Protease-Producing Bacteria Isolated from Beans Effluent-Impacted Soil Habour *apr* and *npr* Protease Encoding Genes

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Abstract

This study employed molecular technique to identify protease-producing bacteria from beans effluent-impacted soil and screened the isolates for the presence of two principal extracellular protease-encoding genes (*apr* and *npr*). Protease-producing bacteria were isolated from beans effluent-impacted soil in Rumuosi, Port Harcourt, Rivers State Nigeria. Protease production was determined by plate screening and enzyme activity assay methods. Three bacterial isolates comprising P2, P3 and P4 were identified based 16S rRNA gene sequences' analyses. These protease-producing strains were further screened for the presence of the two principal extracellular protease-encoding genes *apr* and *npr* using PCR-based technique. Phylogenetic tree analyses of the sequences obtained from the isolates identified the protease producers as *Alcaligenes faecalis* P2, *Serratia marcescens* P3 and *Lysinibacillus* sp. P4. The sequences have been deposited in GenBank under the accession numbers MZ477006.1-MZ477008.1. In addition, PCR results revealed the presence of *apr* gene in *Alcaligenes faecalis* P2 and *Serratia marcescens* P3 while only *Lysinibacillus* sp. P4 harboured the *npr* protease gene. Results of NanoDrop Spectrophometry showed that concentrations of the extracted genomic DNA ranged between 308 and 500 ng/µL with the purity ranging between 1.68 and 1.84. Out of the three isolates, *Serratia marcescens* P3 had the highest protease activity (0.26 U/mL). The study has demonstrated that beans effluent-impacted soil harbour protease-producing bacteria. Furthermore, *apr* and *npr* genes can aid proper characterization of extracellular proteases.

Keywords: Cassava effluent, beans effluent, amylase, protease; 16S rRNA gene.

1.0 Introduction

Proteases or peptidases represent a group of industrially important enzymes with capacity to break peptide bonds. Protease account for approximately 60% of the world global industrial enzyme market (Nadeem *et al*., 2019). Interest in them is due to their application in industrially important products such as foods and detergents. Predominantly, proteases are produced by microorganisms through submerged and solid-state fermentation. Gimenes *et al*. (2019) reported that huge market demand exists for industrial enzymes. The global market demand for industrial enzymes was reported to stand at approximately $5.6 billion, with a predicted increase of 4.9% at a compound annual growth rate, reaching an estimated $7.0 billion in 2023 (Singh *et al*., 2016).

In the industries, proteases are applied in the field of detergent, tanning and food industries, meat processing, cheese production, silver recovery from photographic film, production of digestive enzymes and medical treatment for harmful wound and inflammation as well as in pharmaceutical industry (Muthulakshmi *et al*., 2011).

Many bacteria have been reported as efficient producers of proteases and amylases. Binod *et al*. (2013) reported that *Bacillus* protease was first marketed commercially in 1959 and became big business when Novozymes in Denmark started to manufacture it, with major detergent manufacturers patronizing it around 1965. Most alkaline and neutral proteases have been reportedly produced by bacteria belonging to members of the genus *Bacillus* including strains such as *B. subtilis*, *B. stearothermophilus* etc. (Razzaq *et al*., 2019) however, other bacteria such as *Alcaligenes* sp., *Serratia* sp. etc have also been implicated. *Bacillus clausii* particularly, was recommended for use at a commercial scale for the production of alkaline protease with the use of peptone, Cu, and fructose as the sole source of energy. The optimum pH and temperature recommended was 8 to 9 and 37 to 40 °C, respectively (Vadlamani and Parcha, 2011).

Proteases have been classified based on their tolerance to pH as acidic (2.0–5.0), alkaline (8.0–11.0) and neutral (7.0) (Mukhtar and Haq, 2008). According to Baraniya *et al*. (2016) alkaline metallo-peptidase (apr) and neutral-metallopeptidases (npr) are two key bacterial protease-encoding genes. Vasantha *et al*. (1984) first reported that alkaline and neutral proteases are regulated by *apr* and *npr* genes, respectively. The abundance of these genes in bacteria was reported by Baraniya *et al*. (2016). This study was therefore designed to identify protease-produicng bacteria from beans effluent-impacted soil and screen the isolates for the presence of two principal extracellular protease-encoding genes (*apr* and *npr*).

2.0 Materials and Methods

2.1 Isolation and Screening of Protease-Producing Bacteria

Protease-producing bacteria were isolated from beans effluent-impacted soil from Rumuosi, Obio Okpor L.G.A., Rivers State, Nigeria (Figure 1). The soil was air-dried, sieved to remove debris and serially diluted to 10^{-6} dilution. The diluted sample was plated on skim milk agar plates and incubated at 30° C for 48 h (Anbu *et al*., 2013). After incubation, the colonies with clear zones were selected and inoculated into freshly prepared skim milk agar (Priyadarshini *et al*., 2019**)** plates by spotting them on the agar surface and incubating for 48 h. Development of clear zones on skim milk agar plates confirmed the production of protease. Protease production was further confirmed by casein hydrolysis assays.

