



## Identification and Optimization of Enzymes Extracted from Solid-State Fermentation

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*Received: 23/08/2022, Accepted: 15/07/2023, Available Online: 15/08/2023*

### ABSTRACT

Microbial enzymes produced through solid-state fermentation are essential source of numerous microbial strains due to their higher stability, production rate, biochemical versatility, and availability. These enzymes, especially the thermophilic ones, are capable of withstanding harsh environments, high temperatures, and chemicals used in various industrial processes. This study involved multiple steps. Firstly, bacteria were identified using 16S rRNA gene analysis. Subsequently, solid-state fermented (SSF) enzymes, including amylase, protease, cellulase, and xylanase, were screened using their respective assays. Prior to optimization, the conditions affecting the extraction were evaluated using a one-factor-at-a-time (OFAT) approach. The extraction of SSF enzymes was then optimized using a Face Centered Central Composite Design (FCCCD). Bacterial enzyme identification was performed using Next-generation sequencing, and the following strain families were found: *Enterobacteriaceae*, *Klebsiella*, *Aneurinibacillaceae*, *Atopobiaceae*, *Bacillaceae*, *Burkholderiaceae*, *Clostridiaceae*, *Lactobacillaceae*, *Peptostreptococcaceae*, *Staphylococcaceae*, *Streptococcaceae*, and *Streptomyetaceae*. The highest protein yield was achieved using the one-factor-at-a-time (OFAT) method, with a protein concentration of 6.07 mg/m obtained from 10g of SSF material (in 90 ml of sodium phosphate buffer) at pH 9. The extraction process involved a temperature of 65°C, a duration of 2 hours, and an incubation speed rotation of 250 rpm. Under these optimized conditions, the activities of the SSF enzymes were determined as follows: protease (11.04 U/ml), cellulase (11.59 U/ml), xylanase (174.13 U/ml), and amylase (11.01 U/ml). This research will further contribute to the extraction of palm oil, offering high yield and a promising solution across various fields by replacing the expensive industrial enzymes.

**Keywords:** microbial enzymes, 16S rRNA Amplicon Sequencing, enzymes assay, OFAT, RSM

### INTRODUCTION

Enzyme technology is swiftly evolving and holds great potential to transform various sectors, including the detergent, food bioprocessing, and oil extraction industries. Despite these remarkable prospects, the use of industrial enzymes continues to pose challenges due to their exorbitant cost, stemming from the complexities

involved in their production, limited diversity and quantity, the technically demanding recovery processes, and the difficulties associated with enzyme reuse. Consequently, there is a pressing need to explore novel sources of enzymes to unlock new frontiers in biotechnology. Among these sources, microorganisms such as bacteria, yeast, and fungi, along with their enzymes, have garnered significant attention for their potential in various industrial sectors.

Microbial enzymes have emerged as a preferred alternative to enzymes derived from animal and plant sources. Their superiority lies in their enhanced stability and production rate, their remarkable biochemical versatility, and the wide array of microbial strains readily available for utilization (Liu *et al.*, 2017; Cotta *et al.*, 2023). Notably, thermophilic enzymes, renowned for their ability to generate high-value catalytic products even in harsh conditions, have become a focal point of interest in biorefinery and high-temperature environments. The investigation and characterization of protease and amylase produced by a bacterial strain isolated from food waste (Mohammadipour *et al.*, 2021; Pham *et al.*, 2022) further emphasize the significance of these enzymes and underscore the need for further comprehensive research in this domain.

In line with the diverse applications of enzymes in various fields such as food, cosmetics, detergent, pharmaceuticals, leather, and synthetic biotechnology (Guo *et al.*, 2013), a selection of enzymes including protease, amylase, xylanase, pectinase, and cellulase were identified as key targets for investigation. To determine the microbial source of these enzymes, the 16S ribosomal rRNA gene, widely regarded as the "gold standard" for taxonomic identification and classification of prokaryotic microorganisms, was employed. This gene contains nine hypervariable regions, which provide unique signature sequences facilitating the taxonomic classification of prokaryotes. Instead of analyzing the entire 16S rRNA gene, focusing on individual or multiple hypervariable regions is sufficient to classify microbes at different taxonomic levels, from Phylum to Genus. Careful consideration is given to the choice of hypervariable region and primer set to account for potential primer biases and variations in taxa coverage (Guo *et al.*, 2013; Kumar, 2011). While longer reads generated by technologies like Pac-Bio and Nanopore offer greater taxonomic annotation accuracy, shorter reads from platforms such as MiSeq and NovaSeq6000 still provide a satisfactory resolution for taxa classification (Wang *et al.*, 2007). Next-generation sequencing was employed to analyze the 16S rRNA gene and identify the microorganisms present in the compost sample (Apical, 2022).

The production and extraction of enzymes can be influenced by numerous factors. Optimal conditions are crucial for the extraction of any biological material. In Biotechnology, the most frequently applied operation in obtaining the enzymes in the microbial system is the 'one-at-a-time strategy' which involves optimizing fermentation conditions. Although this approach is time-consuming, it effectively eliminates complex interactions between physicochemical parameters (Vyas *et al.*, 2015). Additionally, response surface methodology (RSM) utilizing factorial design and regression data analysis offers a more comprehensive approach, enabling the precise evaluation of factors and their interactions to identify the optimal conditions and response variables (Li *et al.*, 2020). Physical factors such as pH, temperature, and time have a significant impact on the production of extracellular enzymes in microorganisms (Mustefa *et al.*, 2021). However, the production of extracellular enzymes from a composting mixture has not been extensively explored in recent studies. Therefore, the primary objective of this study was to determine the optimal conditions for the extraction of enzymes while concurrently identifying the bacteria responsible for their production. It is important to note that the subsequent application of these enzymes, specifically in the extraction of palm oil for increased yield using aqueous enzymatic extraction, will not be discussed in this study. The focus will solely be on the enzyme extraction and optimization process.

