

## From fruit waste to enzymes

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### Abstract

Domestic and municipal solid wastes pose environmental concerns and health risks, suggesting the importance of reduced waste disposal. Food waste accounts for the highest percentage of solid waste in Brunei. An effective strategy is to convert food waste into beneficial products such as enzymes and biofertilizers. This study reports utilization of inedible fruit peels as substrates for enzyme production. A fermentation medium prepared by adding fruit peels, brown sugar and water was allowed to ferment over a period of time. Total carbohydrate content was found to be highest in the fermented orange sample ( $37.87 \pm 4.7$  mg/mL) followed by pineapple and banana samples,  $11.98 \pm 1.45$  mg/mL and  $10.60 \pm 0.45$  mg/mL, respectively. Pineapple sample showed the highest concentration of reducing sugar ( $11.93$  mg/mL at week 2 and  $3.31$  mg/mL after 3 months). Enzyme assay showed that citrus fruits like oranges, yield high activities of enzymes like protease ( $0.129$  U/mL),  $\alpha$ -amylase ( $7.261 \pm 0.83$  U/mL) and cellulase ( $0.514 \pm 0.03$  U/mL). This fermentation product of kitchen waste is economical as it uses cheap raw materials, free of chemical additives and hence eco-friendly.

*Index Terms:* food waste management, fruit enzymes, biocleaners, biofertilizer

### 1. Introduction

Increase in urbanization has led to a rapid increase in Municipal Solid Waste (MSW). Landfills are a common destination for MSW, leading to environmental and health effects.<sup>1</sup> According to the Department of Environment, Parks and Recreation, Brunei, approximately 400-500 tonnes of waste goes to Sungai Paku landfill in Tutong District each day.<sup>2</sup> Food waste accounts for the highest percentage of MSW in Brunei followed by paper and plastic. Solid waste reduction can effectively begin on a domestic scale by managing household and kitchen waste. An economic and eco-friendly strategy is converting food waste into beneficial products. One example is increasing soil fertility by microbial activity on food waste added to soil.<sup>3</sup> Secondly, enzymes can be produced by fermentation of food waste with applications in domestic and waste water treatment.<sup>4</sup> Thirdly,

anaerobic digestion is well-known for treating MSW for biogas production<sup>5</sup> consisting of methane (55-65%), carbon dioxide (30-35%) and other trace gases such as hydrogen and nitrogen.<sup>6</sup>

Recently, enzymes production using domestic waste has garnered interest among communities. Enzymes are biological catalysts that facilitate biochemical reactions. Amylases, proteases, lipases, pectinases are widely used in domestic and industrial applications.<sup>7</sup> Crude solution of these hydrolytic enzymes, generally referred to as bioenzymes (or garbage enzymes) can be easily produced in households using vegetable and fruit wastes. The pioneering study in this field is attributed to Rosukon Poompanvong from Thailand, who is known to have developed methodology for production of *garbage enzymes*.<sup>8</sup> United Nations Food and Agriculture Organization recognized her contribution to

organic farming by using fermented organic waste in 2003. In solid-state fermentation, non-soluble solids are used as substrate and source of nutrients for microorganisms.<sup>9,10</sup> Such solid substrates includes fruit peel, agricultural crops, agro-industrial residues and organic waste.<sup>11</sup> In submerged fermentation, a liquid medium is used for homogeneous mixing of nutrients. According to standard protocol, to ten parts of water, one part of brown sugar or molasses and three parts of food waste are added and mixed in an air-tight container. It is then left to ferment for about three months in a cool, dark place.<sup>12</sup> This fermented solution is essentially the crude decoction of hydrolytic enzymes. Among the several uses of bioenzymes recommended by Thirumurugun, some include: i. dish and laundry cleaning, ii. stain and odour removal, iii. fruits and vegetables cleansing, iv. clearing sink and drain blockages (using blended pulp or enzyme sludge), v. natural insect repellent vi. plant fertilizer.<sup>13</sup> Purified enzymes have several applications in industry and agriculture.<sup>14-16</sup> In this study, fruit waste is used as a substrate of fermentation to produce beneficial enzymes. The relationship between total carbohydrate content, reducing sugar, protein content and enzyme production is recorded for use in domestic applications.