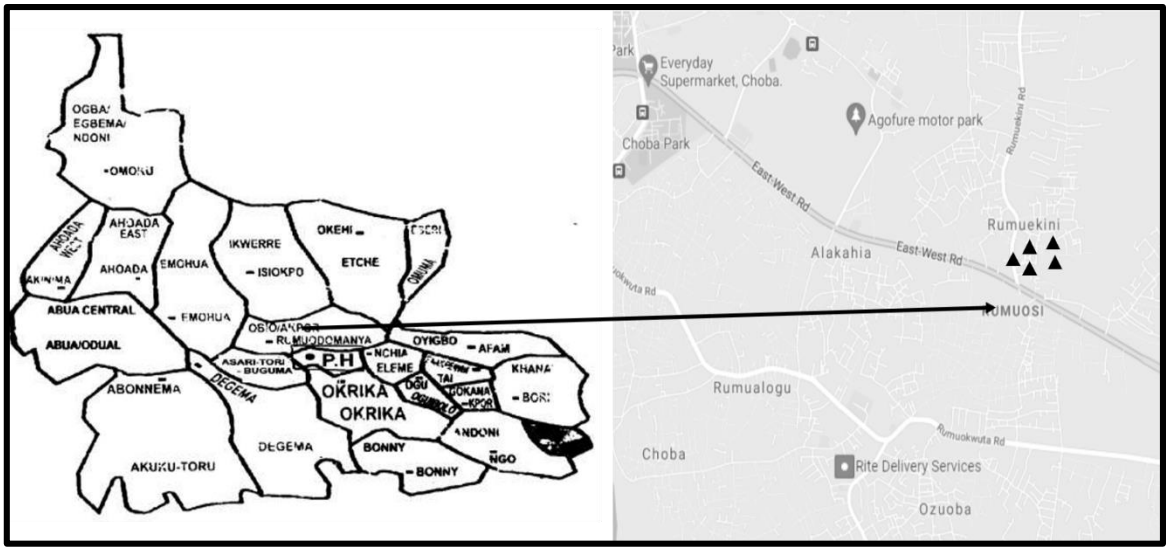

Figure 1: Map of Rivers state with the study area highlighted

2.2 Inoculum development

Pure cultures of the selected bacterial isolates P2, P3 and P4 were inoculated in nutrient broth [consisting of (g L^{-1}): peptone, 5; Beef extract, 3; NaCl, 5] followed by incubation at 37 ℃ for 24 h to get a standardized inoculum (0.5 OD at 600 nm with 3.5 x 10^5 cfu/ml).

2.3 Protease Assay

The selected isolates were inoculated into 20 mL of protease production medium comprising: lactose 10 g; casein 10 g; KH_2PO_4 2 g; K_2HPO_4 2 g; $MgSO_4.7H_2O$ 1 g) and incubated for 48 h on a rotary shaker (200 rpm) at room temperature (Sharma *et al.*, 2014; Saraswathy *et al*., 2013; Tambekar and Tambekar 2013). After incubation, the culture media were centrifuged at 5000 rpm for 20 min and at 4 °C. Cell free supernatants were collected and used as the crude enzyme for protease assay. Protease assay was conducted by a modified method by Cupp-Enyard (2008). Casein dissolved in different pH buffers [acidic (pH:4), neutral (pH:7) and alkaline (pH:9)] were used as the substrate for the assay. The reaction mixture contained casein and the crude enzyme solution. The mixture was incubated at 37 °C for 10 min. Thereafter, the reaction was stopped by addition of 3 ml of 20% ice-cold trichloro acetic acid (TCA). Precipitated proteins were removed by centrifugation and 0.5 ml of the supernatant was mixed with 2.5 ml of 0.5 M Na_2CO_3 and kept for 20 min at room temperature. Finally, Folin's phenol reagent was added to the mixture, kept for 10 min and absorbance was measured at 660 nm against the blank sample. The amount of enzyme required to liberate 1 µmole of tyrosine per ml per minute under the standard conditions defined one unit of protease activity (Hameed *et al*., 1999).

2.4 Selection of protease-producing bacteria

The bacteria (P2, P3 and P4) with highest zones of clearance and highest activities were selected for further studies. Pure cultures of isolates in triplicate were maintained on casein supplemented minimal agar slant in a refrigerator (Haeir Thermocool, China) for future use.