## **MATERIALS AND METHODS**

### **Enzymes preparation and collection**

The solid-state fermented (SSF) material, serving as the enzyme source, was obtained from the International Islamic University IIUM, and stored at 4°C in a chiller. 10 g of the SSF material was weighed and combined

with phosphate buffer at varying pH levels. The mixture was subjected to different temperatures and incubation times within a shaker incubator. Following the incubation period, the samples were filtered and collected in 50 ml tubes, followed by centrifugation.

### **Molecular identification of screened isolates**

The bacteria responsible for producing the SSF enzymes were identified using a highly precise method known as 16S rRNA gene analysis. To begin the process, the purified gDNA that successfully passed the DNA sample quality control was subjected to amplification. This amplification was carried out using specific sequence primers designed for the locus of interest. For the bacterial 16S V3-V4 region, the following primers were utilized:

Forward Primer (16S V3-V4): CCTACGGGNGGCWGCAG

Reverse Primer (16S V3-V4): GACTACHVGGGTATCTAATCC

### **First Part of Library Construction (1st Stage PCR)**

The amplification of the bacterial 16S rRNA gene in the specific regions (16S V3-V4) was accomplished using locus-specific sequence primers with attached overhang adapters. The overhang adapters employed for the forward and reverse primers are provided below:

Forward overhang:

5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG- [locus- specific sequence].

Reverse overhang:

5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG- [locus-specific sequence].

To carry out the PCR reactions, the KOD -Multi & Epi-® enzyme (manufactured by Toyobo) was utilized.

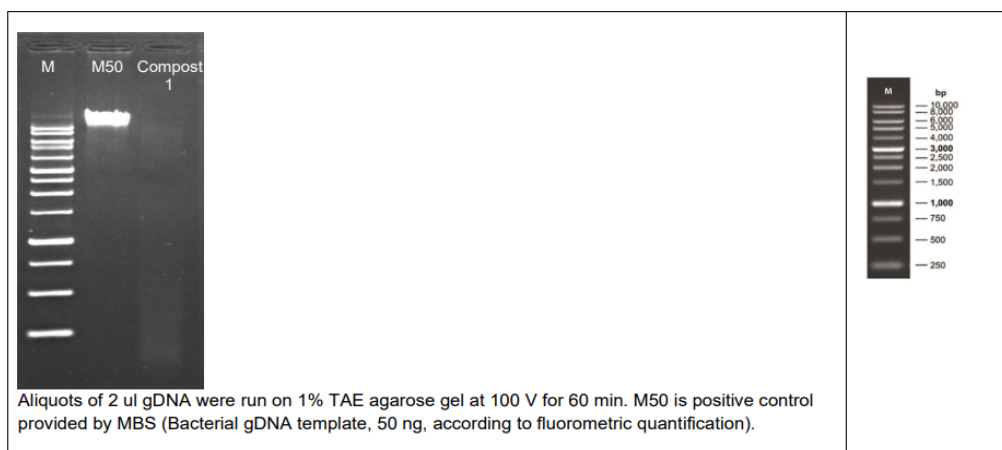
### **Second Part of Library Construction (2nd Stage PCR)**

The amplicon PCR was augmented with dual indices using the Illumina Nextera XT Index Kit v2, following the manufacturer's guidelines. To assess the quality of the libraries, the Agilent Bioanalyzer 2100 was employed. The quality assessment involved utilizing the Agilent DNA 1000 Kit for systemic analysis and fluorometric quantification using the Helixyte Green™ Quantifying Reagent.

### **Next-Generation Sequencing.**

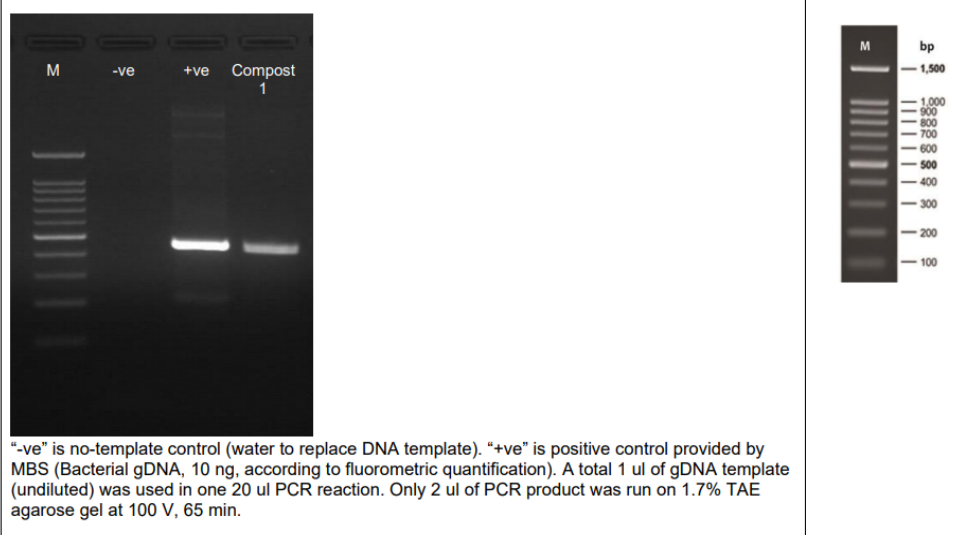
For Next-Generation Sequencing, the libraries were normalized and pooled in accordance with Illumina's recommended protocol. Subsequently, sequencing was performed on the MiSeq platform using 300 PE sequencing (Apical, 2022). Fig 1 illustrates the selection of 16 rRNA gene that will be used to build the library.

