# Sugar Recovery from Bakery Leftovers through Enzymatic Hydrolysis: Effect of Process Conditions and Product Characterization

(Perolehan Kembali Gula daripada Lebihan Bakeri melalui Hidrolisis Berenzim: Kesan Keadaan Proses dan Pencirian Produk)

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ABSTRACT

*This study evaluates the process conditions, (enzyme concentration (120-1200 U/g substrate), temperature (30-60 °C), and pH (3-9)) of enzymatic hydrolysis (EH) for sugar recovery from leftover croissants (LC) and leftover doughnut (LD), and characterizing its residue and hydrolysate. The highest sugar yield recovered from LC was 574.21 ± 0.74 mg/g (840 U/g substrate, 49 °C and pH 3) and for LD was 460.53 ± 0.74 mg/g (1176 U/g substrate, 47 °C and pH 3). The highest fructose and glucose yield for LC and LD were 14.47±0.73 mg/g and 11.84±0.21 mg/g, and 13.26±0.63 mg/g and 10.34±0.11 mg/g, respectively. Morphology analysis (SEM) showed that the structure of LC and LD had changes in its starch granules that indicates hydrolysis process occurrence. The presence of monosaccharides and oligosaccharides were detected from FTIR. HMF was also detected from sugar degradation due to EH, (0.043 ± 0.0334 mg/g for LC) and (0.023 ± 0.0124 mg/g for LD).*

*Keywords: Bakery leftovers; enzyme hydrolysis; glucose; hydroxymethylfurfural; sugar*

ABSTRAK

*Kajian ini menilai keadaan proses (enzim (120-1200 U/g substrat), suhu (30-60 °C) dan pH (3-9)) hidrolisis berenzim untuk perolehan kembali gula daripada sisa kroisan dan sisa donat dan mencirikan hampas dan hidrolisatnya. Hasil gula tertinggi yang diperoleh daripada sisa kroisan adalah 574.21 ± 0.74 mg/g (840 U/g substrat, 49 °C dan pH 3) dan untuk LD adalah 460.53 ± 0.74 mg/g (1176 U/g substrat, 47 °C dan pH 3). Hasil fruktosa dan glukosa tertinggi untuk sisa kroisan dan sisa donat masing-masing adalah 14.47±0.73 mg/g dan 11.84±0.21 mg/g dan 13.26±0.63 mg/g dan 10.34±0.11 mg/g. Analisis morfologi (SEM) menunjukkan bahawa struktur sisa kroisan and sisa donat mempunyai perubahan pada butiran kanji yang menunjukkan berlakunya proses hidrolisis. Kehadiran monosakarida dan oligosakarida telah dikesan daripada FTIR. HMF juga telah dikesan daripada kemerosotan gula yang disebabkan oleh hidrolisis berenzim (0.043 ± 0.0334 mg/g untuk sisa kroisan) dan (0.023 ± 0.0124 mg/g untuk sisa donat).*

*Kata kunci: Glukosa; gula; hidroksimetilfurfural; hidrolisis berenzim; sisa roti*

## INTRODUCTION

Bakery waste that categorizes under cereal is among the most wasted food. As one of the staple food globally and the prime source of carbohydrate, it is a sad fact that the majority of bakery products wasted are still fresh, raw or minimally processed (Ventour 2008). According to Gustavsson et al. (2011), every year around the world, 1.3 billion tonnes of foods are wasted. Bakery waste is part of cereal food category, which this category accumulated approximately 30% of the total food waste in the world (FAO 2013). Bakery products' short shelf life and its sensitivity towards moisture in the environment have contributed to the increasing amount of bakery products being discarded, where it will be ended up in the landfill and/or turn into animal feed. Not only this situation may lead to landfill exhaustion, but the degradation of bakery wastes contributed to the increase of carbon dioxide ($CO_2$) and methane ($CH_4$) gas release, thus generating Green House Gas (GHG) emission. Other environmental impacts are water use and pollution of water, air and soil system (Moult et al. 2018).

However, to its richness in carbohydrate makes it a potential material to be converted into valuable products,

particularly sugar which can be used as food ingredients raw materials in producing biofuel. Sánchez and Cardona (2008) have reviewed fuel ethanol production derived from assorted sugar-rich materials. The authors concluded that the starchy materials available in the present time can cater to market demands. Furthermore, the world could no longer rely on sugar cane as the prime source for sugar; due to limited land space, large scale farming as well as climate change that might affect the sugar production from sugar cane.

Bakery wastes can be a potential source for recovering the sugar through enzymatic hydrolysis (Mohd Thani et al. 2019). According to Akpinar et al. (2009), enzymatic hydrolysis is generally preferable in the food industry due to its simplicity and it will not produce undesirable by-products. Amezcua-Allieri et al. (2017), Demirci et al. (2017), Hudečková et al. (2017), Mahfuzul Islam et al. (2018), and Wang et al. (2011) displayed the potential of enzymatic hydrolysis process to produce sugar from different materials. Regardless of the advantages of enzymatic hydrolysis, to maximize the yield from this process as well as to bring it to a larger production scale, several factors need to be scrutinized and optimized. Some of the factors that important to enzymatic hydrolysis process are enzyme concentrations, process temperature, and pH. The Response Surface Methodology (RSM) was selected for optimization analysis as RSM generated a mathematical model that accurately described the overall process with a significant ability for estimation (Wasswa et al. 2007). Some of the researchers that have employed RSM in their recent works are Fazil et al. (2018), Morais and Rodrigue (2018), Posridee et al. (2018), and Rasli and Sarbon (2018).