2.5 Identification of the Bacterial Isolates

Bacterial genomic DNA extraction was carried using ZR Soil Microbes DNA Mini-Prep extraction kit (Zymo Research Corporation, South Africa). The quantity and purity of the extracted genomic bacterial DNA were analysed using an ND-1000 spectrophotometer (Thermoscientific, Inqaba Biotech, South Africa) and agarose gel electrophoresis system. The genomic DNA was stored at -20 ºC. The amplification of the 16S rRNA gene of the isolates was carried out using primer sets 27F (5^1AGAGTTTGATCMTGGCTCAG-3^1) and 1492R (5^1GGGTTACCTTGTTACGACTT3^1). The PCR reaction was carried out in 25 µL reaction volume, containing 12.5 µL of the Master Mix (Zymo Master Mix), 0.4 µL of each primer, mixed with 5 µ of the DNA template. Sterile nuclease free water of volume, 6.7 µL was added. The following PCR conditions were used: Initial denaturation (95 ºC for 5 min), denaturation (95 ºC for 30 sec.), annealing (52 ºC for 30 sec), extension (72 ºC for 45 sec.), and final extension step (72 ºC for 3 min) and was cooled for 4 ºC. About 5 µL of the amplified products was run on agarose gel electrophoresis at 120 V for 15 min to determine the quality of the products. The amplified products were also purified using DNA clean and concentrator (DCC) kit (Zymo research institute, South Africa) in preparation for sequencing.

PCR products of the bacterial DNA were sequenced using Sanger method of sequencing with 3500 ABI genetic analyser at Inqaba Biotechnical Industries, South Africa. The sequences generated by the sequencer were visualized using Chromaslite for base calling. BioEdit was used for sequence editing, Basic Local Alignment Search Tool (BLAST) was performed using NCBI (National Center for Biotechnology Information) database. Similar sequences were downloaded and aligned with ClustalW and phylogenetic tree drawn with MEGA 6 software.

2.6 Detection of *apr* and *npr* Protease-Encoding Genes

In order to detect the presence of *apr* and *npr* protease-encoding genes, the extracted DNA was subjected to PCR procedure. Primers used for the detection of *apr* and *npr* genes were as reported by Bach *et al*. (2001). The primer sets, their composition, position, melting temperature (T_m), and length of amplicon is given in Table 1.

Table 1: Oligonucleotides used as primers for specific amplification and detection of genes for alkaline metallopeptidases (*apr*), neutral metallopeptidases (*npr*)

Gene	Composition	Position (nt)*	T_m (2AT+4GC) °C	Length of amplicon (bp)
FP *apr* I	5' -TAYGGBTTCAAYTCCAAYAC-3'	808–827	52–60	194
RP *apr* II	5' -VGCGATSGAMACRTTRCC-3'	985–1002		
FP *npr* I	5' -GTDGAYGCHCAYTAYTAYGC-3'	214–233	54–66	233
RP *npr* II	5' -ACMGCATGBGTYADYTCATG-3'	437–446		

3.0 Results and Discussion

3.1 Protease Production by the Bacterial Isolates

Results of plate screening for casein hydrolysis are presented in Table 1. The highest protease producer was isolate P3 with protease activity of 0.26 U/mL.

Table 1: Screening and assay characteristics of the protease-producing bacterial isolates

Isolate code	Halo zone (cm^2)	Tyrosine release (µmol/mL)	Protease activity (U/mL)
P2	4.16	0.176	0.19
P3	5.73	0.232	0.26
P4	2.55	0.202	0.22

3.2 Molecular Identification of Protease Producers

The concentration, purity, quality, and phylogenetic tree construct for the protease-producing bacteria are presented in Table 2 and Figure 2. The 3 protease producers were identified as *Alcaligenes faecalis* P2, *Serratia marcescens* P3 and *Lysinibacillus* sp. P4 .

Table 2: Description of the isolates accession, GenBank closest matches, % identity of the protease- and amylase-producing bacteria

S/N	Isolate Name	Strain	Accession No	GenBank Closest Match	% Identity
1	*Alcaligene faecalis*	P2	MZ477006	*Alcaligene faecalis* IMJ8	98
2	*Serratia marcescens*	P3	MZ477007	*Serratia marcesciens* ZK	99
3	*Lysinbacillus* sp.	P4	MZ477008	*Lysinbacillus* sp. A3-19	99