## (2) GEL PHOTO



## (3) PCR QC

### 16S rRNA gene (V3-V4)



**Fig. 1.** Gel photo ( M50 is positive control provided by MBS (Bacterial gDNA template, 50 ng, according to fluorometric quantification) (2) PCR quality control select the 16S rRNA gene (v3-v4) for further analysis to build the bacterial library(3) (Apical, 2022)

## Enzymes extraction from Solid-State Fermentation

Approximately 10 g of the sample, SSF enzyme was added to 90 ml of sodium phosphate buffer pH 7.0. prepared as per the following composition: 2.021 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , adjusted to pH 7, was added to 250 ml flasks. The flasks were then kept on a rotary shaker (200 rpm/min) for 1 h at room temperature for the extraction of the enzyme from the fermentation solid. At the end of the extraction, the mixtures were transferred into 50 ml centrifuge tubes and centrifuged at 4,000 rpm/min for 10 min. The clear supernatant was designated as the crude enzyme preparation.

## Analytical experiments

### *Protein quantification*

Protein concentration was determined using the Bradford assay, following the microtiter plate protocol described by Bio-Rad protein assay. The dye reagent was prepared by diluting the Bio-Rad dye reagent concentrate in a 1:4 ratio with distilled water, and it was subsequently filtered using Whatman No. 1 filter paper to eliminate any particulates. For generating a standard curve, bovine serum albumin (BSA) was employed as the standard protein. A stock solution of BSA was prepared by dissolving 0.1 g of BSA in 100 ml of 0.05 M PBS buffer at pH 7. Various dilutions of this protein standard were prepared accordingly. To perform the assay, 200 µl of the diluted Bradford Reagent was added to each sample and thoroughly mixed. The microtiter plate was then incubated at room temperature for a minimum of 15 minutes, allowing for adequate color development. Subsequently, the absorbance was measured at a wavelength of 595 nm using a spectrophotometer. Amount of protein was then calculated using Eqn. 1

$$\text{Protein concentration calculation: } x = \frac{\text{abs} - 0.0479}{0.0016} \times 50 \text{ d.f.} \quad \text{Eqn.1}$$

Where: abs are the absorbance value,

0.0016= the constant (a) in the equation of protein standard

d.f: dilution factor

### **Enzyme assay**

#### *Protease Activity Assay*

Protease activity was measured according to the universal procedure described by (Sigma-Aldrich, 2013) (Mahto, *et al.* 2013), in which casein was used as substrate and was measured at a wavelength of 660 nm. The L-Tyrosine standard curve was prepared by varying the tyrosine concentration in a mixture of dH<sub>2</sub>O, sodium carbonate and Folin & ciocalteu's Phenol (F-C) Reagent. To measure the protease activity, 1 mL of the enzyme solution was added to 1 mL of 1% (w/v) casein solution (1 g casein dissolved in 100 mL Tris-HCL), it was then incubated for 20 min in 35°C water bath. To stop the reaction between protease and the substrate, 10% (w/v) Trichloroacetic acid TCA solution was added to the mixture. From this solution, 1 mL was taken and added to Sodium Carbonate Na<sub>2</sub>CO<sub>3</sub>, and 1 mL of Folin & ciocalteu's Phenol Reagent F-C. Equation 2 was applied to measure the protease activity.

$$\text{Protease activity } \left( \frac{\text{Units}}{\text{mL enzyme}} \right) = \frac{(\mu\text{mole of tyrosine released}) * \text{Total volume of assay (mL)}}{\text{volume of used enzyme (mL)} * \text{Time of assay} * \text{volume in cuvette (mL)}}$$

**Eqn. 2**

#### *Cellulase Activity Assay*

To determine sample cellulase activity, the method described by Lone *et al.* (2012) was followed, with slight modifications. The cellulase activity was assessed by measuring the light absorbance at 540 nm, using a glucose standard curve as a reference (Qi *et al.*, 2009). In this assay, 1% (w/v) carboxymethyl cellulose (prepared in 0.01M sodium citrate buffer, pH 4) was added to 0.5 mL of the sample solution. The mixture was then incubated at 40°C for 30 minutes to allow the cellulase to act on the substrate. To stop the reaction, 3 mL of dinitro salicylic acid (DNS) was added, and the mixture was placed in a boiling water bath for 5 minutes. Subsequently, the mixture was diluted with distilled water (dH<sub>2</sub>O) to a final volume of 10 mL.

Cellulase activity was subsequently calculated using Eqn. 3

$$\text{Cellulase activity} \left( \frac{\text{Units}}{\text{mL enzyme}} \right) = \frac{(\mu\text{g glucose released}) \times (\text{Total assay volume ml}) \times \text{Dilution Factor}}{(\text{vol of enzyme used ml}) \times (\text{Vol in the cuvette ml}) \times (\text{time of assay min})}$$

**Eqn.3**

#### *Xylanase assay*

Xylanase activity was determined following the method established by Bailey et al. (1992), which involved measuring the release of reducing sugars from xylan using 3,5-dinitrosalicylic acid (DNS). The crude enzyme extract was used as the source of xylanase and assayed in a reaction mixture of 3.0 mL, comprising 1% xylan (prepared in 0.05 M sodium citrate buffer, pH 5.3), 1.8 mL of 0.05 M citrate buffer, and 1.0 mL of the enzyme source. The reaction was initiated by adding the enzyme source and incubated at 55°C for 25 minutes. To stop the reaction, 3.0 mL of 3,5-dinitrosalicylic acid (DNS) was added to the mixture, which was then boiled for 5 minutes in a water bath (Miller, 1959). After cooling, the developed color was measured at 540 nm (DeVeras *et al.*, 2018).

$$\text{Xylanase activity} \left( \frac{\text{Units}}{\text{mL enzyme}} \right) = \frac{(\mu\text{moles of xylose liberated})(df)}{(30)(1) \text{ slop}} \quad \text{Eqn. 4}$$

Where: df = Dilution factor (10)

