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Concomitant Production of α-Amylase and Protease by *Bacillus Aerius* Strain FPWSHA Isolated from Food Wastes

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Abstract: Amylase and protease are enzymes that have potential applications in the food industry, detergent formulation, pharmaceuticals, waste degradation, and the dehairing process in the leather-making industry. In the current study, fifty-six bacteria were isolated from food wastes and screened for amylase and protease production. Biochemical tests and 16S rRNA gene sequencing were used to characterize and identify bacterial strains. Ten potential isolates with high extracellular enzyme secretion were selected. Among ten isolates, strain FPWSHA was ideal for α-amylase and protease production based on its larger hydrolytic zone of clearance under casein and starch agar than other isolates. This strain was identified as *Bacillus aerius* FPWSHA (Accession number in GenBank: OM258619) and utilized to produce protease and α-amylase enzymes using potato peels powder (PPP) waste as a cheap substrate under submerged fermentation. Under culture conditions optimization, 16.9 U/mL amylase and 12.3 U/mL proteases were achieved within 48 h of fermentation. *Bacillus aerius* was isolated from food wastes for the first time to produce α-amylase and protease enzymes. In the future, the bacterial strain described here should be improved for hydrolytic enzyme production in commercial and biotechnological applications.

Keywords: amylase; protease; food waste; bacteria isolation; 16S rRNA; *Bacillus aerius* FPWSHA; potato peels powder; submerged fermentation; culture conditions optimization.

1. Introduction

Amylase and protease were the leading contributors to the enzyme market in 2019. Their demand is projected to continue in the following years, with the global enzyme market expected to reach 14,506 million USD in 2027 [1]. Amylase and protease are essential in the food industry, detergent formulations, bioethanol, pharmaceuticals, feeds, and biotechnology [2]. For example, when used in detergents, the proteases remove protein stains while amylases remove starchy food stains[3]. Enzymes are produced mainly by mammals, microbes, and plant systems [2-4]. Microbial enzyme production is advantageous since microorganisms can easily manipulate and be made in bulk in fermentation [5-7]. Some studies have explored the

microbial production of α-amylase and protease in a separate fermentation medium by bacteria and fungi [8,9]_ENREF_6. Few studies have reported on the spontaneous production of amylase and protease.

Moreover, one of the significant challenges in the industrial production of enzymes is the substrates' cost, estimated to be 30-40% of the whole process [10]. Agricultural-wastes products, such as those derived from maize, potatoes, sugar canes, and the dairy industry, could serve as inexpensive fermentation carbon sources in solid-state and submerged fermentation [10-12]. These agriculture and food wastes comprise Carbon and Nitrogen sources required to deliver the nutrients for the growing and breakdown of microorganisms as the substrate. The bacterial population colonizing agricultural and food waste can be considered a source of enzymes and biochemical compounds [13,14]. Previously *Bacillus aerius* ssp was researched for probiotic properties and increased fish immunity [15]. Antifungal activity has also been detected in *Bacillus aerius* [16]. No study reported the production of amylase and protease by that bacterial strain. The present study aimed to isolate and investigate the production of extracellular amylase and protease by bacteria associated with kitchen wastes in a single production medium. Furthermore, it optimized the culture conditions and nutritional parameters for efficient amylase and protease production under submerged fermentation.

2. Materials and Methods

2.1. Chemicals and reagents.

The chemicals included starch soluble (High purity laboratory Chemical Pvt. ltd India), casein hydrolysate (Duchefa Biochemie Netherlands), Folin-Ciocalteau (Sigma-Aldrich, Co, St Louis), DNSA from HiMedia (Mumbai, India) were used. All other reagents and materials were analytical grade and commercially available.

2.2. Samples collection and bacteria isolation.

Nine food kitchen wastes were collected from three restaurants in Juja town, Kiambu County, Kenya, including starchy wastes (potato peels and Ugali) and proteinaceous wastes (meat, milk, and fish). The samples were stored at 4°C before use. The serial dilution method was utilized to isolate bacterial strains [17].

2.3. Biochemical characterization of isolates.

The primary biochemical tests were carryout to characterize the isolates, Including the Indole test, Oxidase test, catalase test, starch hydrolysis, lipid hydrolysis, motility, Citrate utilization test, hydrogen sulfide test, and methyl red (MR) and Voges–Proskauer [18,19].

2.4. Screening Isolates for extracellular hydrolytic enzyme production.

The bacterial isolates were screened for the production of extracellular protease and amylase enzymes. The colony of each isolate was streaked on agar media containing a suitable substrate specific for each enzyme activity. The starch and casein were used to screen amylase and protease-producing bacteria.

A loop full of the bacteria sample was streaked in one line and plated onto nutrient agar (1.0% starch, 5g/L peptone, 1.5g/L beef extract, to find bacterial strains that produce amylase 1.5g/L yeast extract, 5g/L NaCl, 15g/L agar) incubated at 37°C for 48 hours. The plates were

overlaid with iodine reagent solution (0.01 M I_2-KI) [20,21]. The clear zone formed around the isolates' growth identified the amylase enzyme produced. The positive isolates were sub-cultured several times for purity and maintained in nutrient agar slants (-20°C).

For protease-producing bacteria isolates, the skim milk agar with 1% casein medium 100 mL of distilled water (% w/v) composed of yeast extract 0.1, NaCl 0.5, $MgCl_2$, 0.03, agar 1.5, $MgSO_4.7H_2O$, 0.02, milk powder, casein hydrolysate 1, pure glucose 1, at pH =7.2 was used as screening medium [22]. To confirm the extracellular protease-producing bacteria, the clear zone of casein hydrolysis around any colony was observed after 48 hours of incubation at 37℃. No zone of clearance formation was evaluated as no protease and amylase production.