## 2. Materials and Methods

### 2.1 Sample collection and fermentation

Household fruit waste such as fruit stalks and peels were collected and fermented in suitable bottles for enzyme production. In this study, peels of orange, pineapple, banana and mixed fruit (pomelo, watermelon and melon) were used as substrates. Mixed fruit sample containing a random selection of fruit peels are observed for a comparative analysis of enzyme concentration with respect to individual selected fruits. In case of comparable enzyme activities in both cases, it would be convenient, in a domestic household, to ferment different fruit peels together instead of segregating them. Two additional fermentation media were prepared for the mixed fruits; one with the addition of baker's yeast (*Saccharomyces cerevisiae*) and another with yoghurt (Lactic Acid Bacteria). At the end of the

incubation period, the respective solutions were analysed for various parameters. All experiments were conducted in duplicate, and the results are shown as the mean  $\pm$  1 standard deviation.

### 2.2 Estimation of moisture content

Fruit peels were sliced, placed on a glass petri dish, weighed to record the initial mass and oven-dried at 80 °C for 24 hours. After 24 hours, the dry fruit peels were weighed. The dried peels were left in the oven for another 1-2 hours and weighed again to ensure a constant weight is reached. Percentage of moisture in the peels was calculated. Moisture content (%) is defined as the quantity of water content found in a solid sample. The formula to calculate moisture content is shown in *Equation 1*:<sup>17</sup>

$$\text{Moisture content (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad (1)$$

### 2.3 Measurement of pH

Aliquots of respective fermentation solutions were taken every two weeks up to three months for measurement of pH. The pH of solutions was measured using a lab pH meter (Thermo Scientific, Orion Star A211). During fermentation, the solutions turn acidic and hence a drop in pH is expected.

### 2.4 Estimation of total carbohydrate content

Total carbohydrate was tested using a modified protocol.<sup>18</sup> An aliquot of fermented sample solution was mixed with 1 mL of 5 % aqueous phenol, followed by the addition of 5 mL concentrated sulphuric acid. The mixture was allowed to stand for 10 minutes, then mixed using a vortex mixer for 30 sec and incubated in water bath at 25 °C for 20 min. Absorbance was measured at 490 nm and the concentration of total carbohydrate was calculated.

### 2.5 Estimation of reducing sugar using DNS method

Reducing sugar content was analysed using the dinitrosalicylic (DNS) colorimetric method described by Miller.<sup>19</sup> 3 mL of fermented sample was mixed with 3 mL of DNS reagent and heated

in a boiling water bath for 5 min. The tubes were then cooled under running tap water, before measuring the absorbance of each sample at 575 nm. Sugar content was calculated using glucose standard.

### 2.6 Estimation of total protein content

Protein estimation was done by using the method described by Bradford.<sup>20</sup> Coomassie dye solution was prepared by mixing 10 mg of Coomassie Brilliant Blue G-250, 10 mL of 88 % phosphoric acid, 4.7 mL of absolute alcohol and diluting the mixture with 100 mL of distilled water. 1 mL of fermented sample solution was mixed with 3 mL of Coomassie dye solution. The mixture was incubated in dark for 5 min and absorbance was measured at 595 nm. Protein content was calculated using the standard curve of bovine serum albumin (BSA).

### 2.7 Qualitative enzyme assay

Protease was screened using casein agar plate. One litre of the media was composed of 28 g skim milk powder, 5 g casein enzymic hydrolysate, 2.5 g yeast extract, 1 g dextrose, 15 g agar powder and a final pH of  $7.0 \pm 0.2$ . The mixture was autoclaved at 121 °C for 15 minutes and poured into petri dishes. Fermented solution containing a mixture of crude enzymes was streaked on the plate and incubated for 2 to 3 days at 37 °C. Zone of clearance on skim milk agar indicates hydrolysis of casein by proteases.

Cellulase was screened using cellulose agar plate. Nutrient agar (NA) was supplemented with 0.2 % Carboxymethylcellulose sodium salt (Sigma-Aldrich 21902), sterilized at 121 °C for 15 minutes and poured into agar plates. The fermented solution was then streaked onto the solidified agar plate for 2-3 days at 25 °C. The plates were flooded with 0.3 % Congo-red solution for 10 minutes followed by de-staining with 1 M Sodium Chloride solution. The presence of cellulase is indicated by a translucent zone around the colony.<sup>21</sup>

### 2.8 Quantitative enzyme assay

Protease assay was carried out using L-tyrosine as standard.<sup>22</sup> 2.5 mL of casein-potassium

phosphate buffer (0.65 % casein dissolved in 50 mM potassium phosphate buffer, pH 7.5) was mixed with 0.5 mL enzyme solution and incubated for 10 minutes at 37 °C. To arrest the reaction, 5 mL of 10 % Trichloroacetic acid was added to the mixture and incubated at room temperature for 30 minutes. The reaction mixture was filtered to remove precipitate and 2 mL of the filtrate was mixed with 5 mL of 500 mM sodium carbonate buffer. Absorbance was measured at 280 nm after incubation for 30 minutes. One unit (U) per mL of enzyme is defined as the amount of enzyme required to convert one micromole of substrate into product per minute under given temperature and pH.<sup>23</sup>