30 = Time of assay (in minutes) as per Unit Definition

1 = Volume ml of enzyme used, Slop= 0.0046

#### *Amylase assay*

In this study, the Bernfeld (1955) method was used for amylase catalytic activity. Briefly, starch (1% w/v) was dissolved in 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M NaCl by heating and stirring until all substrate was completely dissolved. A 0.5 ml of enzyme diluted solution was added into a glass tube, while a blank tube was set up with 0.5 mL of phosphate buffer. The tubes were equilibrated at 25°C for 5 minutes. Next, 0.5 mL of pre-incubated starch solution was added to each glass tube containing the enzymes, followed by incubation for exactly three minutes at 25°C. To stop the reaction, 1 mL of dinitrosalicylic acid (DNS) reagent was added to each tube, and the tubes were then placed in a boiling water bath for 5 minutes. Afterward, the tubes were allowed to cool to room temperature before adding 10 mL of distilled water (Rani et al., 2012; Vyas *et al.*, 2015). The absorbance of the resulting solution was measured at 540 nm, and a standard curve was prepared using a maltose stock solution with varying concentrations.

$$\text{Amylase activity} \left( \frac{\text{Units}}{\text{mL enzyme}} \right) = \frac{(\text{mg of Maltose released}) \times (\text{Dilution factor})}{\text{volume of used enzyme (mL)}} \quad \text{Eqn. 5}$$

### **Optimization of the extraction of enzymes from Solid State Fermentation**

The experiment was to optimize the factors for the extraction of SSF for the high activities of enzymes using one-factor-at-a-time (OFAT). Five significant parameters were tested in this design. The first, varying the ratio of SSF enzymes, (5, 10, 15 gram), second, pH of buffer (6,7,8,9) and third, varying temperature of the incubation (37, 45, 50,60,70°C), fourth the time of incubation (1, 2, 3h), and last rotation speed (100, 150,200,250 rpm).

### **RESULTS AND DISCUSSION**

The production of enzymes was carried out under the solid-state and submerged fermentation method. The enzymes were collected from renewable resources which are bio-compatible, and the enzymatic catalyst reactions are carried out under milder conditions than the conventional chemical catalyst. These types of enzyme sources are advantageous due to the consistent production and cost-effective method which are both simpler

to extract and simultaneous application that are also reported by (Raveendran et al., 2018). The enzymes that were screened are amylase, protease, xylanase and cellulase.

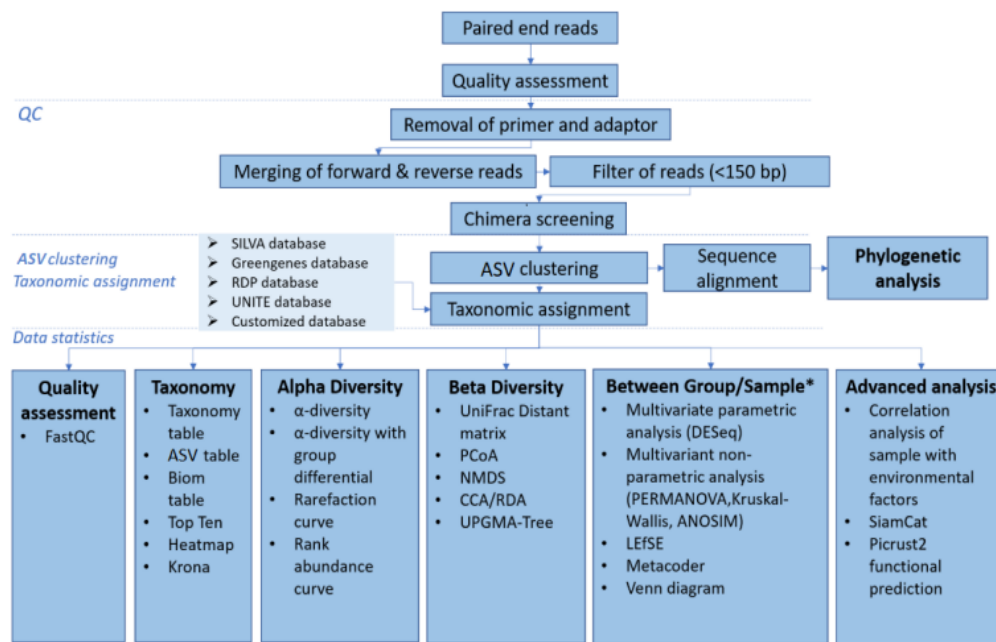
The identification of the bacteria was done to know the types that are present in the solid state fermented material (SSF) using next generation sequencing (NGS), that was detailed next.