2.5. Identification of the promising isolates by 16S rRNA and amplification of genes encoding amylase and protease.

The isolated bacteria with a promising extracellular hydrolytic enzyme production feature were selected and identified by 16SrRNA sequencing. The extracted DNA of the isolate was subjected to amplification for target genes encoding amylase and protease. The DNA isolated from the bacterial isolate was used as the template in the PCR process for genotypic characterization. 2 µL of DNA was used to amplify 16S rRNA genes using universal 27F and 1492R primers 5'-AGAGTTTGATCCTGGCTCAG-3', 5'-GGTTACCTTGTTACGACTT-3' [23,24]. The amplification was carried out in a total volume of 50µl. The following (PCR) steps were used: Denaturation at 94: 1 min at 94 ℃, 30 cycles in 30 secs denaturation at 94 ℃, 30 secs of primer annealing at 52℃, 1 min of extension at 72 ℃, and final extension at 72℃ for 5 min. The concentration and purity of samples were determined using a NanoDrop spectrophotometer. Obtained products were sent to Macrogen (Holland) for sequencing. The sequences obtained from Macrogen were compared with the GenBank database. The similarity rate was designated, and the GenBank accession number was received. For the genes encoding amylase and protease, two sets of primers used AmyE-F-5'-ATGGACGAAAAAAATGAACAGC-3' and AmyE-R-5'-TCAATGGGGAAGAGAACCG-3, clpP-F-5'-ATGGACGAAAAAAATGAACAGC-3'and clpP-R-5'-CATTGGATCAGCCCCTCG-3'. The PCR temperature conditions included initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturing at 94°C for 1 minute, annealing at 55°C (amyE), 58℃ (clpP) for 1min and extension at 72°C for 2 min. Finally, the final extension was allowed at 72°C for 5 minutes. The PCR products were separated on an agarose gel electrophoresis at 1% (w/v) [25].

2.6. Construction of phylogenetic tree.

For phylogenetic analysis, the 16S rRNA sequence of the isolates and reference sequence retrieved from the NCBI-GenBank database were aligned with MUSCLE. The resulting alignments were analyzed with MEGA 11 to construct the phylogenetic tree. The neighbor-joining method inferred the phylogenetic tree, and sequence divergence among the strains was quantified using the Kimura-2-parameter distance model. One thousand bootstrap replications were calculated to evaluate the tree topology [26].

2.7. Determination of amylase and protease enzyme activity.

The amylase enzyme activity was performed following the method developed by Simair with minor modifications [27]. 1 mL of crude enzyme and an equal volume of substrate (1.0%

w/v starch soluble) were mixed thoroughly, and the mixture was incubated at 37°C for 10 min in a water bath. After 10 min, the reaction was stopped by adding 2.0 mL of DNS reagent, and the color changed due to the reduced sugar when the reaction tubes were kept in boiling water for 5 min. The samples were cooled on running tap water, and the absorbance of the developed red-brown color was analyzed using a spectrophotometer at 540 nm against an enzyme blank. The blanks were prepared by replacing the sample with the same amount of water. The enzyme activity of amylase was calculated from the glucose standard curve. Amylase activity is the amount of enzyme which released 1µmol of D-glucose/min under the assay conditions [28].

Protease enzyme activity was determined using 1% casein as a substrate following the method developed by Tolbert in 2019 with minor modifications [29]. 5 mL of casein containing phosphate buffer pH 7.4 was mixed with 1mL of diluted crude enzyme. The samples were incubated at 40°C in a water bath for 10 mins. The reaction was stopped by adding 5 mL of 110mM trichloroacetic acid (TCA) and setting it at room temperature for 30min. 2 mL of supernatants were collected after centrifuging the samples at 10000 rpm for 10min. 5 mL of 500 mM Na_2CO_3 was added to 2 mL and 1mL of Folin-Ciocalteau reagent. The samples were incubated at 40°C for 30 min, and then the absorbance was measured using a UV double beam spectrophotometer at 660nm. The standard curve was prepared using tyrosine solution (0.055-0.553 µmol/mL). Protease activity (U) was defined as the enzyme quantity required to liberate one µmol tyrosine in 1 min under the specified assay conditions.

2.8. Agricultural waste substrate preparation.

Various agricultural wastes (potato peels, orange peels, banana peels) and corn starch soluble were used as a carbon source to produce amylase and protease. All wastes were locally collected, cleaned, and dried at 65°C in an oven. The products were milled using a blender before use to obtain the fine powder. One substrate was selected for efficient amylase and protease production through fermentation. Table 1 shows the approximate compositions of agricultural wastes.

Table 1. The chemical composition of agricultural wastes [13].

Agricultural wastes	Chemical composition
Fresh banana peels powder	Protein 3.7%, fat 0.85%, water 0.28, and carbohydrate 66.51%, others 30%
Fresh orange peels powder	Protein 7.8%, starch 4.5%, 1.0%, 12.6%, and moisture 2.9% others 72,4%
Fresh potatoes peel powder	Moisture 11.2%, sugar 3.45%, carbohydrate 64.47%, and protein 13.52% 12.2%

2.9. Submerged fermentation of Bacillus aerius FPWSHA.

The *Bacillus aerius* F.P.W.S.H.A. was used for extracellular amylase and protease production in shake-flask culture using basal medium (10g/ L Soluble starch, 2g/L yeast extract, 5 g/L peptones, 0.5 g/L $MgSO_4$, 0.5 g/L KH_2PO_4, 1.5 g/L NaCl and 0.5 g/L $CaCl_2$ pH=6.5 [8]. The flasks were plugged with cotton wool and sterilized at 121°C for 20 minutes at 15 pounds/cm². After 48h of cultivation, the fermented medium was harvested and centrifuged at 10.000 rpm for 10 min to determine periodically hydrolytic enzyme activity.