For  $\alpha$ -amylase, the enzyme solution (0.5 mL) was incubated at 25 °C for 3 minutes, then 0.5 mL of starch-sodium phosphate buffer (1 % starch, w/v dissolved in 0.02 M sodium phosphate buffer, pH 6.9) was added. The reaction mixture was incubated at room temperature for 5 minutes before 1 mL DNS reagent was added to stop the reaction. The terminated reaction mixture was heated in a boiling water bath for 5 minutes and left to cool. This mixture was then diluted with distilled water to 10 mL. Absorbance was measured at 540 nm and the concentration of enzyme was calculated using a glucose standard.

Cellulase activity was measured using DNS method.<sup>24</sup> The substrate, Carboxymethylcellulose (CMC), was mixed with sodium acetate buffer (1 % CMC, w/v 0.1 M sodium acetate buffer, pH 4.8). 0.5 mL of enzyme solution was added to the CMC-buffer mixture and incubated for 1 hour at 40 °C. The reaction was arrested by the addition of 1 mL DNS reagent. Absorbance was measured at OD<sub>540</sub> and values were calculated using glucose standard.

## 3. Results and Discussion

### 3.1 Effect of moisture content

The significance of measuring moisture content of food is to determine its freshness, nutritive value and quality. Food possessing high moisture decay rapidly as high moisture provides optimal conditions for mold and microbial growth.<sup>25</sup>

Estimated moisture range in selected fruit peels was approximately 70-95% (as shown in *Table I*). The higher the water content present in fruit peel, the greater the rate of deterioration in a given time.

**Table I:** Percentage moisture content of selected fruit peel samples.

| Code | Sample     | Differences in weight (g) |       | Moisture content (%) |
|------|------------|---------------------------|-------|----------------------|
|      |            | Initial                   | Final |                      |
| A    | Orange     | 19.48                     | 5.32  | 72.69                |
| B    | Pineapple  | 36.09                     | 6.97  | 80.69                |
| C    | Banana     | 9.22                      | 1.59  | 82.75                |
| D    | Pomelo     | 19.32                     | 4.13  | 78.62                |
| E    | Watermelon | 34.15                     | 4.13  | 87.91                |
| F    | Melon      | 35.51                     | 3.28  | 90.76                |

### 3.2 Effect of pH

Experimental results in *Figure 1* show the pH value of the solutions gradually decrease over time, to a range of pH 3.0-4.0. The drop in pH of the fermentation medium is due to the conversion of sugars present in the mixtures to acids, ethanol and carbon dioxide. Hence, reduction in pH is an indicator of completion of fermentation and corresponds to higher levels of volatile acids and enzymes.<sup>25</sup>

### 3.3 Total carbohydrate content

*Figure 2* summarizes the change in total carbohydrates content after fermentation. The fermented orange sample contained of  $37.87 \pm 4.7$  mg/mL of total carbohydrates which is the highest among the fruit peel samples tested. The estimated carbohydrate content for the pineapple and banana samples were  $11.98 \pm 1.45$  mg/mL and  $10.60 \pm 0.45$  mg/mL, respectively. The addition of Baker's yeast ( $4.30 \pm 0.48$  mg/mL) or yoghurt ( $8.80 \pm 1.48$  mg/mL) into the mixed fruits sample causes a rapid reduction of total carbohydrate content compared to the mixed fruits sample alone ( $13.10 \pm 0.78$  mg/mL). During the first two weeks of fermentation, the initial carbohydrate concentrations for all fruit samples were higher. Existing microbes in various fruit peels grow aerobically in the culture solution for the conversion of these

carbohydrates into soluble sugars and other metabolites. Carbohydrates present in the fermented solution may be a mixture of soluble (glucose, fructose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) broken down by a consortium of microorganisms present in the fruit peel. The presence of carbohydrates at the end of the fermentation period are remains of any unfermented sugars.

Orange peels, which were the only citrus fruit peels used in this study, contain a terpenoid chemical compound called D-limonene, which is an inhibitor of yeast fermentation. High D-limonene content in citrus fruit waste can inhibit microbial growth.<sup>26</sup> Limonene is the major constituent of citrus fruits' essential oil that are reported to have anti-bacterial and anti-inflammatory properties.<sup>27</sup> This further explains the high concentration of carbohydrates measured in the orange peel sample. Another possible reason for the high carbohydrate concentration is the presence of glucose, fructose, galactose, arabinose, xylose and galacturonic acid in the fermentation medium which could have been produced during enzymatic hydrolysis of orange peels.<sup>28</sup> Fermented solutions containing yeast have lower carbohydrate concentration as yeast contains enzymes that catalyse the breakdown of glucose to ethanol and carbon dioxide. The microbial diversity in the fruit peels accompanied with the addition of yeast further contributes to the utilization of carbohydrates for growth and production of useful metabolites.