## Identification of bacteria

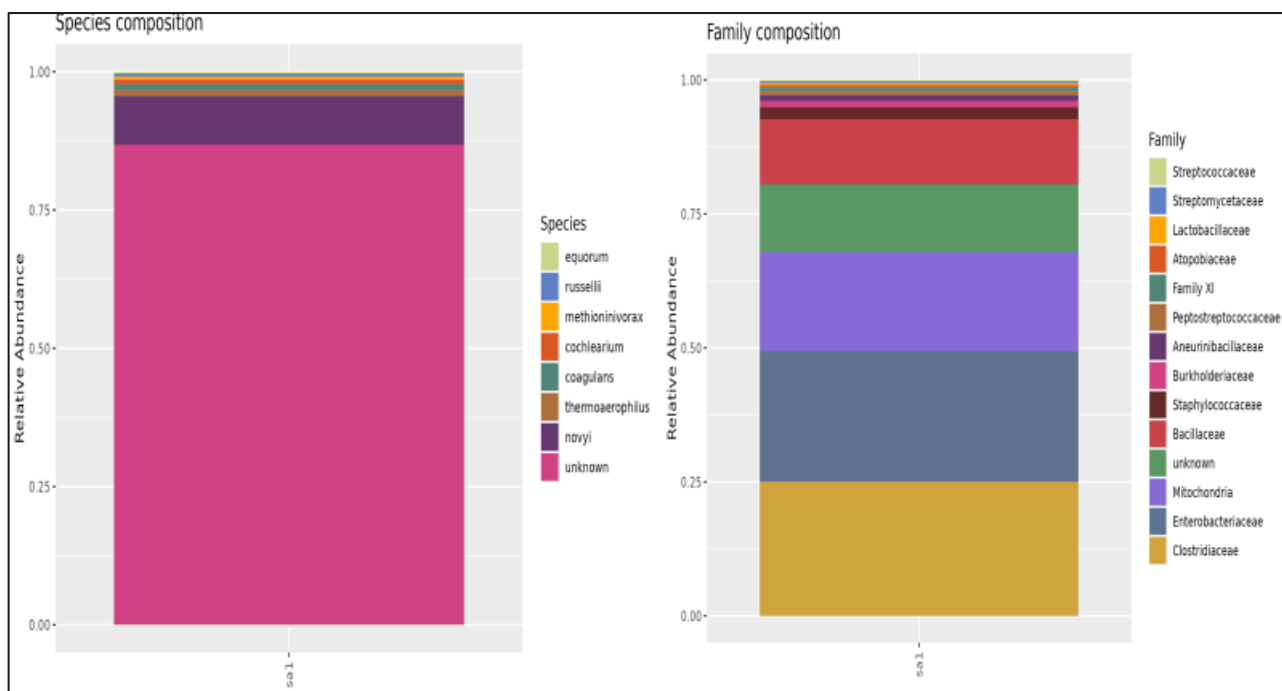
Using Agilent Bioanalyzer and fluorometric quantification, quality of the constructed amplicon libraries was assessed. Then with Illumina MiSeq platform, to avoid any contamination the libraries that pass the Quantification and quality control (QC) of next generation sequencing NGS were selected.

The raw data was then processed and analysed according to the bioinformatics pipeline in Fig 2. (Apical Scientific Sdn. Bhd., 2022). The strain families that were found are: *Enterobacteriaceae*; *Klebsiella*, *Aneurinibacillaceae*, *Atopobiaceae*, *Bacillaceae*, *Burkholderiaceae*, *Clostridiaceae*, *Lactobacillaceae*, *Peptostreptococcaceae*, *Staphylococcaceae*, *Streptococcaceae*, *Streptomyetaceae*.

The main genes of bacteria identified were *Aneurinibacillus*, *Bacillus*, *Clostridium sensu stricto* 15, 18, and 7, *Hathewaya*, *Klebsiella*, *Lactococcus*, *Ligilactobacillus*, *Paraclostridium*, *Peptoniphilus*, *Peptostreptococcus*, *Peptostreptococcus*, *Ralstonia*, *Staphylococcus*



**Fig. 2.** 16S/ ITS/ 18S Amplicon Sequencing Analysis Pipeline (Nova Lifetech, Singapore)



**Fig. 3.** Identified Species and families present inside the solid-state fermented material (SSF)

### Extraction from different Solid-State Enzymes

To start the extraction of protein, the selection of solvent/buffer, distilled water and sodium phosphate buffer was chosen. It was observed that sodium buffer used at pH 7 and 50mM gives a higher concentration of protein 8.42 mg/ml more than that with distilled water which was only 3.41 mg/ml. As also reported by (Lim and Kang, 2013), the highest protein was obtained with 50 mM sodium phosphate (pH 7).

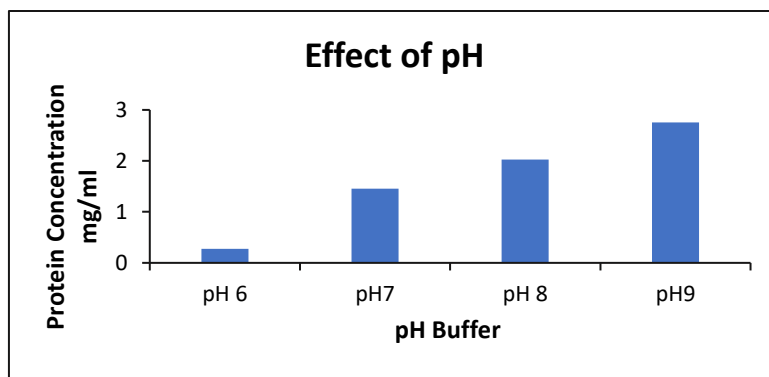
### Optimization of the extraction of enzymes from Solid-State Fermentation

#### *Study of operating conditions of extraction of enzymes from compost (OFAT Analysis)*

Using OFAT one factor at a time, the parameters that can affect the activity of enzymes were selected. Five significant parameters were tested in this design. The first, varying the pH of buffer (6,7,8,9) and second, temperature of the incubation (37, 45, 50°, 60, 65, and 70°C), third, the time of incubation (30 min, 1, 2, 3h)

#### *Effect of pH buffer*

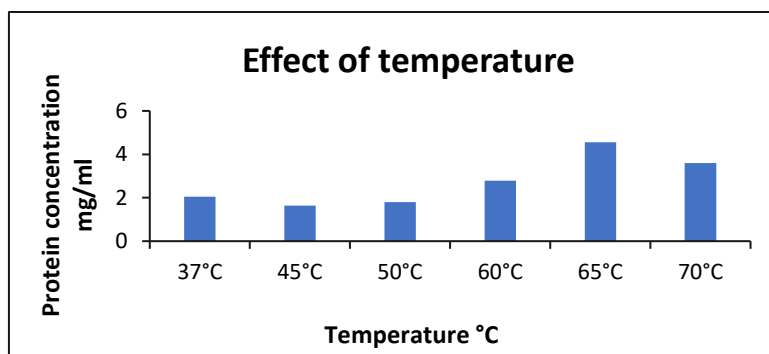
The second varied factor was pH phosphate buffer (6, 7, 8, 9 pH) and others are fixed to 10gram compost, 37°C incubation temperature, 1h incubation time, and speed of 200 rpm. The highest protein concentration is 2.75 mg/ml in pH 9. Like showed in other works, the average enzymes activity extracted from compost was initially at pH 8.5 (Wang and Liang , 2021).