2.10. Culture conditions optimizations.

The effect of different parameters on amylase and protease production was studied by changing one parameter at a time. The pH, temperature, substrates and concentrations, nitrogen sources, inoculum size, incubation period, and rotation speed were adjusted for optimum

enzyme production. The influence of the pH of the production medium on enzyme formation was examined by changing the pH of the fermentation medium from 3.0 to 12 (phosphate buffer adjusted by NaOH and HCl). Nitrogen sources such as urea, tryptone, ammonium phosphate, ammonium nitrate, Ammonium sulfate, yeast extract, sodium nitrate, and peptone were examined. Optimum inoculum volume for enzyme production was evaluated using a 0.5 to 4% inoculum. The optimal incubation period of enzyme production was investigated by incubating the inoculated media for up to 3 days or 72 h; the sample was harvested after 12 h of incubation and analyzed. The optimum temperature for amylase and protease production was determined by varying the incubation temperatures from 20 to 60°C. Furthermore, the effect of rotation speed was set from 100 to 250rpm.

2.11. Statistical analysis.

The growth of bacteria analyzed the cell density by checking optical density (OD_{600nm}) using a spectrophotometer. The hydrolytic enzymatic activities assay of the isolated strains was performed in triplicates with three independent replicates. The results are reported as mean ± standard deviation. Origin pro-2021 versions assessed the statistical significance of the differences between the mean values. The values were considered significantly different at $P < 0.05$ [30].

3. Results and Discussion

3.1. Isolation and screening of hydrolytic enzyme bacteria.

The initial research goal was to find a bacterial strain that could produce protease and amylase simultaneously. Nine food kitchen waste samples from restaurants were used as isolation sources of enzyme-producing bacteria. Soluble starch and skim milk powder with casein were used as specific substrates in the isolation and screening medium. Bacteria on isolation media were purified after serial dilution. Those whose colony structure and morphology differed were selected for quality enzyme activity analysis. Out of the 56 pure colonies obtained, 19.3% showed protease, 28.1% showed amylase as individual extracellular enzymes, and 17.5% of the isolate showed the production of both amylase and protease enzymes. In contrast, 35.1% of the bacteria did not show either activity (Figure 1A).

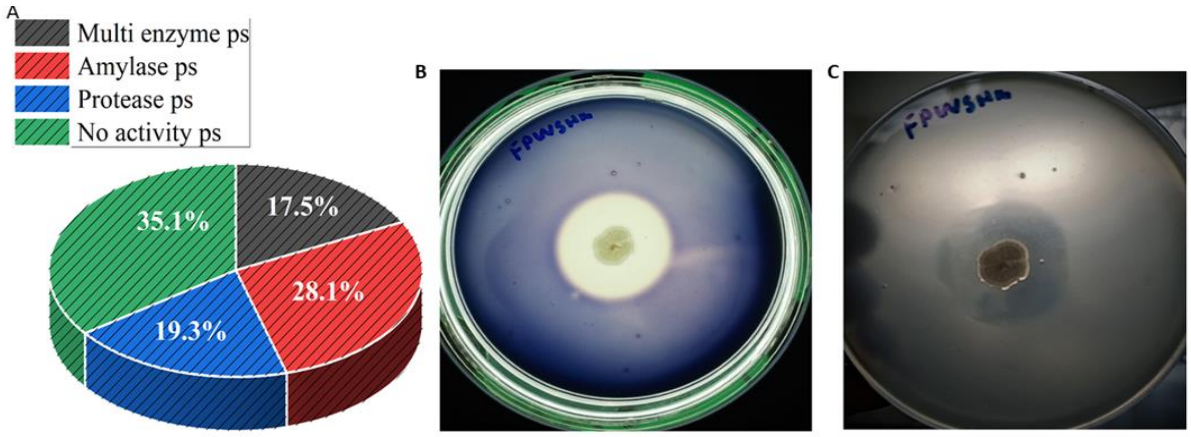

Figure 1. (A) Pie chart of 56 isolates and their amylase and protease enzymes distribution. **(B)** The clearance zone shows starch hydrolysis around the bacteria growth. **(C)** Clear zones indicate casein hydrolysis; Ps stands for producing strain.

When separate colonies were screened for their ability to produce alkaline α-amylase and protease, only ten were found to secrete a significant amount of these enzymes on agar plates. They were biochemically characterized in (Table 2). Among these ten strains, the highest extracellular α-amylase-producing strain (FPWSHA), capable of producing a clear halo zone of more than 10mm diameter, was selected for amylase and protease production (Figure 1B&C).

Table 2. Biochemical characterization of isolates from food wastes.

Characteristics /strains	Catalase	Oxidase	Citrate utilization	MR	H2S production	Indole	Starch hydrolysis	Casein hydrolysis	Lipid hydrolysis	VP (Voges Proskauer)
FPWMt	+	-	+	+	-	-	-	+	-	-
FPWH	+	+	+	+	-	-	+	+	-	+
FPWU3	+	-	+	+	+	-	-	+	-	-
FPWF4sw	-	+	+	+	-	+	+	-	-	+
FPWF3w2	+	-	+	-	-	-	+	-	-	+
PROT	+	-	+	+	+	+	-	+	-	-
FPWF2W3	+	-	+	-	-	-	+	+	+	+
FPWSHA	+	-	+	-	-	-	+	+	+	+
F22W4	+	+	+	+	-	+	+	+	+	-
PP4	+	+	+	+	-	-	-	+	+	-

(+)positive ,(-) negative

3.2. Molecular characterization of bacterial isolates and genes encode enzyme amplification.

16S rRNA amplification and nucleotide sequencing confirmed the size of the purified PCR product at 1492bp (Figure 2A). Amplification of genes was done using primers targeting regions encoding amylase and protease. The fragment for protease (clpP: EC 3.4.21.92) gene 594 bp and 1980 bp alpha-amylase (AmyE, EC: 3.2.1.1) was successfully amplified as confirmation of protease and amylase genes in genomic DNA of *Bacillus aerius* FPWSHA isolated from food waste (Figure 2B).