### 3.4 Reducing sugar content

*Figure 3* shows the reducing sugar content at two and three months of fermentation. The highest concentration of reducing sugar was 11.93 mg/mL (at week 2) and 3.31 mg/mL (at 3 months) for the pineapple sample. In contrast, banana sample had the lowest concentration among other samples (without addition of yeast or yoghurt), with 5.41 mg/mL and 2.17 mg/mL of reducing sugar at week two and at three months, respectively. It is estimated that fermentation caused an overall 60-80% reduction in reducing sugar content during the ten-week period.

Reducing sugars have reactive aldehyde or ketone groups. Glucose, fructose and sucrose are known to be the main carbohydrates present in fruit peels. Sucrose is a non-reducing sugar. The percentage of reducing sugar is about 30 % of total carbohydrate content for most fruit peels. However, orange peels only contain 6 % reducing sugar of total carbohydrates. During enzymatic hydrolysis of orange peels, the desired

reaction is the release of monosaccharides from cellulose and hemicellulose.<sup>25</sup> The accumulation of limonene disrupts microbial growth and hence the release of sugar monomers into the fermented solution could be reduced. It was reported that inhibition occurs even at very low limonene concentration resulting in complete failure of fermentation at higher concentrations.<sup>25</sup>

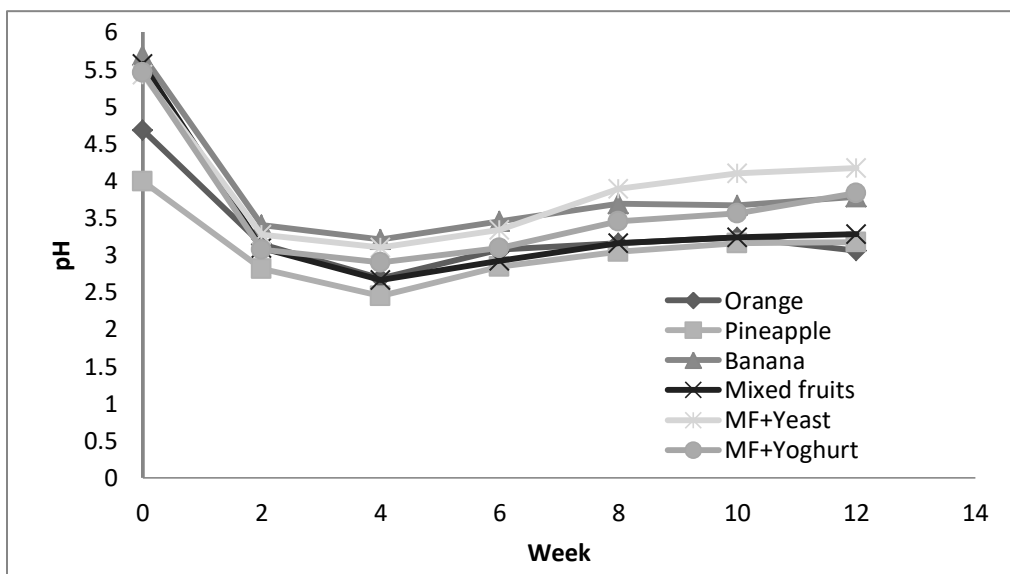


Figure 1. pH recorded for different fermented solutions from week 0 to 12.