**Fig .4.** Effect of pH buffer, highest pH was 9

### Effect of Incubation Temperature

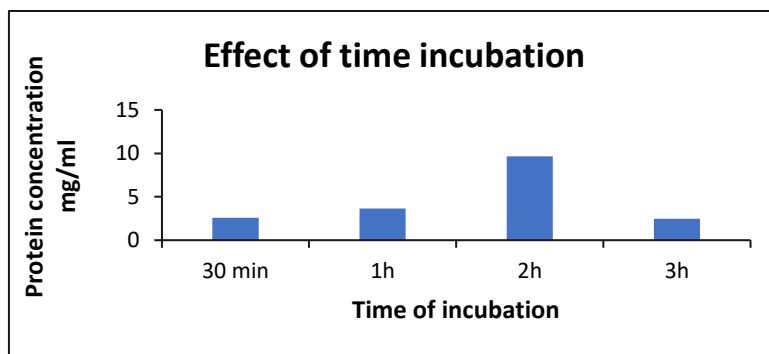
The variation factor was made in the temperature of incubation that was varied between (37, 45, 50, 60, 65, and 70°C). Other factors were fixed to 10 grams of sample, pH 9 buffer, and time 1h at 200 rpm rotation. The highest protein concentration was at 65°C with 4.55 mg/ml. As reported by (Wang and Liang, 2021), the temperature of 62°C of Group 6 reached the maximum.



**Fig.5.** Effect of temperature of incubation, the highest temperature was 65°C.

### Effect of Incubation Time

The incubation time is important and influences the concentration of protein. It was varied at different time range (30min, 1h, 2h, 3h), the other factors fixed to; 10 grams of sample, pH buffer to 9, and incubation temperature 65°C at 200 rpm rotation speed. The optimum time was at 2h the concentration of protein was 9.64 mg/ml and at 30 min was at 7.12 mg/ml as its illustrated in Fig 6 below. As reported by Lim and Kang (2013) the SMC of *P. sajor-caju* was maximal with 2-3 hr incubation at room temperature (Singh et al., 2003).



**Fig. 6.** Effect of incubation time, the optimum time was 2h.

#### *Optimization of enzymes using OFAT*

In order to create the experiment and analyze the results, Design Expert software (version 7.0.0) was used. Based on the Response Surface Method, the Face-Centered Central Composite Design (FCCCD) with  $\alpha = 1$  was selected. Six replicates at the center point level were used in a total of 20 runs. In Table 1 below, the experimental plan for maximizing the enzymes xylanase, amylase, protease, and cellulase from the compost is presented.

**Table 1.** The optimization of the conditions of the extraction process using FCCD.

| Run | pH | Temperature °C | Time h | Cellulase U/ml | Amylase U/ml | Protease U/ml | Xylanase U/ml |
|-----|----|----------------|--------|----------------|--------------|---------------|---------------|
| 1   | 8  | 60             | 1      | 6.38           | 8.22         | 6.33          | 119.35        |
| 2   | 10 | 60             | 1      | 5.82           | 7.85         | 8.63          | 122.32        |
| 3   | 8  | 70             | 1      | 5.44           | 6.61         | 4.23          | 113.23        |
| 4   | 10 | 70             | 1      | 6.55           | 7.55         | 4.61          | 105.03        |
| 5   | 8  | 60             | 3      | 6.58           | 7.91         | 4.81          | 79.03         |
| 6   | 10 | 60             | 3      | 5.07           | 7.85         | 5.57          | 95.86         |
| 7   | 8  | 70             | 3      | 4.88           | 6.26         | 6.14          | 94.11         |
| 8   | 10 | 70             | 3      | 5.18           | 8.04         | 5.53          | 98.16         |
| 9   | 8  | 65             | 2      | 6.84           | 9.28         | 7.15          | 140.04        |
| 10  | 10 | 65             | 2      | 7.14           | 9.58         | 7.24          | 144.02        |
| 11  | 9  | 60             | 2      | 9.75           | 9.77         | 9.33          | 125.2         |
| 12  | 9  | 70             | 2      | 9.28           | 8.88         | 7.54          | 122.96        |
| 13  | 9  | 65             | 1      | 9.16           | 10.17        | 9.34          | 149.13        |
| 14  | 9  | 65             | 3      | 10.01          | 9.96         | 8.64          | 125.12        |
| 15  | 9  | 65             | 2      | 10.15          | 10.21        | 9.41          | 165.82        |
| 16  | 9  | 65             | 2      | 11.59          | 10.12        | 11.04         | 174.13        |
| 17  | 9  | 65             | 2      | 10.83          | 10.04        | 10.49         | 156.52        |
| 18  | 9  | 65             | 2      | 10.22          | 10.23        | 10.85         | 154.56        |
| 19  | 9  | 65             | 2      | 11.04          | 10.69        | 10.61         | 152.43        |
| 20  | 9  | 65             | 2      | 10.71          | 11.01        | 10.79         | 153.61        |

The statistical data were examined using the results of the Analysis of Variance (ANOVA). The statistical significance for each of the tested coefficients was determined using the p-Value from the ANOVA table. The  $\alpha$ -level was set at 0.05 intervals, so p-values below this value were considered significant. In addition, DOE software offers R<sup>2</sup> results, which are crucial for assessing the regression model's good of fit. R<sup>2</sup> is crucial for determining how much of the variation in the observed response values can be accounted for by the experimental factors and their interactions. According to Samoo *et al.* (2019), R-squared values closer to 1 generally indicate better experimental models. The ANOVA results are shown in Tables 3 and 4 below.