The nucleotide homology and phylogenetic analysis of 16S rRNA gene sequencing and BLAST results revealed that isolated bacteria belong to Bacillus, Aeromonas, Proteus, and Wohlfahrtiimonas genera. The identified bacteria species were *Bacillus aerius, Bacillus subtilis, Bacillus pumilus, Bacillus Tequilensis, Bacillus cereus, Proteus mirabilis, Proteus cibarius, Aeromonas cavaie, and Wohlfahrtiimonas chitiniclastica* were found to be the nearest homology of isolates from food processing wastes (Figure .3A**)**. Nucleotide-nucleotide BLAST in NCBI results showed that strain FPWSHA has more than 99% sequence similarity with the first hit of *Bacillus aerius*. It showed 99.58% sequence similarity to *Bacillus aerius strain* N602 (MK629828). According to the results reported above, the FPWSHA strain was identified as *Bacillus aerius*. Its sequence of 1456 bp was submitted to GenBank and was designated *as Bacillus aerius* FPWSHA (accession number OM258619). Phylogenetic analysis was based on nucleotide sequence homology. A rooted phylogenetic tree was constructed using the neighbor-joining method for all ten isolates, and a figure was drawn for *Bacillus aerius* FPWSHA (Figure 3B). The distances were computed based on Kimura-2 parameters [26,31].

The isolate FPWSHA was selected for further experiments to optimize the amylase and protease production.

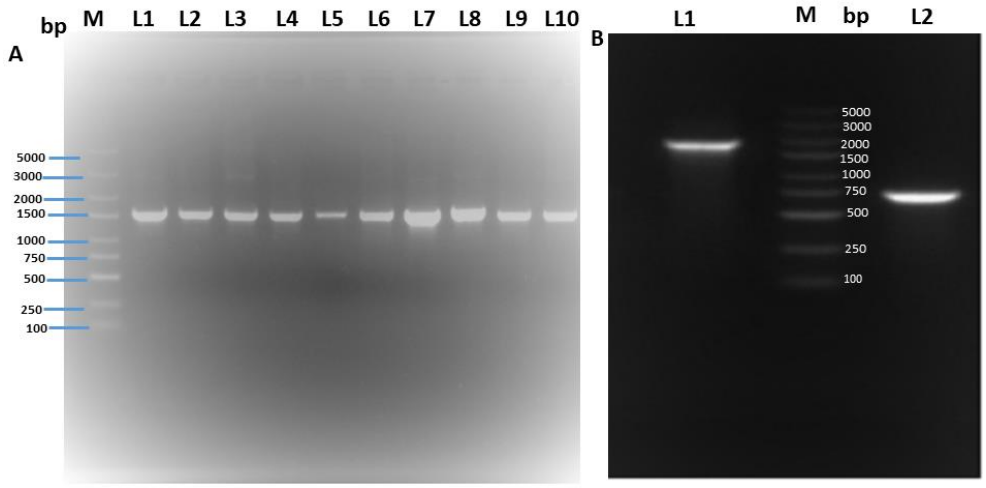

Figure 2. **A)** Gel electrophoresis showing polymerase chain reaction (PCR) amplification of the 1.5 kb of 16S rRNA amplicons. Lane M: 2000bp plus DNA marker; PCR products of 16S rRNA of 10 isolates M: 1kb ladder, L1: FPWmt, L2:FPWH, L3: FPWU3, L4: PROT, L5: FPWSHA, L6: FPWF4SW, L7: W3SFR5, L8: FPWF3w2, L9: F22W4, L10:PP4. **B)** Gel electrophoresis shows polymerase chain reaction amplification of protease and amylase encoding genes. Lane M; 2000bp plus DNA marker; amyE amylase gene, clpP protease gene.