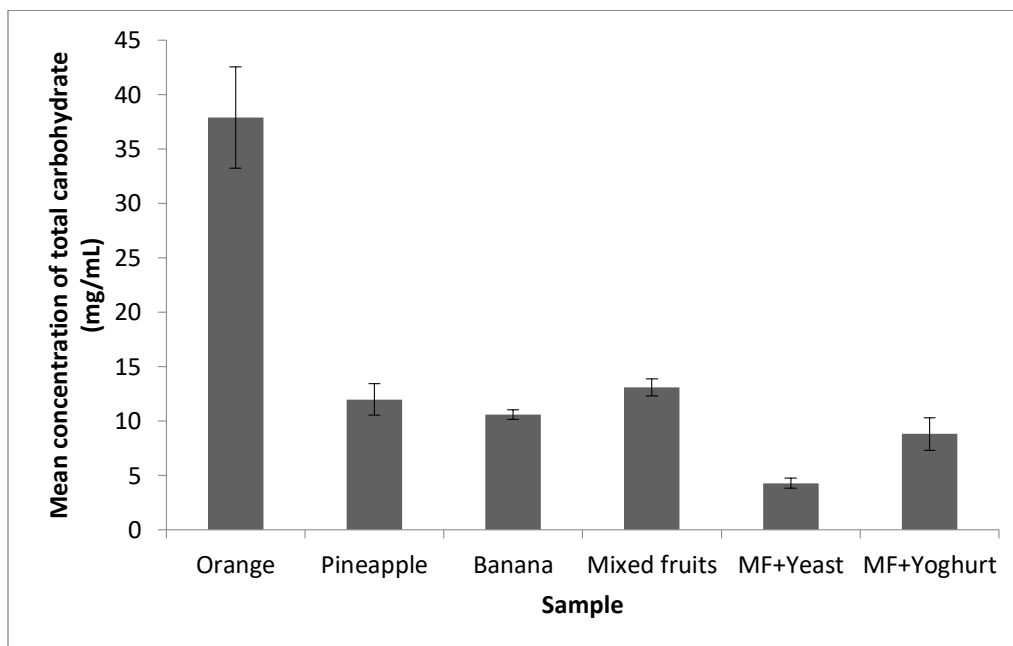
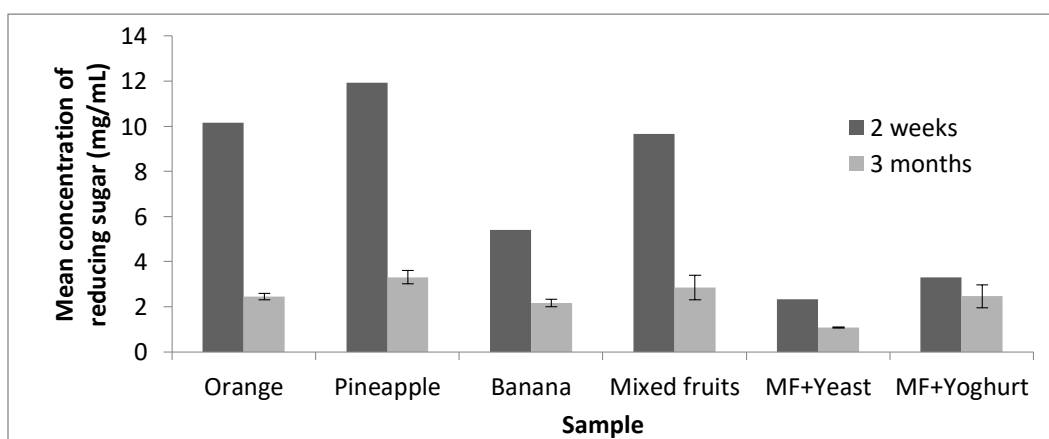


Figure 2. Total carbohydrates concentration at OD<sub>490</sub> after fruit peels were fermented for three months.