The aims of this research were to: study the effect of process conditions of enzymatic hydrolysis of LC and LD on the sugar yield, optimize the process conditions of enzymatic hydrolysis in order to obtain maximum sugar yield from LC and LD, and to analyze the morphology of the residue and it products after EH at optimal conditions in terms of its chemical composition, and quantification of its by-product; hydroxymethylfurfural (HMF) content).

## MATERIALS AND METHODS

### SAMPLE PREPARATION

For this study, croissants and doughnuts were chosen due to its famousness among consumers, whereby in Malaysia, majority of bakeries sell croissants and doughnuts in their outlets. Croissants and doughnuts were taken from a local bakery shop in Selangor, Malaysia. The samples were plain croissants and doughnuts without coating or filling. Prior to enzymatic hydrolysis, all the samples undergo yeast and mould analysis, where the results showed that all the samples were still in the admissible limit ($5 \times 10^3$ CFU/g), indicating that the products were in the acceptable and safe conditions to be consumed (Hozová et al. 2002). After that, leftover croissants (LC) and leftover doughnuts (LD) were crushed using a knife mill, vacuum packed and finally stored at -20 °C for later analysis. The other chemicals, solvents, and standard sugar all were analytical grades (Fisher Scientific, UK). From preliminary experiment, the carbohydrate content of leftovers croissants (~52% wet basis) was higher than leftovers doughnuts (~47% wet basis) (Mohd Thani et al. 2020).

### ENZYMATIC HYDROLYSIS REACTION

Amyloglucosidase produced from *Aspergillus niger* (Sigma 10113) was purchased from Megazymes. The enzyme unit is 120 U/mg. For the enzymatic hydrolysis, 1.5 g of the substrate was mixed with 30 mL of distilled water in the Erlenmeyer Flask. The range of pH was from pH 3 until 9 for the slurry, and it was adjusted according to the experimental runs using 1 mol L$^{-1}$ H$_2$SO$_4$ or NaOH solutions. Different levels of enzyme concentrations (120-1200 U/g substrate). The selection range was based on previous studies that had used amyloglucosidase on carbohydrate-rich materials for enzymatic hydrolysis (Demirci et al. 2017; Han et al. 2016). The flasks were placed in the incubator shaker (200 rpm) for 24 h at temperatures of 30-60 °C. The enzymatic reaction was ended by heating the suspension at 80 °C for 5 min. The mixture was immediately cooled and centrifuged at 9000 rpm for 30 min. The supernatant (hydrolysate) was filtered and collected for further analysis.

### TOTAL SUGAR YIELD ANALYSIS

The determination of sugar yield in the hydrolysate was based on a method by Nielson (2010). 1 mL of 5% phenol solution, 5 mL of sulfuric acid (H$_2$SO$_4$), and 1 mL of the hydrolysate were mixed well and vortexed for 5 minutes. By using Ultrospec 3100 Pro UV/visible spectrophotometer, the absorption of the solution was recorded at 490 nm, in accordance with glucose as the standard. The total sugar yield was defined as in (1).

$$\text{Total Sugar Yield } \left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{Weight of total sugar (mg)}}{\text{Weight of substrate (g)}} \quad (1)$$

## SUGAR PROFILE ANALYSIS

Hydrolysate obtained after enzymatic hydrolysis were analyzed for identification and quantification of the sugar using high-performance liquid chromatography (HPLC) (Jasco, Japan), consisted with an auto sampler, a column heater, and a pump. The column used was Agilex Hi-Plex H column (300 × 7.7 mm), at 65 °C with distilled water as the mobile phase. 20 μL of the sample was injected at a flow rate of 0.3 mL/min. Quantification of sugar compounds was compared to calibration curves of glucose, fructose, mannose, galactose, and arabinose standards. The yield of monosaccharide was calculated using (2), where $M_{mono}$ is concentration of monosaccharides, $V_{aq}$ is the volume of aqueous phase, and $W_{substrate}$ is mass of substrate.

$$\text{Yield of monosaccharides (mg/g substrate)} = \frac{M_{mono} \text{ (mg/L)} \times V_{aq} \text{(L)}}{W_{substrate} \text{(g)}} \quad (2)$$

## EXPERIMENTAL DESIGN AND ANALYSIS FOR OPTIMIZATION

The response surface methodology (RSM) method was used to optimize and predict the optimum process condition. Prior to process conditions optimization, a one-time factor was carried out in very broad range, which to evaluate the effect of process conditions on hydrolysis processes. Based from the one-time factor results, three significant levels of each parameter were selected in order to obtain maximum (optimum) total sugar yield. The selected levels were then used to design the optimization stage using RSM.

A three-level three-factor face-centered central composite design (CCD) generated using Design Expert® software (Stat Ease Corp, USA) was employed to study the interaction of the independent variables; enzyme concentration (U/g substrate), process temperature (°C) and pH on total sugar yield as the response from LC and LD. The independent variables were employed at three levels (-1, 0, 1), whereby the range of enzyme concentration was selected at 840-1200 (U/g substrate), process temperature at 40-60 °C and pH at 3-5.5. A total of 20 experimental runs were generated using CCD (Table 1), and the experimental runs were done in a randomized manner. The experimental results or response (total sugar yield) for each run are also presented in Table 1.