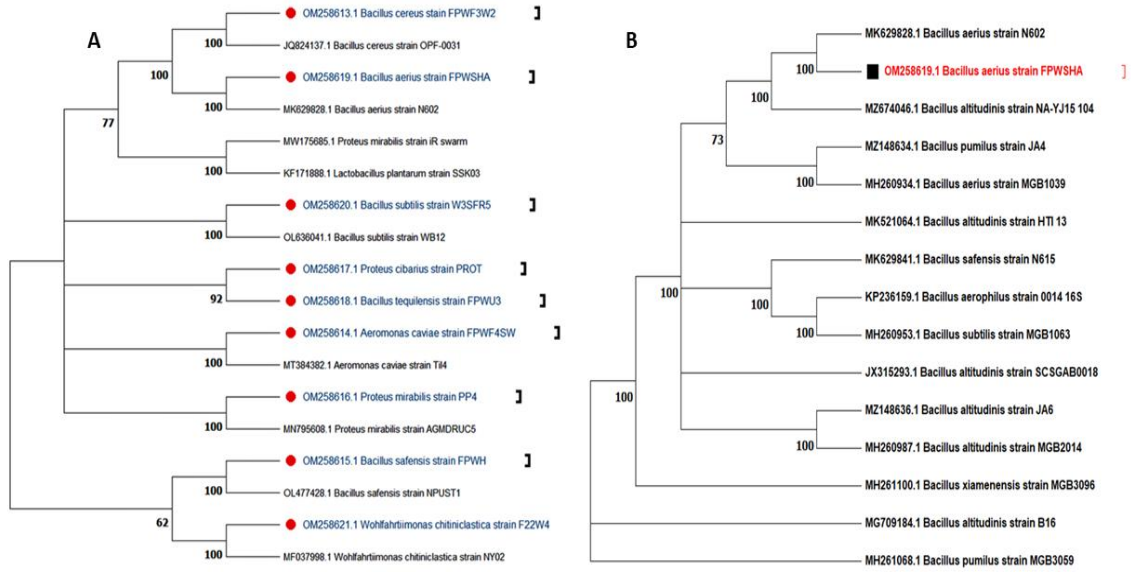

Figure 3.(A) A phylogenetic tree of bacterial isolates relied on the nucleotide sequences of 16S rRNA genes constructed by the neighbor-joining method. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA11) software. The phylogenetic tree of nine isolates from food wastes with their accession numbers and similar bacteria from the bacterial NCBI database. **(B)** A phylogenetic tree constructed by the Neighbor-joining method based on 16S rRNA sequences of the genus *Bacillus aerius*. The number next to each node displays the percentage bootstrap value of 1000 replicates. The highlighted strain in red is *Bacillus aerius* FPWSHA isolated from food wastes and its accession number. GenBank accession numbers of the bacteria are presented before each bacterial strain.

3.3. Optimization of culture condition for amylase and protease production by Bacillus aerius FPWSHA.

3.3.1. Effect of different agricultural wastes as carbon sources and concentrations for amylase and protease production by *Bacillus aerius* FPWSHA.

The nature and concentration of substrates may affect the growth and yield of microbial enzyme production; there is also a need to increase enzyme production by using cheap

substrates and wastes reported to have nutrients for bacteria growth. The effect of different carbon was performed using agricultural wastes: potato peels (PPP), orange peels (OPP), and banana peels (BPP). 1% of prepared powder was utilized as a carbon source in the inoculum medium. The starch soluble (SSC) was added to the medium as a control. The potato peel powder showed highly amylolytic and proteolytic activity, followed by the orange peel powder. Banana peel powder showed less enzyme activity for both enzymes (Figure 4A). The potato peel powder has nutrients for the growth of the bacterial cells because it contains carbohydrates, sugars, and proteins, as aforementioned in (Table 2). The cells were grown in the basal medium by replacing commercial starch soluble with potato peel powder. The PPP was added to the medium in various quantities ranging from 5 to 30 g/L (Figure 4B). When 20 g/L of PPP was employed as the substrate, 10.5 U/mL of Amylase and 7.6 U/mL of protease were obtained after 36 h of incubation. This concentration was chosen for the subsequent experiment since it gave high activity to both enzymes. The isolation, screening, and identify bacterial strains are of interest for enzymes and biochemical production. In addition, for economical biosynthesis, using cheap substrates in fermentation may yield a valued added product. The current work screened and optimized the bacteria isolates for two enzymes produced under agricultural wastes in submerged fermentation. The results showed that high levels of enzyme production were achieved when potato peel waste powder was used as substrates. Banana peels and orange peel powder were also used in solid-state fermentation [32]. PPP was reported to increase protease and alpha-amylase by *Anoxybacillus rupiensis* T2 [25]. Pomegranate was also used as an agricultural waste substrate for amylase production by an isolated *Aspergillus terreus* [14]. Our study contrasts with the study reporting that consuming commercial starch soluble as a carbon source was the best substrate for enzyme production for amylase [33]. But it was in good agreement with the study, which used potato peels for amylase enzyme production by *Bacillus amyloliquefaciens* [24].

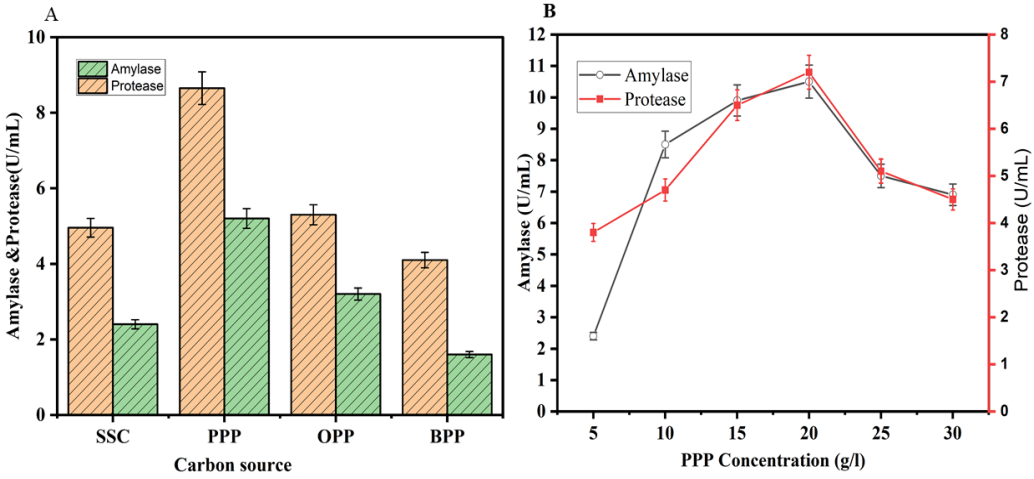

Figure 4. (A) Effect of different agricultural wastes as carbon sources. **(B)** The result of different concentrations of PPP for amylase and protease production by *Bacillus aerius* FPWSHA SSC starch soluble, PPP: potato peels powder, OPP: Orange peels powder, BPP: banana peels powder. The culture conditions were temperature 37°C, pH= 7, incubation of 36 h, and rotation speed of 150 rpm.