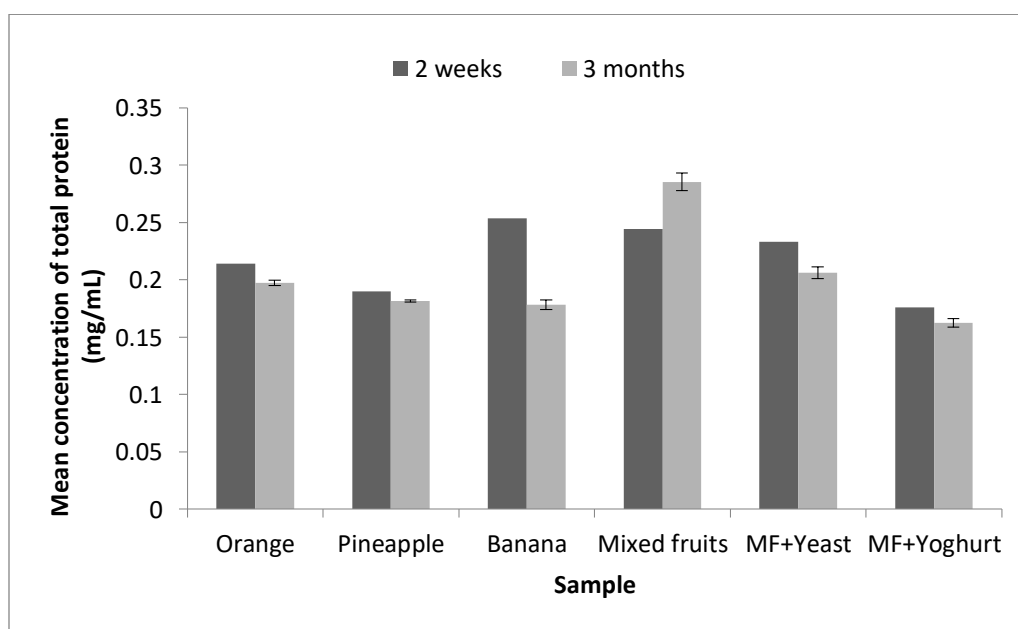
### 3.5 Total protein content

From **Figure 4**, the concentration of protein in all fruit waste samples were observed to be low, ranging from 0.176 mg/mL for the mixed fruit sample supplemented with yoghurt, to 0.253 mg/mL for banana peel (two week fermentation medium). After three months of fermentation, the amount of protein is the lowest in the growth medium containing mixed fruits with yoghurt (0.163 mg/mL) and the highest in control mixed fruits (0.285 mg/mL). There is no major decrement in protein concentration from the initial fermentation medium to that after a period of three months. An exception is the mixed fruits

fermentation medium control which showed increased protein content. This may be due to different amounts of protein content from a mixture of fruit wastes being released into the fermentation medium which remain underutilized by microbes. Fruit peels with high protein concentration could serve as a possible alternative substrate for cultivation of fungi.<sup>28</sup> Protein is an essential nutrient required for microbial growth and production of enzymes, which might be the reason for the decrease in protein content in the samples.<sup>28,29</sup>



**Figure 3.** Reducing sugar content at OD<sub>575</sub> for fruit peels fermented for two weeks and three months.



**Figure 4.** Total protein content at OD<sub>595</sub> for various fruit peels fermented for two weeks and three months.

### 3.6 Screening of protease

**Figure 5** shows protease activity on skimmed milk agar after incubation at 37 °C. The release of protease was observed on each agar plate after three days of incubation. From the zone of clearance observed, all samples contained protease enzymes. Fermented orange solution had the highest protease activity because white-coloured skimmed milk agar turned translucent which signifies complete utilization of the protein by protease-producing microbes. The addition of yoghurt into the fermentation medium introduced probiotic bacteria which, together with the existing microbes in the fruit peels, lead to greater secretion of protease enzymes. The protease activity of fermented samples is shown in **Figure 6**. Protease concentration was highest in the orange peels sample (0.129 U/mL) and lowest in the mixed fruit with yeast sample (0.041 U/mL). Other fermented samples had a range of protease produced in the solution with 0.046 U/mL for pineapple peels, 0.054 U/mL for banana, 0.081 U/mL for mixed fruits (control) and 0.073 U/mL for mixed fruits supplemented with yoghurt.



**Figure 5.** Qualitative screening of protease from the mixed fruits with yoghurt sample, after incubating at 37 °C on skimmed milk agar plate. Zone of clearance indicates protease activity.

The relationship between microbial growth and protease production has been previously reported<sup>22</sup> where protease secretion by

microorganism can be partially inducible in nature to produce extracellular proteases under most growth conditions. However, proteolytic activity tends to be relatively low in yeast. A possible reason for low proteolytic activity in mixed fruits with yeast could be due to ethanol toxicity in yeast from the accumulation of ethanol as the by-product during fermentation, which causes the inactivation of yeast enzymes.