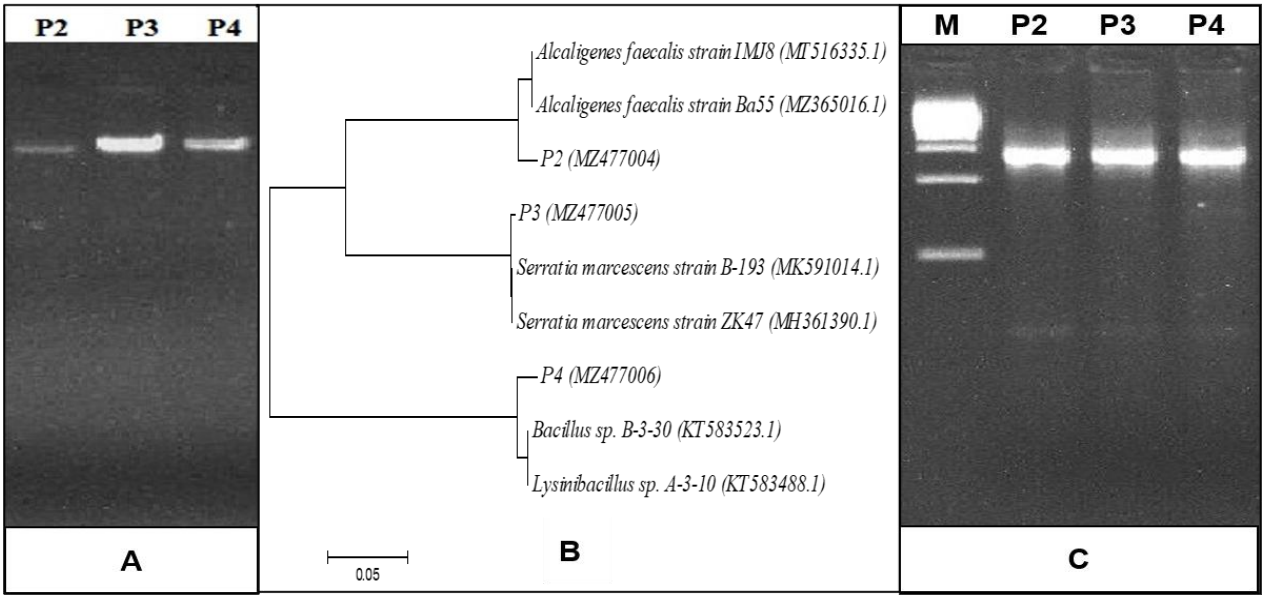

Figure 2: Genomic DNA, phylogenetic tree and PCR products from the protease-producing isolates

3.3 Discussion

This study was designed to identify protease-producing bacteria from beans effluent-impacted soil and screen the isolates for the presence of two principal extracellular protease-encoding genes (*apr* and *npr*). PCR techniques have shown high reliability in targeting specific regions of microbial DNA. In this present study, PCR technique was employed in the identification of protease-producing bacteria and in the determination of the presence of alkaline metallo-peptidase (*apr*) and neutral-metallopeptidases (*npr*) genes. The protease-producing bacteria were identified as *Alcaligenes faecalis* P2, *Serratia marcescens* P3 and *Lysinibacillus* sp P4. Their sequences have been deposited in GenBank under the accession numbers: MZ477004.1- MZ477006.1. This study is unique as it is the first report to the best of our knowledge on the isolation of protease-producing bacteria from beans effluent-impacted soil. However, few studies have reported protease production from protein rich wastes (Saxena and Singh, 2011; Yazid *et al*., 2016)

Alcaligenes faecalis, *Serratia marcescens* and *Lysinibacillus* sp. have all been previously implicated in protease production. Thangam *et al*. (2000) reported that *Alcaligenes faecalis* isolated from tannery soil produced protease efficiently. Similarly, *Alcaligenes faecalis* was reported as an efficient alkaline protease producer by Marathe *et al*. (2018). The findings of this study corroborate these claims as the *Alcaligenes faecalis* strain P2 used in this present study produced protease under alkaline condition. The best protease-producing bacterium was *Serratia marcescens* P3 with protease activity of 0.26 U/mL. *Serratia marcescens* has been reported to produce efficient protease by other studies. Jo *et al*. (2008) reported high protease production by the same bacterium. Iqbal *et al*. (2018) also reported efficient alkaline protease production by *Serratia marcescens*. *Lysinibacillus* sp. P4 used in this study produced neutral protease. Many *Bacillus* species have been reported as efficient producers of both alkaline and neutral proteases (Akhavan-Sepahy and Jabalameli, 2011; Amira and Eida, 2016).

Functional PCR analysis of the DNA isolated from the isolates revealed protease-encoding genes: alkaline metallo-peptidase (*apr*) in *Alcaligenes faecalis* P2 and *Serratia marcescens* P3 and neutral-metallopeptidases (*npr*) in *Lysinibacillus* sp. Baraniya *et al*. (2016) reported that these basic protease-encoding genes are widely distributed among bacteria. This study corroborates Baraniya *et al*. (2016) assertion as all the bacterial isolates harboured either of the genes. The presence of these genes may have conferred on the isolates their pH preferences.

Conflict of Interests

Authors have declared that no conflicting interests exist.

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