TABLE 1. Central composite design (CCD) values of enzymatic hydrolysis conditions, and result data for leftover croissants (LC) and leftover doughnuts (LD)

| Run order | Independent variables | | | Response | |
| | $X_1$ = Enzyme concentration (U/g substrate) | $X_2$ = Temperature (°C) | $X_3$ = pH | Total sugar yield (mg/g) | |
| | | | | LC | LD |
|---|---|---|---|---|---|
| 1 | 840 | 40 | 3.0 | 502.74 ± 0.60 | 136.63 ± 0.60 |
| 2 | 840 | 60 | 3.0 | 435.89 ± 0.45 | 79.79 ± 0.60 |
| 3 | 840 | 40 | 5.5 | 449.26 ± 0.89 | 90.63 ± 0.74 |
| 4 | 840 | 60 | 5.5 | 414.32 ± 0.30 | 63.26 ± 0.45 |
| 5 | 840 | 50 | 4.0 | 574.84 ± 0.74 | 214.95 ± 0.89 |
| 6 | 1020 | 40 | 4.0 | 217.16 ± 0.89 | 254.32 ± 0.30 |
| 7 | 1020 | 60 | 4.0 | 252.42 ± 0.89 | 227.79 ± 0.89 |
| 8 | 1020 | 50 | 3.0 | 362.63 ± 0.45 | 346.53 ± 0.89 |
| 9 | 1020 | 50 | 5.5 | 283.68 ± 0.74 | 267.47 ± 0.45 |
| 10 | 1020 | 50 | 4.0 | 342.32 ± 0.89 | 326.11 ± 0.60 |
| 11 | 1020 | 50 | 4.0 | 347.58 ± 0.89 | 324.32 ± 0.45 |
| 12 | 1020 | 50 | 4.0 | 351.26 ± 0.74 | 324.11 ± 0.74 |
| 13 | 1020 | 50 | 4.0 | 350.95 ± 0.60 | 326.32 ± 0.30 |
| 14 | 1020 | 50 | 4.0 | 348.21 ± 0.89 | 327.47 ± 0.74 |
| 15 | 1020 | 50 | 4.0 | 346.21 ± 0.74 | 324.21 ± 0.30 |
| 16 | 1200 | 40 | 3.0 | 229.26 ± 0.60 | 441.89 ± 0.89 |
| 17 | 1200 | 60 | 3.0 | 192.53 ± 0.74 | 361.79 ± 0.45 |
| 18 | 1200 | 40 | 5.5 | 201.47 ± 0.30 | 410.63 ± 0.45 |
| 19 | 1200 | 60 | 5.5 | 157.79 ± 0.45 | 350.79 ± 0.30 |
| 20 | 1200 | 50 | 4.0 | 246.32 ± 0.60 | 453.47 ± 0.89 |

### SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

The characterization of morphological properties of LC and LD was executed using Scanning Electron Microscope (SEM) Hitachi S-3400N Tabletop Microscope. The aim of this analysis was to observe any changes, particularly of the surface of the residue due to enzymatic hydrolysis. The hydrolysates taken from enzymatic hydrolysis of LC and LD were dried in the oven at 105 °C for 24 hours prior to SEM viewing. Next, the samples were attached on stubs and sputter-coated with a layer of gold. By using an accelerating voltage of 10.0 kV, the SEM micrographs of LC and LD were viewed.

### FOURIER TRANSFORM-INFRARED (FT-IR) ANALYSIS

FTIR analysis was carry out for identifying the presence of functional groups or chemicals bonds in the hydrolysates from LC and LD through enzymatic hydrolysis, by using PerkinElmer SpectrumTM 100 FT-IR spectrometer (PerkinElmer) with spectral range of 400-4000 cm$^{-1}$ at 200 scans per second. A plot of infrared radiation intensity versus wave number was recorded for LC and LD sugar recovered at optimal condition to qualitatively identify the functional groups of the samples.

### HYDROXYMETHYLFURFAL (HMF) ANALYSIS

The detection and quantitative evaluation of hydroxymethylfurfural was conducted according to the method by Bogdanov (2009). Using High Performance Liquid Chromatography (HPLC), with a mobile phase of water: methanol (90:10 by volume) and a C18-reversed phase material column. 20 µL of sample was injected at the flow rate of 1.0 mL/min, and HMF was detected using UV detection at 285 nm. The quantification of HMF in the hydrolysate was determined by calculating the corresponding peak areas of the samples.

## RESULTS AND DISCUSSION

### EFFECT OF PROCESS CONDITIONS ON TOTAL SUGAR YIELD AND SUGAR PROFILE

Amyloglucosidase hydrolyzes α-1,4 glycosidic and α-1,6 linkages bonds from the non-reducing ends of starch, producing sugar (Keeratiburana et al. 2020). Furthermore, according to Ma et al. (2020), there are two ways on how amyloglucosidase hydrolyzed; 'outside-in' and 'inside-out' pattern. For 'outside-in' pattern, amyloglucosidase molecules penetrate from specific areas towards granule matrices. Meanwhile, for 'inside-out' pattern amyloglucosidase molecules bind to channels and penetrate towards the central cavity or granule matrix. Considering the fact there are a few factors that can affect enzymatic hydrolysis, thus, this section would discuss the effect of process conditions (enzyme concentration, temperature, and pH) on total sugar yield and sugar profile analysis.