3.3.2. The effect of temperature and initial pH on amylase and protease production by *Bacillus Aerius* FPWSHA.

The effect of temperature was assayed at different temperatures ranging from 20-60°C at optimum pH. In the present study, to determine the optimum temperature for enzyme production, fermentation was carried out at different temperatures from 20 to 60°C. Enzyme

production gradually increased with increasing bacterial growth and temperature. Figure 5A shows that the maximum enzyme activity production was achieved at 40°C and considered an optimum temperature. The lower activity was observed at a higher temperature above 40°C due to long-term exposure resulting in reduced enzyme activity. In this study, 40°C was an optimum temperature that provided high bacteria growth and enzyme activity. This study agrees with research conducted by Mukhtar and coauthors, where 37°C was an optimum for amylase enzyme synthesis and 40°C for protease by *Bacillus subtilis* [34].

Amylase and protease production by microbial strains depends on the extracellular pH because culture pH strongly influences the growth of the cell and many enzymatic processes. Transporting various components across the cell membranes supports bacterial cell growth and associated product production [11]. A pH range from 4 to 12 was used to study the effect of pH on protease and amylase activity (Figure 5B), and optimum pH was found at pH= 8 for amylase and protease enzyme activity. The enzyme activity was lower in the high alkaline pH range than in acidic pH. The extracellular pH controls a number of enzymatic processes as well as the transport of various components across cell membranes, allowing cells to grow and produce products. The extracellular pH affects the production of amylase and protease by microbial strains. The Bacillus enzyme producers have been studied, and most have alkaline enzyme producers, including *Bacillus subtilis* [35], *Bacillus licheniformis* [36], and *Bacillus safensis* [37]. This strain is unique because it has shown a higher enzyme activity in an acidic culture medium compared to high alkaline culture. Still, the bacteria showed a moderate production of both enzymes at pH =4, possibly due to the strain's acidophilus properties.

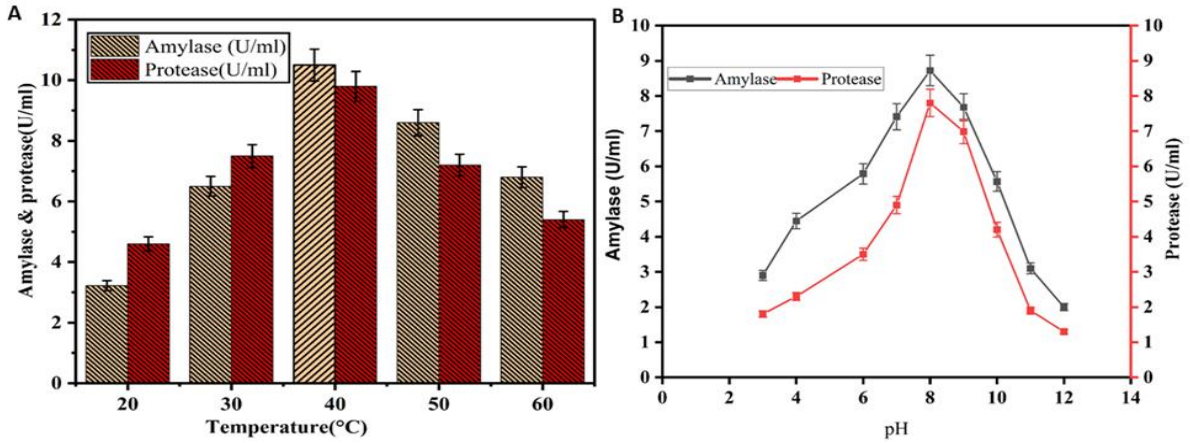

Figure 5. (A) Effect of temperature on protease and amylase production by *Bacillus aerius* FPWSHA Culture parameters: shaking speed 150 rpm, initial pH 7.0, 20 g/L PPP, incubation time 36 h. **(B)** Effect of pH on protease activity by *Bacillus aerius* FPWSHA. The culture medium conditions were temperature 40°C, shaking speed 150 rpm, and varying the initial pH from 3 to 12.

3.3.3. The effect of inoculum volume and rotation speed on amylase and protease production by *Bacillus aerius* FPWSHA.

The inoculum size was optimized from 0.5 to 4 %. 2% inoculum volume was the optimum for amylase and protease production, as shown in (Figure 6A). The effect of inoculum size on enzyme production for enzyme and cell growth may depend on bacterial efficiency, stability, type, and extent [38]. The production of enzymes gradually decreased when the inoculum level increased further. It could be because the bacteria overgrow at high inoculum levels, and the nutrients in the media are insufficient to stop the bacteria from growing [39]. Thus, amylase and protease production was affected at a higher inoculum concentration than 2%. Our findings support previous studies that report that microbial biomass cannot rapidly

increase and that, as a result, substrate breakdown is slowly decreasing metabolite biosynthesis [40, 41]. Similar results said that depleting nutrients and oxygen available in the growth medium may have an inhibitory influence on enzyme synthesis at a large inoculum size [42].

The rotation speed was reported to ensure a homogeneous mixture of nutrients in the fermentation broth and dissolved oxygen [43]. The rotation speed of the incubator was optimized by incubating the bacterial strain at different rates of 100 to 250 rpm. The optimum rotation speed favorable for maximum amylase and protease production was 180 rpm; beyond this rotation speed, enzyme activity was low, as shown in (Figure 6B). Shaking the culture flasks at a moderate rate lets the bacteria get the nutrients they need and ensures enough oxygen, which helps them make a lot of enzymes [39]. The decrease in enzyme activity in this work may be due to the effect of shear on the studied *Bacillus aerius* above 180 rpm.