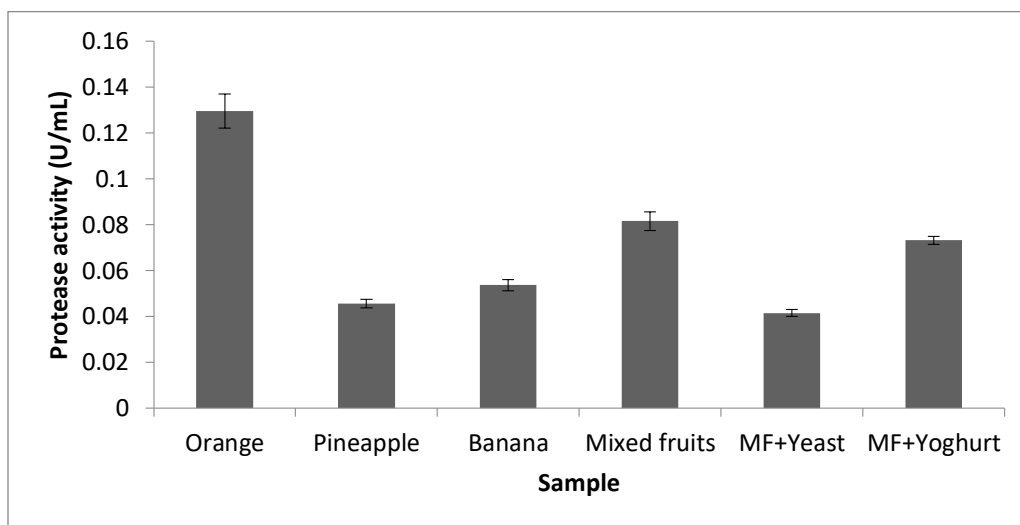
### 3.7 $\alpha$ -amylase content

The activity of  $\alpha$ -amylase was determined by analysing the hydrolysis of soluble starch spectrophotometrically. **Figure 7** shows the  $\alpha$ -amylase enzymatic activities of the six fermented solutions. As seen from the figure, fermented orange peel sample was found to contain the highest  $\alpha$ -amylase concentration with  $7.261 \pm 0.83$  U/mL which was approximately 3 to 4 times higher compared to the other samples. In contrast, the mixed fruit with yeast sample had the lowest  $\alpha$ -amylase activity ( $0.615 \pm 0.07$  U/mL).

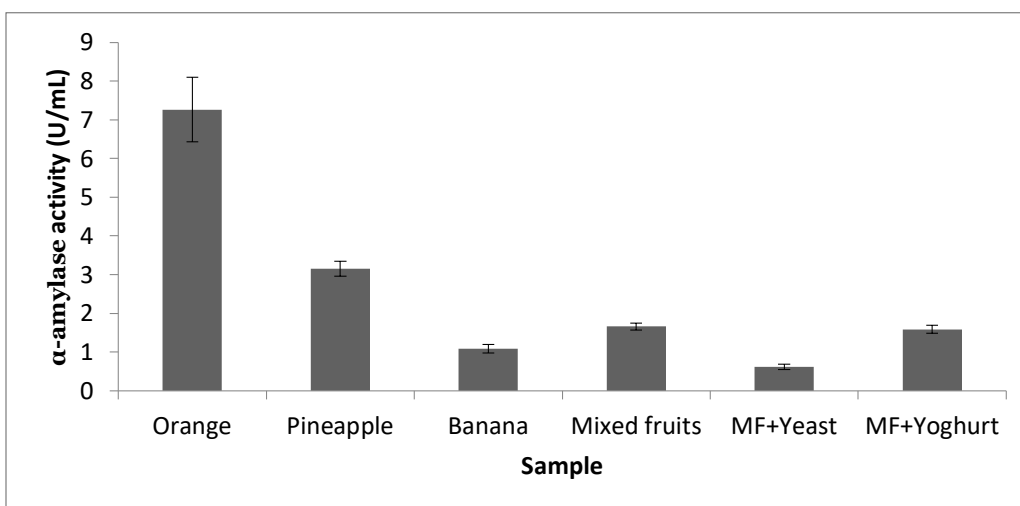
Orange peel offers a rich source of carbon for fermentation as it is rich in fermentable sugars (glucose, fructose and sucrose).<sup>23</sup> Hence it contains sufficient carbon for microbial growth and enzyme production. As reported in literature,<sup>30</sup> extra-cellular  $\alpha$ -amylase production is affected by pH. The pH of the fermented orange sample in the present study was pH 3 (**Figure 1**). Starch used as substrate in enzyme analysis, acts as an inexhaustible carbon source for the synthesis of  $\alpha$ -amylase in comparison with other carbon sources due to its enzyme stabilization ability.<sup>31,32</sup>

A low  $\alpha$ -amylase activity in yeast was observed in this study (**Figure 7**). Little research has been done on  $\alpha$ -amylase production in yeast, *Saccharomyces cerevisiae*. One study<sup>33</sup> reports that yeast exhibit no effect on  $\alpha$ -amylase production. Most studies reported that the production of fungal amylase is limited to a number of species, particularly *Aspergillus* and *Penicillium* species.<sup>34</sup> *Aspergillus oryzae* has received much attention in research interest due to its capability of producing high amounts of

valuable proteins and industrial enzymes such as  $\alpha$ -amylase.<sup>35</sup>