The enzymatic hydrolysis of LC and LD was carried out at (120-1200 U/g substrate) enzyme concentration fixed at 50 °C and pH 5.5. The total sugar yield of hydrolysate from LC and LD after enzymatic hydrolysis (EH) was presented in Figure 1.

Different process conditions showed different trends of total sugar yield for both LC and LD (Figure 1). From Figure 1(a), the enzyme concentration had a great effect on the total sugar yield. The amount of total sugar yield for LC increased from 120 to 840 U/g substrate until it yielded a maximum value of 275.16±0.19 mg/g (at 840 U/g substrate), but later decreased from 840 to 1200 U/g substrate. It can be observed that the amount of total sugar yield recorded for LC (until 720 U/g substrate) was higher than that of the LD. The different behaviour of hydrolysis between LC and LD might be due to the availability of the substrate (in the LC and LD) during the process. Further addition of enzyme concentration (840 - 1200 U/g substrate) has caused a decrease in sugar recovery in LC, which might be due to the excess of enzymes with limited substrates to be attacked and hydrolyzed the saccharides. Demirci et al. (2017) observed positive trends in recovering sugar when the enzyme concentration increased. Azmi et al. (2017) mentioned if enzyme concentration is insufficient, it will resulted a slower rate and low yield, whereas if enzyme concentration is in excess it can lead to underutilized of the enzymes. Thus, it can be concluded that a sufficient amount of enzyme (amyloglucosidase) was essential to catalyze the hydrolysis reaction, which breakdown the polysaccharides (carbohydrate) to sugars (oligo-, di- and monosaccharides), as well as facilitates in gaining high sugar yield.

The process temperature was observed to have a significant effect towards enzymatic hydrolysis process as each enzyme has its own optimum temperature for it to work efficiently. As the temperature increased, the rate of enzyme catalysed reaction increases due to the increase of enzyme activity. At a temperature from 30 °C to 50 °C, the hydrolysates of LC and LD have recorded an increase in total sugar yield. The highest value for both LC and LD were recorded at 50 °C, with 311.53±0.18 mg/g of total sugar yield for LC; and 308.89±0.28 mg/g for LD. Further increment in temperature (from 50 °C to 60 °C) has caused the sugar recovered from LC and LD to decrease.

a)
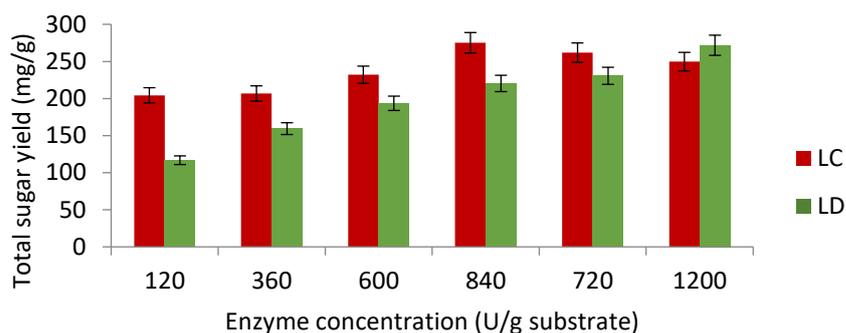


b)
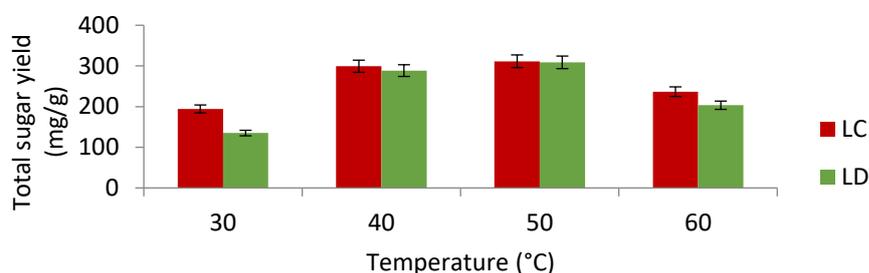


c)
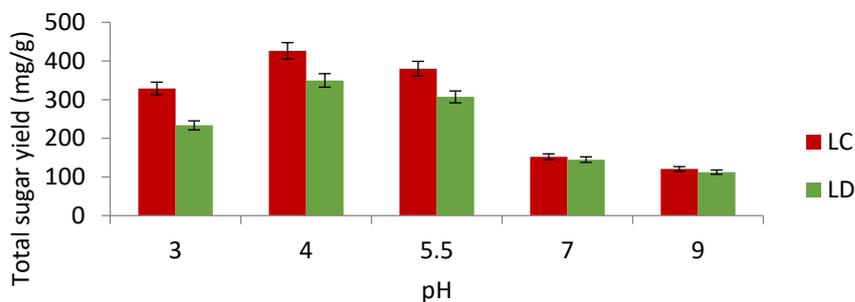


FIGURE 1. Total sugar yield (mg/g) of leftover croissant (LC) and doughnut (LD)
hydrolysates prepared by enzymatic hydrolysis under various process conditions (a) Enzyme
concentration (120-1200 U/g substrate at 50 °C min and pH 5.5), (b) Temperature (30-60 min
at 840 U/g substrate (LC), 1200 U/g substrate (LD) enzyme concentration and pH 5.5), and (c)
pH (3-9 at 840 U/g substrate (LC), 1200 U/g substrate (LD) enzyme concentration and 50 °C)