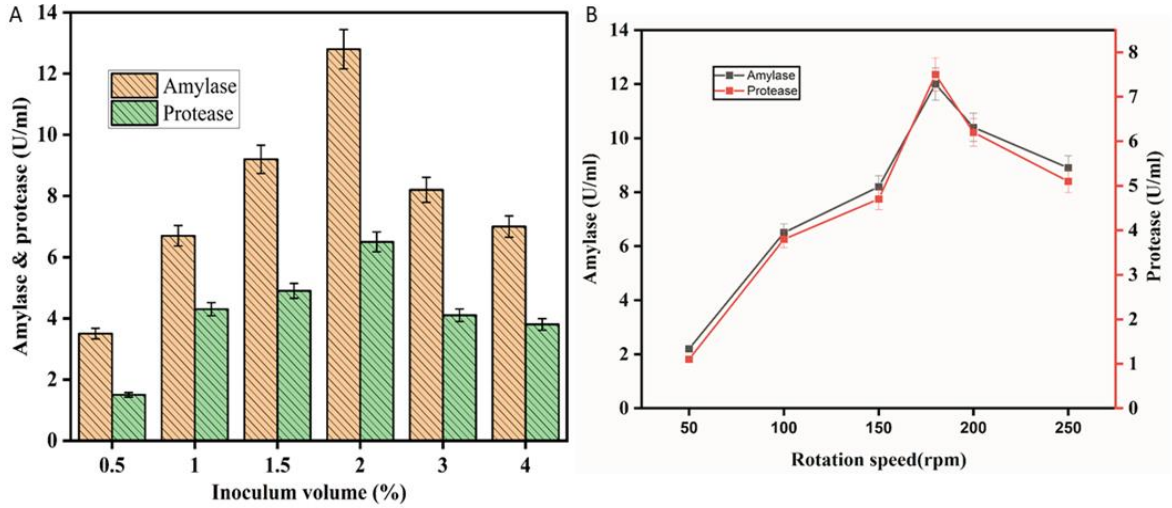

Figure .6. (**A**) Effect of inoculum volume on amylase and protease production by *Bacillus aerius* FPWSHA Culture parameters: shaking speed 150 rpm, initial pH 8.0, temperature 40°C and 20 g/L of PPP, incubation time 36 h. **(B)** Effect of rotation speed on amylase and protease production by *Bacillus aerius* FPWSHA. The cell culture conditions were 40°C, pH=8, Peptone 0.3%, PPP 20 g/L, 36 h of incubation, and the rotation speed settings from 100 to 250rpm. The error bars show the averages and standard deviations of three independent replicates.

3.3.4. The effect of nitrogen sources on amylase and protease production by *Bacillus aerius* FPWSHA.

The nature and relative concentration of nitrogen sources in the growth medium are essential in amylase and protease production. The present study results mentioned that peptone was the best nitrogen source. The effect of organic nitrogen sources on amylase and protease production was analyzed using organic and inorganic nitrogen sources at 0.3%. Organic nitrogen was used, including yeast extract, peptone, tryptone, and urea. While sodium nitrate, ammonium sulfate, ammonium nitrate, and ammonium nitrate were used as inorganic nitrogen sources (Figure 7). Sodium nitrate was found to be an optimum for the growth of the cell with enzyme activities compared to another inorganic nitrogen source. Among the different organic nitrogen sources tested, peptone was an excellent nitrogen source for amylase and protease production by *Bacillus aerius* FPWSHA. Peptone and sodium nitrate were considered the nitrogen sources in a subsequent experiment. Although other organic nitrogen sources such as yeast extract, tryptone, and urea could result in a reasonable amount of enzyme production in this study, they were less effective than peptone and inorganic nitrogen or urea. Sodium nitrate was the only inorganic nitrogen source influencing growth and amylase/protease production.

In addition to acting as a nitrogen source, most organic nitrogen sources supply vitamins, minerals, and additional growth factors in the cultivation medium that enhance bacterial growth and enzyme production.

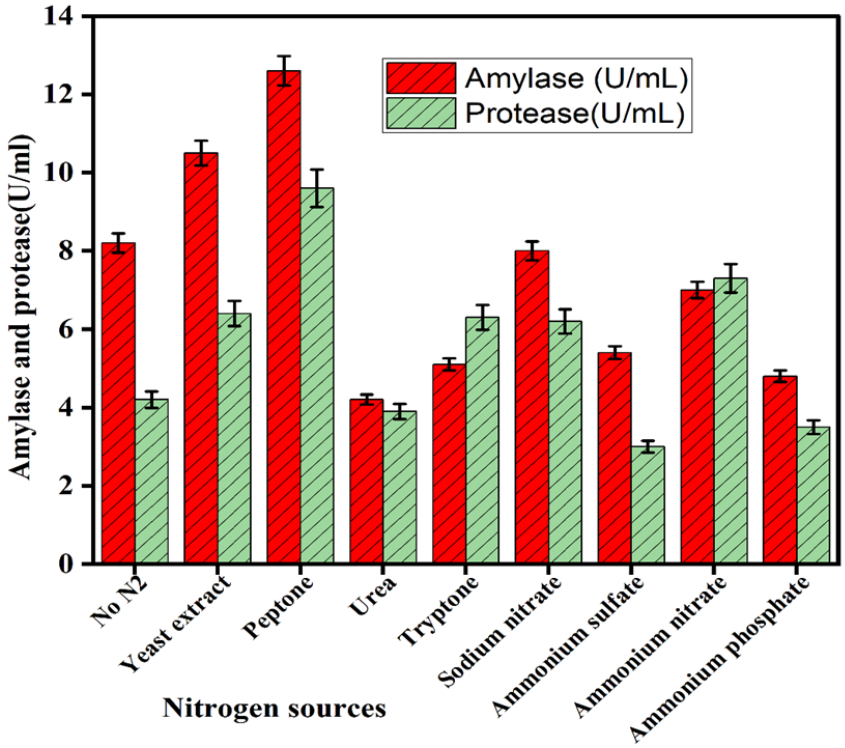

Figure 7. Effect of nitrogen sources on amylase and protease production by *Bacillus aerius* FPWSHA medium without any nitrogen source addition was taken as the control. Different organic nitrogen sources (0.3% w/v) in 100 mL of medium. The bars show the averages and standard deviations of three replicates.

