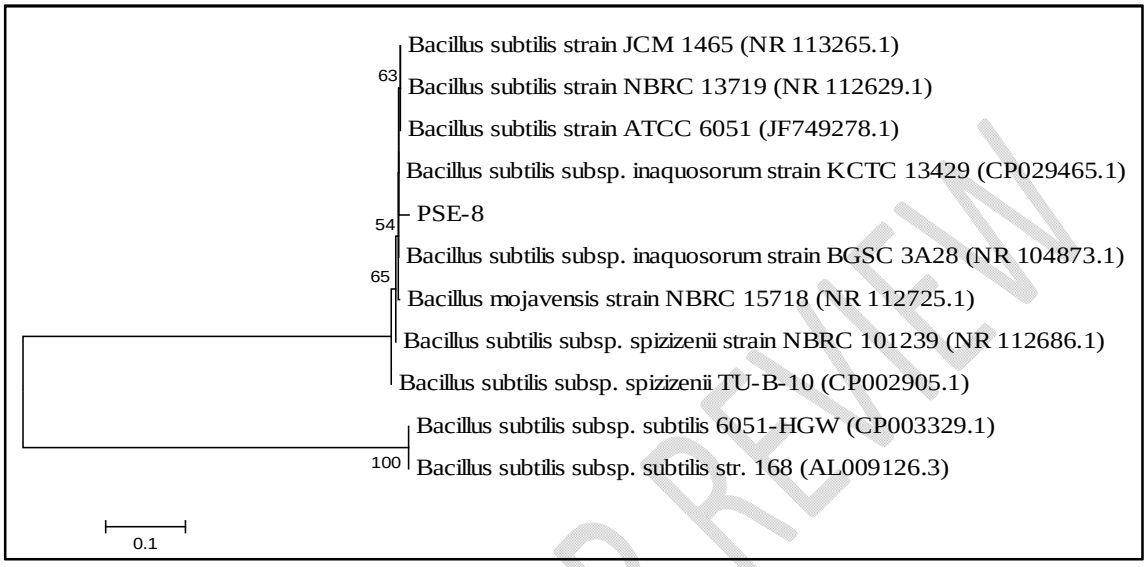

Figure 2: Phylogenetic tree relationships of isolates with GenBank Closest matche**s**

4. Discussion

This study investigated the production of pectinase from *Bacillus subtilis* PSE-8 using cassava peel as substrate and the optimization of the cultural conditions for improved yield. Pectinase is one of the important industrial enzymes with wide application in food industry (Sharma and Satyanarayana, 2012), animal feed (Praveen and Suneetha, 2014), processing of textile material (Rebello et al., 2017), paper and pulp industry (Agrawal et al., 2016), and wastewater treatment (Praveen and Suneetha, 2014). These wide applications of pectinase justify the interest in pectinase enzymes. Moreover, the extensive distribution of pectin molecules in plants makes pectinases indispensable in successfully utilizing plant wastes as feedstock for various industrial processes.

Pectinase production has been attempted on a variety of substrates. In this study, cassava peel was used as substrate for pectinase production. Cassava peel has been sparsely used as substrate for pectinase production; however, a few studies have investigated its effectiveness. Mukesh et al. (2012) studied the production and optimization of pectinase from *Bacillus* sp. MFW7 using cassava waste as substrate and reported high pectinase yield. Chemical content of cassava peel makes it a suitable candidate for pectinase production. Chemical analysis of the cassava peel used in this study revealed high dry mass percentage with glucose representing 88.2% of the total dry mass. Such high sugar content mainly available as starch (49.33% dry mass) makes cassava a good substrate for enzyme production.

Preliminary result showed that submerged fermentation was the preferred route of pectinase production by the bacterial isolate employed in this study. Many studied have

reported preference of submerged fermentation to solid state fermentation by bacterial isolates for the production of enzymes. Subramaniyam and Vimala (2012) reported that bacteria, which have higher water activity requirements than fungi, prefer submerged fermentation to solid state fermentation. Therefore, the use of submerged fermentation for pectinase production by the bacterial isolate in this study agrees with other findings (Kashyap et al., 2000); Kashyap et al., 2003).