The reaction rate reached a maximum at a temperature of 50 °C and above this temperature, the enzyme becomes denatured. Hudečková et al. (2017) reported in their work that 55 °C is the optimum temperature to obtain sugar from waste bread before fermentation. However, Pietrzak and Kawa-Rygielska (2015) who also employed amyloglucosidase to hydrolyze bread waste has found that 59 °C was the best temperature in their work.

The pH condition during enzymatic hydrolysis was also crucial to ensure maximum sugar recovery from the LC and LD. According to Khanna (2010), pH can effect the ionization state of acidic or basic amino acids, where

if its altered it may lead to enzyme's inactivity. From Figure 1(c), the enzymatic hydrolysis of LC and LD using amyloglucosidase seem to favour acidic condition where it is obvious that between pH 3 and pH 5.5, the process has yielded a high value of total sugar as compared to neutral (pH 7) and alkaline pH condition (pH 9). At pH 4, the highest total sugar yield was obtained at 426.21±1.93 mg/g (LC) and 349.79±1.94 mg/g (LD). Han et al. (2012) reported that the changes in pH value may result in the loss of enzyme activity or dissociation between substrate and catalytic group of enzyme active center, leading to the reduced speed of enzymatic reaction. Hudečková et al. (2017) also had similar findings where the highest sugar obtained from enzymatic hydrolysis of waste bread was found to be at pH 4.2.

Sugar profile analysis (Table 2) was carried out to scrutinize the hydrolysate from enzymatic hydrolysis of LC and LD using HPLC, where the two peaks

corresponding to glucose and fructose were detected. From Table 2, the sugars recovered (glucose and fructose) from EH of LC and LD has significant effect ($p < 0.05$) towards processing conditions. As enzyme concentration increased, both glucose and fructose also increased, as observed on the total sugar yield. The highest fructose and glucose concentrations detected at 840 U/g substrate (LC) were 9.46±0.16 mg/g and 11.13±0.62 mg/g, respectively, and 10.10±0.12 mg/g and 12.76±0.18 mg/g, respectively, for LD at 1200 U/g substrate. However, glucose and fructose started to slowly decrease for LC as enzyme concentration increased from 840 to 1200 U/g substrate. The reduction of glucose and fructose may be due to enzyme saturation. When the enzyme concentration increased but the amount of substrate (LC) remains the same, less substrate is available for enzyme to attack. As this happened, the enzymatic hydrolysis efficiency will decrease, consequently reducing the total sugar yield.

TABLE 2. The monosaccharide contents of the hydrolysate obtained from the enzymatic hydrolysis of leftover croissants (LC) and doughnuts (LD) with various process conditions ($p < 0.05$)

| Process conditions | | Leftovers croissants (LC) | | Leftovers doughnuts (LD) | |
|---|---|---|---|---|---|
| | | Glucose (mg /g substrate) | Fructose (mg /g substrate) | Glucose (mg /g substrate) | Fructose (mg /g substrate) |
| Enzyme concentration (U/g substrate) at 50 °C and pH5.5 | 120 | 5.80±0.59 | 6.10±0.70 | 4.44±0.64 | 4.63±0.27 |
| | 360 | 5.87±0.59 | 6.20±0.69 | 6.50±0.62 | 5.77±0.57 |
| | 600 | 9.50±0.74 | 8.96±0.53 | 8.62±0.42 | 7.40±0.71 |
| | 840 | 11.13±0.62 | 9.46±0.16 | 8.73±0.31 | 8.21±0.12 |
| | 720 | 10.85±0.78 | 8.83±0.72 | 12.09±0.55 | 9.38±0.26 |
| | 1200 | 10.17±0.60 | 8.60±0.24 | 12.76±0.18 | 10.10±0.12 |
| Temperature (°C) at enzyme concentration 840 U/g substrate (LC) 1200 U/g substrate (LD); and pH 5.5 | 30 | 8.91±0.13 | 9.56±0.03 | 3.85±0.24 | 2.75±0.66 |
| | 40 | 10.10±0.28 | 10.54±0.21 | 9.23±0.92 | 9.99±0.48 |
| | 50 | 10.71±0.15 | 12.57±0.08 | 10.86±0.09 | 12.08±0.95 |
| | 60 | 9.37±0.47 | 9.65±0.02 | 8.02±0.37 | 7.67±0.41 |
| pH at enzyme concentration 840 U/g substrate (LC) 1200 U/g substrate (LD); and 50 °C | 3 | 9.24±0.10 | 11.82±0.01 | 8.67±0.3 | 11.06±0.06 |
| | 4 | 11.84±0.21 | 14.47±0.73 | 10.34±0.11 | 13.26±0.63 |
| | 5.5 | 11.2±0.29 | 12.42±0.39 | 9.89±0.03 | 11.51±0.03 |
| | 7 | 8.28±0.95 | 10.71±0.25 | 3.84±0.12 | 5.16±0.05 |
| | 9 | 2.68±1.03 | 0.86±1.13 | 1.13±0.35 | 1.85±0.28 |