Under the same optimization conditions as this enzymes study, many similarities were demonstrated by other studies. According to Panesar *et al.* (2012), the alkaline pH was advantageous to produce cellulose because there was less conversion of glucose into gluconic acid. This results in higher cellulose production. Earlier studies (Rajkumar *et al.*, 2011; Qamar *et al.*, 2020) used *B. megaterium* to create an alkaline protease that was most active at pH 9 to 10 and 60 °C. These studies also discovered that the protease enzyme from alkalophilic bacterial sources has an optimal pH range in the range of 9 to 9.5. The highest activity for xylanase was 189.6 mU at 72 hours and pH 8.5 (Wang *et al.*, 2007).

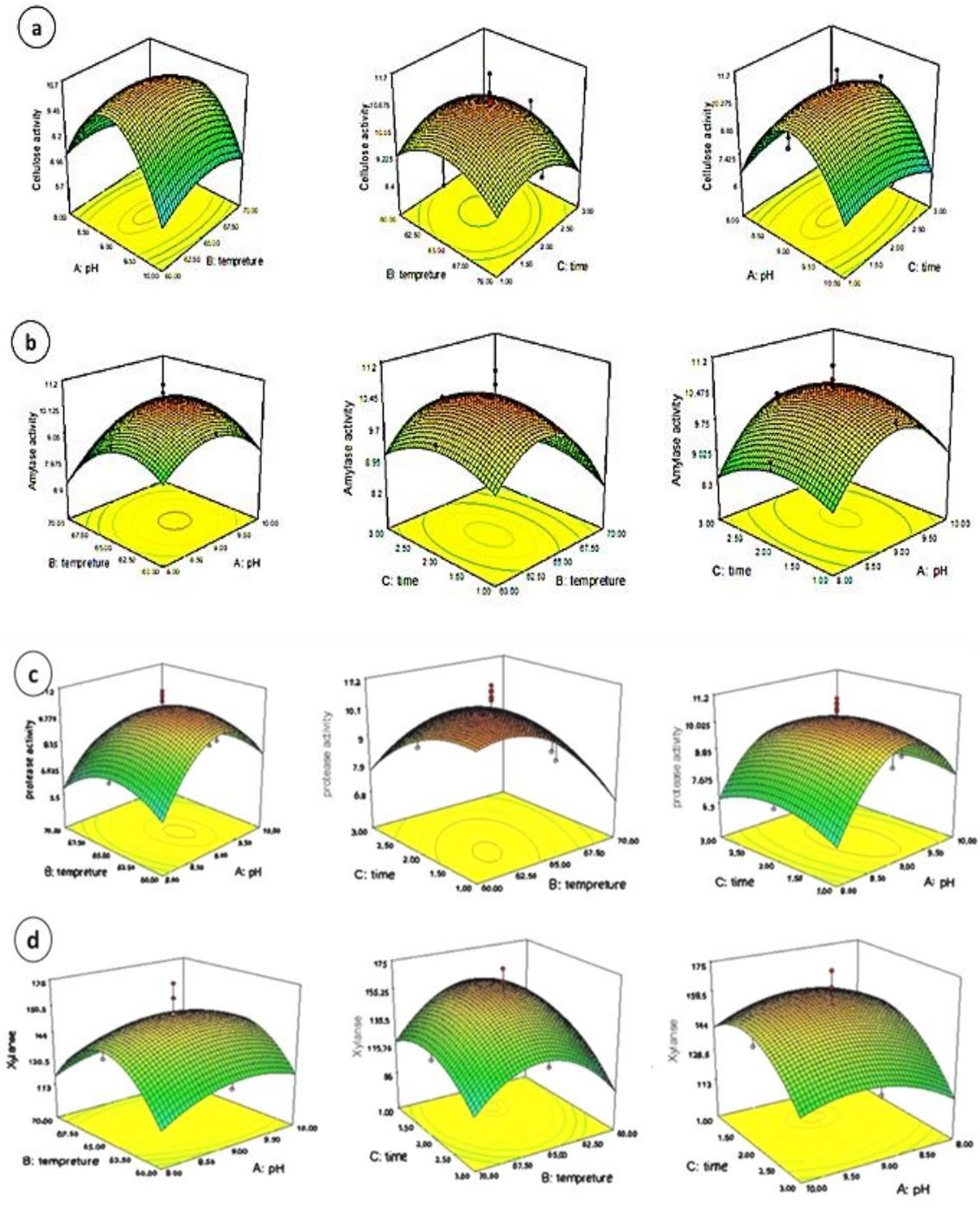
**Table 2.** The ANOVA results of RSM

|               | Cellulase |         | Amylase |         | Protease |         | Xylanase |         |
|---------------|-----------|---------|---------|---------|----------|---------|----------|---------|
|               | F-Value   | p-value | F-Value | p-value | Value    | Prob> F | F-Value  | p-Value |
| Model         | 37.36     | <0.0001 | 42.56   | <0.0001 | 181.33   | <0.0001 | 25.93    | <0.0001 |
| A-pH          | 0.043     | 0.839   | 7.28    | 0.0224  | 0.27     | 0.6157  | 2.07     | 0.1803  |
| B-temperature | 1.73      | 0.2179  | 19.7    | 0.0013  | 214.56   | <0.0001 | 10.66    | 0.0085  |
| C-time        | 0.89      | 0.3673  | 0.16    | 0.7005  | 740.36   | <0.0001 | 1.46     | 0.2546  |
| AB            | 5.08      | 0.0479  | 13.46   | 0.0043  | 145.97   | <0.0001 | 3.29     | 0.0997  |
| AC            | 1.3       | 0.2809  | 1.79    | 0.21    | 19.57    | 0.0013  | 1.95     | 0.1931  |
| BC            | 0.8       | 0.3924  | 0.27    | 0.6116  | 93.48    | <0.0001 | 16.7     | 0.0022  |
| A^2           | 103.23    | <0.0001 | 38.89   | <0.0001 | 263.08   | <0.0001 | 43.37    | <0.0001 |
| B^2           | 6.2       | 0.032   | 46.38   | <0.0001 | 1.29     | 0.2827  | 11.41    | 0.007   |
| C^2           | 5.18      | 0.046   | 7.65    | 0.0199  | 0.52     | 0.4879  | 3.77     | 0.0807  |
| Residual      |           |         |         |         |          |         |          |         |