For optimal production of pectinase by the bacterial isolate, cultural and nutritional conditions were optimized using response surface methodology (RSM). This was necessary as low yield has been identified as one factor that challenge pectinase production with local substrate. RSM technique employed in this study was central composite design (CCD), and the model obtained from the design effectively predicted the pectinase production by the bacterial isolate. Based on RSM technique employed the following factors (pH, temperature, incubation time, and cassava peel concentration) were optimized and data obtained showed the highest pectinase production (117.5 U/mL) at pH 9, temperature 45^{o}C, incubation time 3 days, and cassava peel concentration 3 % w/v. Yugandhar et al. (2008) and Ajayi et al. (2018) similarly investigated the effectiveness of RSM techniques in optimizing cultural and media conditions for improved pectinase production.

The maximum pectinase activity (117.5 U/mL) reported in this study is higher than the maximum pectinase activity of 87. 5565 U/mL from *Enterobacter* sp. PSTB-1 reported by Reddy and Saritha (2016) and 10.65 U/mL from *Bacillus licheniformis* reported by Hou et al. (2011). However, this was lower than the maximum pectinase activity of 367.92 U/mL *Aspergillus niger* reported by Suresh and Viruthagiri (2010) using a combination of wheat bran and sugarcane bagasse as substrate under solid state fermentation.

The pH and temperature of culture medium play important roles in enzymes production, as they affect the growth and function of the enzyme producing microorganisms. *Bacillus subtilis* PSE-8 used in this study produced pectinase over a temperature range of 25-65^{o}C and pH range of 5-9, but maximum yield was at 45^{o}C and pH of 9. Madu (2015) reported the same pH value for pectinase production by *Bacillus lichenoformis* using cassava waste dump and an optimum temperature of 40ºC for pectinase production with an enzyme activity of 0.1669 µmol/ml/min which decreased by 40% when the temperature was raised to 45ºC. However, Dey (2010) reported a lesser pH (6) and temperature (37) in pectinase production by *Bacillus subtilis*.

The incubation time in this study was 3 days for maximum pectinase yield. This time is less than the 96 h reported by Kaur et al. (2011) for maximum pectinase production by different isolates of *Bacillus* sp. but higher than 24-48 h reported by Madu (2015). Our result is consistent with the findings of Majumder et al. (2020) who reported that *P. ostreatus* performed much better in submerged fermentation for the production of exo-polygalacturonase. Majumder et al. (2020) obtained highest production of exo-polygalacturonase activity (6160 Ul^{-1}) on the 4th day of cultivation. This differed with the result of this present study.

The pectinase producing bacterium used in this study was identified as *Bacillus subtilis* PSE-8 based on 16S rRNA gene analyses. *Bacillus* spp have been highly implicated in the production of pectinases by different researchers. Reena et al. (2016) produced alkaline pectin lyase from *Bacillus cereus*. Nizamudeen and Bajaj (2009) and Hou et al. (2011) also reported the production of pectinase from different *Bacillus* spp.

Conclusion

Pectinase-producing bacterium with high pectinase yield was isolated from a dump sites located at a fruit market in Oyigbo LGA, Rivers State. The bacterial isolate showed zone of inhibition of 9 mm when screened on Agar plate using pectin as sole carbon source. Temperature, pH, incubation time, and cassava peel concentration affected the ability of the bacterium to produce pectinase. These factors (pH, temperature, incubation time, and cassava peel concentration) were optimized using RSM and the data obtained showed the highest pectinase production at pH 9, temperature 45^{o}C, incubation time 3 days, and cassava peel concentration 3 % w/v. This pectinase-producing bacterium was identified as *Bacillus subtilis* PSE-8 using 16S rRNA gene sequences. The outcome of this study demonstrated that cassava peel can be used as carbon source to produce pectinase by *Bacillus subtilis* PSE-8. Thus, will help to reduce the environmental pollution posed by the usual indiscriminate disposal of the waste as well as encourage reduction in cost of pectinase production being an inexpensive substrate. More so, the pectinase produced by this bacterium could be applied in various food processing and other allied industries.

Ethics approval and consent to participate
Not applicable.

Consent for Publication
Not applicable.

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