The enzymatic hydrolysis was carried out at an enzyme concentration of 840 U/g substrate and 1200 U/g substrate, for LC and LD, respectively, at temperature 30-60 °C and pH 5.5. Different types of enzyme require different temperature to achieve high efficiency. From Table 2, glucose and fructose increased as the temperature increased for both LC and LD. Highest glucose and fructose yield were recorded for LC and LD at temperature 50 °C, with 10.71±0.15 mg glucose/g and 12.57±0.08 mg fructose/g for LC and 10.86±0.09 mg glucose/g and 12.08±0.95 mg fructose/g for LD. During enzymatic hydrolysis, in order to convert LC and LD into glucose and fructose, the enzyme (amyloglucosidase) must collide and bind to the substrate at its active site. An increase in temperature during the process will increase the number of collisions of the enzyme and substrate per unit time. Thus, the rate of an enzyme-catalysed reaction increases as the temperature is raised. Han et al. (2016) has recorded 55 °C as the optimum temperature for enzymatic hydrolysis of food waste using amyloglucosidase. Nonetheless, when the temperature further increased to 60 °C, the glucose and fructose started to decrease, indicating that the temperature may be too high for amyloglucosidase and the denaturing phase has started.

In this work, the enzymatic hydrolysis of LC and LD was carried out with pH of 3-9, temperature of 50 °C and enzyme concentration of 840 U/g substrate (LC) and 1200 U/g substrate (LD). From Table 2, glucose and fructose were observed to increase from pH 3 until pH 4. However, glucose and fructose started to decrease as the condition shifted to neutral and alkaline (pH 5.5- 9). The highest glucose and fructose reported at pH 4 for LC and LD with glucose and fructose of 11.84±0.21 mg/g and 14.47±0.73 mg/g, and 10.34±0.11 mg/g and 13.26±0.63 mg/g, respectively. In enzymatic hydrolysis, optimum pH is the point where the enzyme is most active, where higher or lower pH values than the optimum pH values can affect the stability and loss of enzyme activity. Optimum pH value varied according to the type of enzyme used. pH values played great influence during the binding process of enzyme and substrate at the active site, where the ions (from the surrounding) may affect the chemical bonding of enzyme and substrate. In this work, pH 4 was the most suitable for enzymatic hydrolysis of LC and LD. A study by Hudečková et al. (2017) on waste bread using amyloglucosidase has also reported pH 4.2 as the optimal pH value for enzymatic hydrolysis.

OPTIMIZATION OF PROCESS CONDITIONS USING RSM

The best three levels of each factor (enzyme concentration at 0.7-1.0% (w/w), process temperature at 40-60 °C and pH at 3-5.5.) are selected for designing the optimization of process conditions using Response Surface Methodology (RSM). The RSM was used to predict the optimum condition, which the hydrolysate and residue obtained at this condition were further characterize (these results will be discussed in the following section).

Analysis of variance (ANOVA) for the response of total sugar yield from LC and LD are presented in Table 3. ANOVA was used to investigate the influence of each independent variable (enzyme concentration ($X_1$), temperature ($X_2$) and pH ($X_3$)) in order to construct an empirical model. The model is then used to maximize the total sugar yield recovered from LC and LD through EH. The significance of each condition was obtained by examining its respective p-value. If the p-value < 0.05, it indicates significance towards the response variable (total sugar yield).

Both LC and LD models were found to be significant (p < 0.0001). Enzyme concentration and pH appeared to be the important variables for LC, as these variables were found to be significant (p < 0.0001). Meanwhile, for LD, all of the independent variables (enzyme concentration, temperature and pH) were found to be significant (p < 0.0001). For quadratic factor term, enzyme concentration ($X_1X_1$) and temperature ($X_2X_2$) were significant (p = 0.0005 and p=0.0001) for LC and for LD only temperature ($X_2X_2$) was significant p<0.0001, while the other variables were not significant. For interaction factor term, all the variables were found not significant for both LC and LD. The coefficients of determination ($R^2$) for sugar yields of LC and LD were 0.9699 and 0.9882, respectively, which indicate the experimental results were well represented by the model. Multiple regression analysis on the experimental data was performed, and the model for the response variable (total sugar yield) can be expressed using the following quadratic equation (shown in Table 3) in the form of coded values, after exclusion of the insignificant terms.

To illustrate the effects of the process conditions on sugar recovery of LC and LD via EH, 3D contour graphs of the response surface and experimental models are presented in Figures 2 and 3. From the graph, the enzyme concentration had a great effect on the total sugar yield. However, for LC, enzyme concentration has positive effect only to a certain value (840 U/g substrate) but later exhibit a negative effect where the increment in the amount of enzyme present during the process resulted in lowering total sugar yield. Positive trend was recorded for LD, where an increasing enzyme concentration had a positive effect on total sugar yield. Nonetheless,

despite the negative effect of enzyme concentration for LC samples, it still had higher total sugar yield than LD. The divergent in the effect of enzyme concentration on total sugar yield might be due to the carbohydrate content on raw materials, whereby LC has higher carbohydrate content when compared to LD.