3.4. Time course production of amylase and protease by Bacillus aerius FPWSHA.

Bacillus aerius FPWSHA was subjected to fermentation under optimum pH 8, temperature 40°C, PPP 20 g/L, peptone 0.3% as nitrogen source, incubation time of 48 h, and 2% inoculum volume. The incubation time effect was determined by incubating the samples for up to 72 h (Figure 8). The maximum activities of both enzymes could be reached at 48 h; 16.9 U/mL amylases were obtained, while 12.3 U/mL proteases were registered. The results above showed an increase of 1.3 fold compared to the non-optimized inoculum medium.

The incubation time was optimized for enzyme production in different research. In this study, the incubation period influenced the amylase and protease activity from 12 to 48 h, and beyond that time, there was a decrease in enzyme activity. The reduction in enzyme production after an optimum incubation period can be explained by cell autolysis, nutrient (proteins and carbohydrates), and some metal ions that support enzyme activities and cell growth. The more explanation for an increase in incubation time decreases the enzyme activity, which may be due to an interaction of synthesized enzymes with other components in the medium or a decrease and cell death [44]. The same results were observed when *Anoxybacillus rupiensis* T2 was used in the fermentation medium for amylase production [25]. The protease activity is less than the amylase activity in most experiments where the bacteria enzymes are simultaneously produced. This difference in enzyme production by the *Bacillus* species may depend on the genetic differences for each bacterium [38].

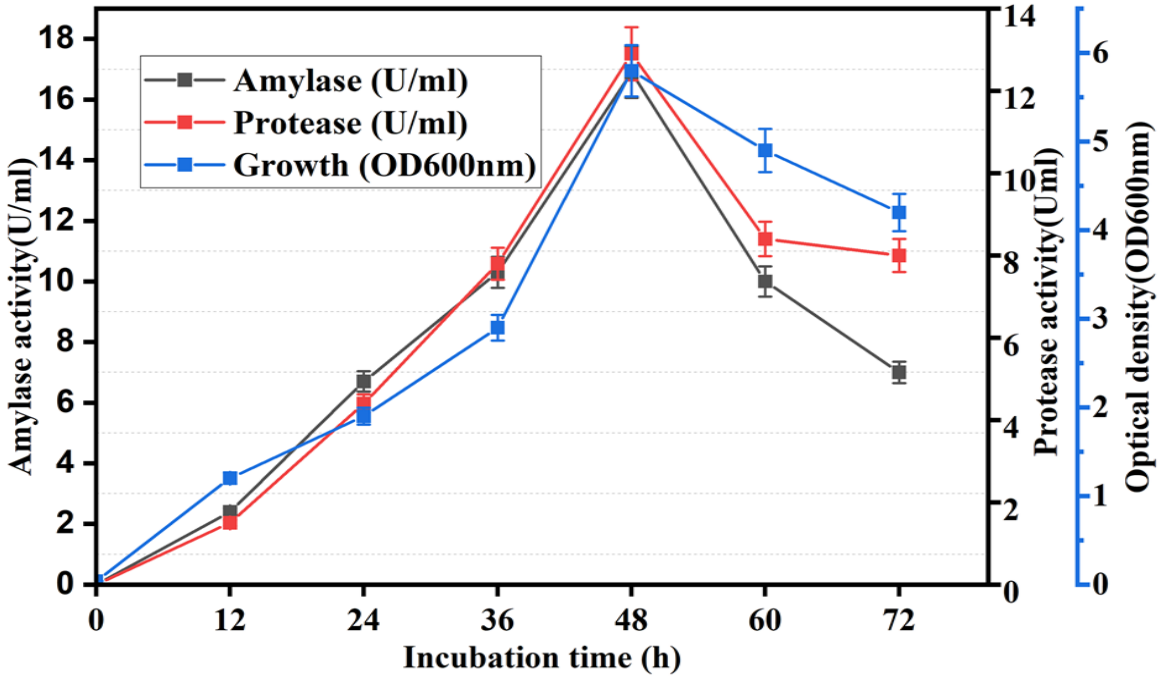

Figure 8. Effect of incubation time on cell growth with protease and amylase production by *Bacillus aerius* FPWSHA. The culture medium was 20 g/L PPP, 0.3% peptone, Temperature 40°C, pH=8 for amylase and protease, inorganic salts, and incubated under 180 rpm as rotation speed. The bars show the averages and standard deviations of three replicates.

4. Conclusions

Isolation and identification of novel bacterial species are necessary, especially for industrial enzyme production. In addition, there is a need to increase enzyme production by using more inexpensive substrates for economical manufacturing. In this study, 56 different bacteria were isolated from food processing wastes for enzyme production. A strain of *Bacillus aerius* FPWSHA was screened successfully for concurrent amylase and protease production under cheap substrates PPP in submerged fermentation. Improving this strain may yield better industrial and biotechnological for microbial amylase and protease production. Our study presents the first successful report on isolating and culture conditions optimization of α-amylase and protease enzyme productions from *Bacillus aerius* FPWSHA.

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Conflicts of Interest

The authors declared no conflict of interest.

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