**Figure 6.** Protease activity for different fermented solution after three months of fermentation.



**Figure 7.**  $\alpha$ -amylase activity for different fruit peels samples after three months of fermentation.

### 3.8 Cellulase assay

Qualitative screening of cellulase activity was carried out on nutrient agar plate containing carboxymethylcellulose (CMC) as substrate. Screening for cellulase was done by staining with congo-red solution for clear observance of enzyme activity. A faint zone of clearance surrounding the culture growth indicates the presence of cellulase enzyme. The appearance of the halo zone around the bacterial or fungal colony provides strong evidence that microbes produced cellulase in order to degrade CMC

which is used as the sole source of carbon in the media. After screening for the cellulolytic potential of enzymes produced during fermentation of fruit wastes, spectrophotometric analysis was carried out to quantify cellulase activity. Aliquots of crude enzyme were obtained from each sample solution to analyse the cellulase concentration, as described in the protocol. According to **Figure 8**, the orange peels showed the highest cellulase activity, reaching  $0.514 \pm 0.03$  U/mL, after three months of fermentation. The three fruit peels namely



pineapple, banana and mixed fruits with yeast showed similar enzymatic activities of  $0.123 \pm 0.01$  U/mL,  $0.134 \pm 0.00$  U/mL and  $0.101 \pm 0.01$  U/mL, respectively. Enzyme activities depend on the release of acidic by-products in the fermentation media.<sup>36</sup> Another possible reason for the decrease in cellulase activity after three months of fermentation is the loss of moisture content during fermentation and inactivation by reduction in pH.<sup>37</sup> The optimum pH of cellulase is between pH 5 to 6.<sup>38</sup>

The fermented solution serves as a crude mixture of hydrolytic enzymes like amylases and proteases. The solution can be filtered and stored in bottles for domestic use. The enzyme filtrate can be used in domestic cleaning and stain removal applications. By optimising their use in domestic cleaning one can reduce the use of commercial chemical detergents. The residue obtained after filtering the solution can either be used for the next batch of fermentation or used as fertilizer.

#### 4. Conclusion

Domestic wastes, mainly food arise in large quantities due to growing demands of increasing population, thereby posing challenges of waste disposal. Hence it is imperative to devise

strategies for reduced waste by converting them into useful products like enzymes and fertilizers. Moreover, over the years, there is a roused public concern regarding the potential ecological disadvantages of using synthetic cleaning agents which release toxic chemicals that disrupt environmental pH balance. Fermentation of certain food wastes into enzymes can serve as useful cleaning products due to their specificity, high biodegradability and low toxicity.

The present investigation attempts such an economical and eco-friendly alternative to managing kitchen waste like inedible parts of fruits by converting them into beneficial enzymes. Orange peels are seen to be a promising source of enzymes among the fruit wastes investigated. The crude enzyme solutions were seen to be useful in domestic cleaning and stain removal. Further investigation and repeatability of the established protocols is needed to yield reproducible results. Further trials are needed to scale up processes by optimizing fermentation parameters and developing suitable formulations with enhanced appeal and shelf-life of enzyme based cleaning products.

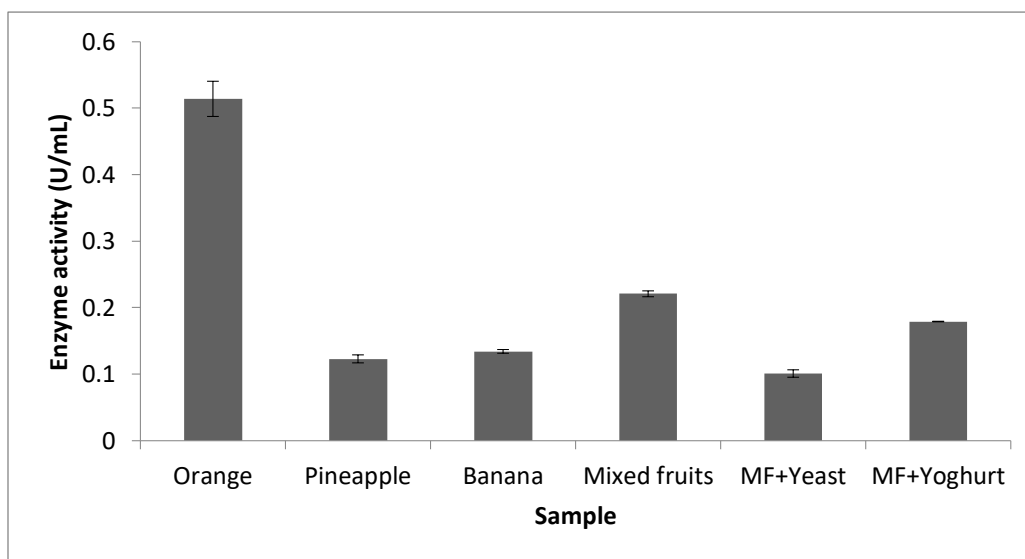


Figure 8. Cellulase activity for various fruit peels samples after three months of fermentation.

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