TABLE 3. Analysis of variance (ANOVA) model for the total sugar yields of the leftover croissants (LC) and leftover donuts (LD) via EH

| Source | LC | | | | | Comment | LD | | | | | Comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sum of squares | DF | Mean square | F-value | p-value | | Sum of squares | DF | Mean square | F-value | p-value | |
| Model | 2.216E+005 | 9 | 24619.38 | 35.74 | <0.0001 | Significant | 2.500E+005 | 9 | 27779.08 | 93.17 | <0.0001 | Significant |
| Linear | | | | | | | | | | | | |
| $X_1$ | 1.815E+005 | 1 | 1.815E+005 | 263.49 | <0.0001 | Significant | 2.055E+005 | 1 | 2.055E+005 | 689.06 | <0.0001 | Significant |
| $X_2$ | 2137.39 | 1 | 2137.39 | 3.10 | 0.1086 | | 6160.39 | 1 | 6160.39 | 20.66 | 0.0011 | Significant |
| $X_3$ | 4688.52 | 1 | 4688.52 | 6.81 | 0.0261 | Significant | 3380.08 | 1 | 3380.08 | 11.34 | 0.0072 | Significant |
| Quadratic | | | | | | | | | | | | |
| $X_1X_1$ | 18028.38 | 1 | 18028.38 | 26.18 | 0.0005 | Significant | 437.13 | 1 | 437.13 | 1.47 | 0.2538 | |
| $X_2X_2$ | 24725.97 | 1 | 24725.97 | 35.90 | 0.0001 | Significant | 17841.62 | | 17841.62 | 59.84 | <0.0001 | Significant |
| $X_3X_3$ | 12.30 | 1 | 12.30 | 0.0018 | 0.8963 | | 324.67 | | 324.67 | 1.09 | 0.3213 | |
| Interaction | | | | | | | | | | | | |
| $X_1X_2$ | 57.14 | 1 | 57.14 | 0.083 | 0.7792 | | 388.23 | 1 | 388.23 | 1.30 | 0.2804 | |
| $X_1X_3$ | 72.85 | 1 | 72.85 | 0.11 | 0.7517 | | 110.85 | 1 | 110.85 | 0.37 | 0.5556 | |
| $X_2X_3$ | 17.95 | 1 | 17.95 | 0.026 | 0.8750 | | 251.21 | 1 | 251.21 | 0.84 | 0.3803 | |
| Residual | 6887.53 | 10 | 6887.75 | | | | 2981.68 | 10 | 298.17 | | | |
| Pure Error | 54.66 | 5 | 10.93 | | | | 9.88 | 5 | 1.98 | | | |
| $R^2$ | 0.9699 | | | | | | 0.9882 | | | | | |
| Model Equation | Total Sugar Yield = 336.26 – 134.85$X_1$ – 14.63.56$X_2$ -21.56$X_3$ +2.67$X_1X_2$+3.01$X_1X_3$+1.49$X_2X_3$+ 80.97$X_1X_1$- 94.82$X_2X_2$-2.22$X_3X_3$ | | | | | | Total Sugar Yield = 320.67 + 143.48$X_1$ - 24.84$X_2$ – 18.39$X_3$ – 6.97$X_1X_2$ +3.71$X_1X_3$ +5.58$X_2X_3$ +12.61$X_1X_1$ -80.55$X_2X_2$-11.38$X_3X_3$ | | | | | |

Both LC and LD display similar trends of total sugar yield for the effect of temperature (Figures 2(b) and 3(b)), where both contour model favour a middle level of temperature (50 ºC) to obtain highest total sugar yield. Previous studies by Demirci et al. (2017) and Hudečková et al. (2017) also recorded that medium temperature (55-60 ºC) produce a high yield of sugar from bread waste using the same type of enzyme (amyloglucosidase). At lower temperature (40 °C), the heat available may not be sufficient for the reaction rate to achieve optimal hydrolysis process, while higher temperature (60 °C) may cause the denaturation phase to occur. Hence, in this work, it was found that 50 °C was the optimum temperature for enzymatic hydrolysis of LC and LD.