**Table 3:** Analysis of Variance (ANOVA) for enzymes activities in RSM

| Enzymes        | Cellulase | Amylase | Protease | Xylanase |
|----------------|-----------|---------|----------|----------|
| R-Squared      | 0.9711    | 0.9746  | 0.9589   | 0.9605   |
| Adj R-Squared  | 0.9451    | 0.9517  | 0.9219   | 0.9249   |
| Pred R-Squared | 0.8862    | 0.9291  | 0.8588   | 0.9141   |
| Adeq Precision | 14.651    | 19.097  | 13.552   | 15.214   |

Finally, the DOE software enabled to generate of the contour, 3D surface plots to observe the responses with graphical variation as shown in Figure 7 below. In this case, it was the interaction between pH, time, and temperature. The plots illustrated four different enzymes, cellulase, amylase, protease, and xylanase.



**Fig. 7.** Plots in Response to the Interaction between Samples: pH (A) Temperature (B) Time(C) for (a) cellulase, (b) amylase, (c) protease, and (d) xylanase enzymes

#### *Validation of enzymes extraction*

To confirm the model's accuracy in predicting the response variations, model validation was conducted. The criterion for the numerical solution was assessed by setting the goal at maximum for the cellulase, amylase, protease, and xylanase activity, while the three other factors were set as in range as shown in Table 4. Triplicates

from random runs were selected from this experiment to validate the results. The percentage of errors were calculated by comparing the response obtain between predicted and experimental values.

**Tables 4.** The validation of the optimization of enzymes: protease, amylase, xylanase, and cellulase

| Number | pH   | Temperature °C | Time h | Cellulase activity<br>predicted | Cellulose activity<br>actual | Error % |
|--------|------|----------------|--------|---------------------------------|------------------------------|---------|
| 1      | 8.97 | 64.54          | 1      | 11.42                           | 11.56                        | 1.21    |
| 2      | 8.97 | 64.47          | 1      | 11.42                           | 11.47                        | 0.43    |
| 3      | 9.01 | 64.65          | 1      | 11.42                           | 11.75                        | 2.78    |
| Number | pH   | Temperature °C | Time h | Amylase activity                | Amylase actual               | Error % |
| 1      | 8.98 | 65.23          | 1      | 10.71                           | 11.043                       | 2.98    |
| 2      | 8.98 | 65.18          | 1      | 10.72                           | 10.923                       | 1.83    |
| 3      | 8.99 | 65.11          | 1      | 10.73                           | 11.01                        | 2.54    |
| Number | pH   | Temperature °C | Time h | Protease activity<br>predicted  | Protease activity<br>actual  | Error % |
| 4      | 9    | 64.96          | 1      | 11.44                           | 11.76                        | 2.72    |
| 5      | 8.94 | 65.25          | 1      | 11.52                           | 11.71                        | 1.62    |
| 6      | 8.94 | 65.51          | 1      | 11.62                           | 11.65                        | 0.25    |
| Number | pH   | Temperature °C | Time h | Xylanase activity               | Xylanase actual              | Error % |
| 6      | 8.94 | 65.51          | 1      | 174.48                          | 179.01                       | 2.53    |
| 7      | 8.96 | 64.76          | 1      | 174.27                          | 175.21                       | 0.53    |
| 8      | 8.95 | 65.79          | 1      | 173.93                          | 174.21                       | 0.16    |

It is clear from the results that the predicated model's value was marginally under that of the experiment. This slight difference in values could be due to the experimental procedure, where tests were conducted in an area that was on the edge of the technological domain that FCCCD was interested in. As a result, the FCCCD model was accurate and trustworthy in predicting the enzyme activity.

## CONCLUSION

The main genes of bacteria identified were *Aneurinibacillus*, *Bacillus*, *Clostridium sensu stricto* 15, 18, and 7, *Hathewaya*, *Klebsiella*, *Lactococcus*, *Ligilactobacillus*, *Paraclostridium*, *Peptoniphilus*, *Peptostreptococcus*, *Peptostreptococcus*, *Ralstonia*, *Staphylococcus*. Microbial enzymes are a productive source of catalysis that can be applied in a variety of industries. Knowing the ideal extraction conditions from solid-state fermented material, which included using sodium phosphate buffer at pH 9, a ratio of 10 grams of SSF, temperature incubation at 65°C for 2 hours, and speed of 250 rpm. Protease 11.04 U/ml, cellulase 11.59 U/ml, xylanase 174.13 U/ml, and amylase 11.01 U/ml were the enzyme activities that were measured for each. Using aqueous enzymatic extraction, the immobilization technique will be used to further study, stabilize, and optimize this enzyme for high-yield palm oil immobilization technique will be used to further study, stabilize, and optimize this enzyme for high-yield palm oil extraction using aqueous enzymatic extraction. The price of the commonly used industrial enzymes will be reduced thanks to this source of enzymes. The extraction process is simple and safe for the environment, and they can be produced in large quantities.

## ACKNOWLEDGMENTS

The authors are grateful to the Department of Biotechnology Engineering of the International Islamic University Malaysia (IIUM) for providing the laboratory facilities.

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