a)
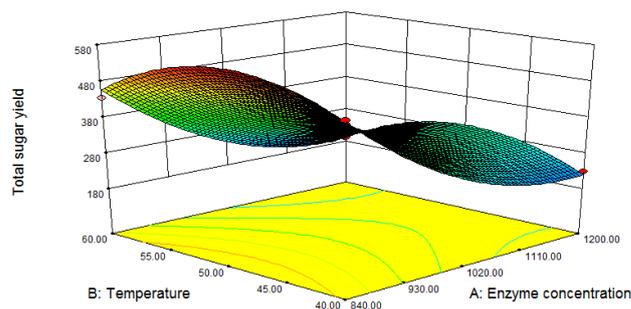


b)
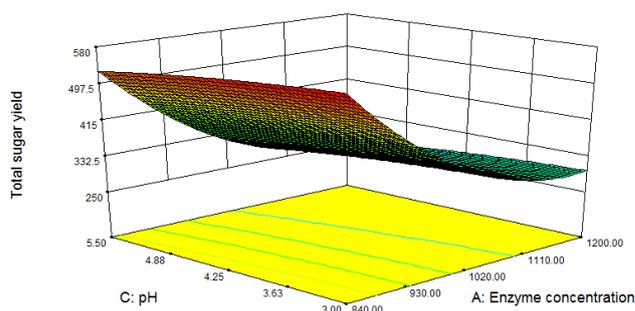


c)
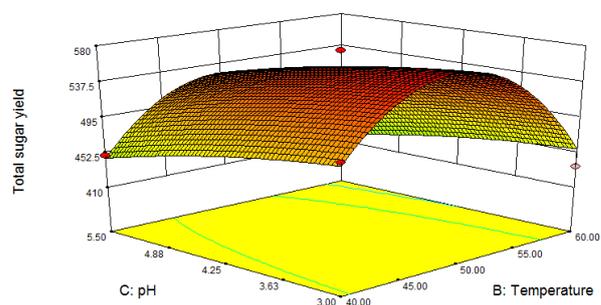


FIGURE 2. Response surface and contour graphs for total sugar yield of the
leftover croissants (LC) as function of enzyme concentration (U/g substrate),
temperature and pH *via* enzymatic hydrolysis

Finally, the other parameter that affects total sugar yield from LC and LD is the pH of the solution. From previous section, it is stated that enzymatic hydrolysis of LC and LD using amyloglucosidase seem to favour acidic condition (pH 3 to 5.5). Figures 2(b), 2(c), 3(b) and 3(c) show that between this pH range, small changes on the total sugar yield was observed. Hudečková et al. (2017) also had the same finding where the optimum

sugar yield from wheat-rye bread through enzymatic hydrolysis where at acidic condition (pH 4.2).

Optimization process was carried out to determine the optimal process condition of all parameters (enzyme concentration, temperature and pH), with the aim to obtain a maximum total sugar yield from LC and LD. Table 4 displays the predicted optimal process conditions suggested by RSM and also comparison between the

actual validation experimental results of LC and LD. The response (total sugar yield) was also presented. Insignificant difference (< 1%) was observed between the predicted and actual data for both LC and LD. This indicates the validity of the RSM model to confirm that the response model is adequate for predicting optimisation parameters of EH.

a)
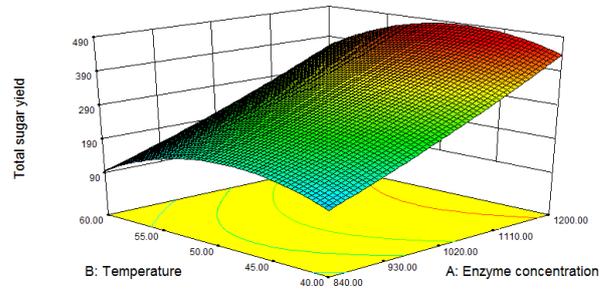


b)
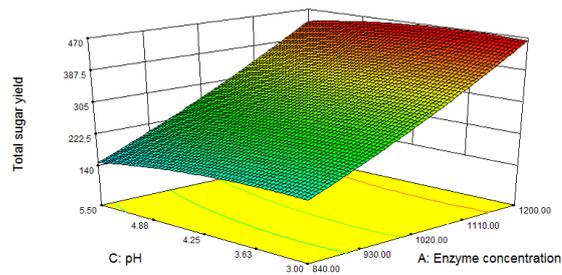


c)
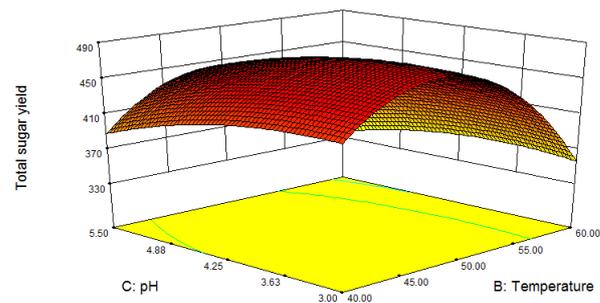


FIGURE 3. Response surface and contour graphs for total sugar yield of the leftover doughnuts (LD) as function of enzyme concentration, temperature and pH *via* enzymatic hydrolysis

TABLE 4. Response surface methodology (RSM) predicted and actual experimental process conditions with total sugar yield of the leftover croissants (LC) and leftover doughnuts (LD)

| | | Enzyme concentration (U/g substrate) | Temperature (°C) | pH | Total sugar yield (mg/g) | Difference (%) |
|---|---|---|---|---|---|---|
| LC | Predicted | 840 | 48.53 | 3.01 | 575 | 0.14 |
| | Actual | 840 | 49 | 3 | 574.21 ± 0.74 | |
| LD | Predicted | 1176 | 46.93 | 3.19 | 460.04 | 0.11 |
| | Actual | 1176 | 47 | 3 | 460.53 ± 0.74 | |

## OPTIMIZATION ON PROCESS CONDITIONS FOR SUGAR RECOVERY FROM THE LEFTOVERS

SEM analysis was carried out on the residue of LC and LD after EH, where the effects of enzymatic hydrolysis can be determined through assessing the morphological changes. SEM analysis is crucial to this study as it provides images and examined the structure and surface of the molecules before/after EH, considering that enzymatic hydrolysis involved gelatinization and saccharification of starch molecules that leads to alteration on the structure of the molecule. Figure 4 shows the cell surface of the solid sample after EH at optimum condition.
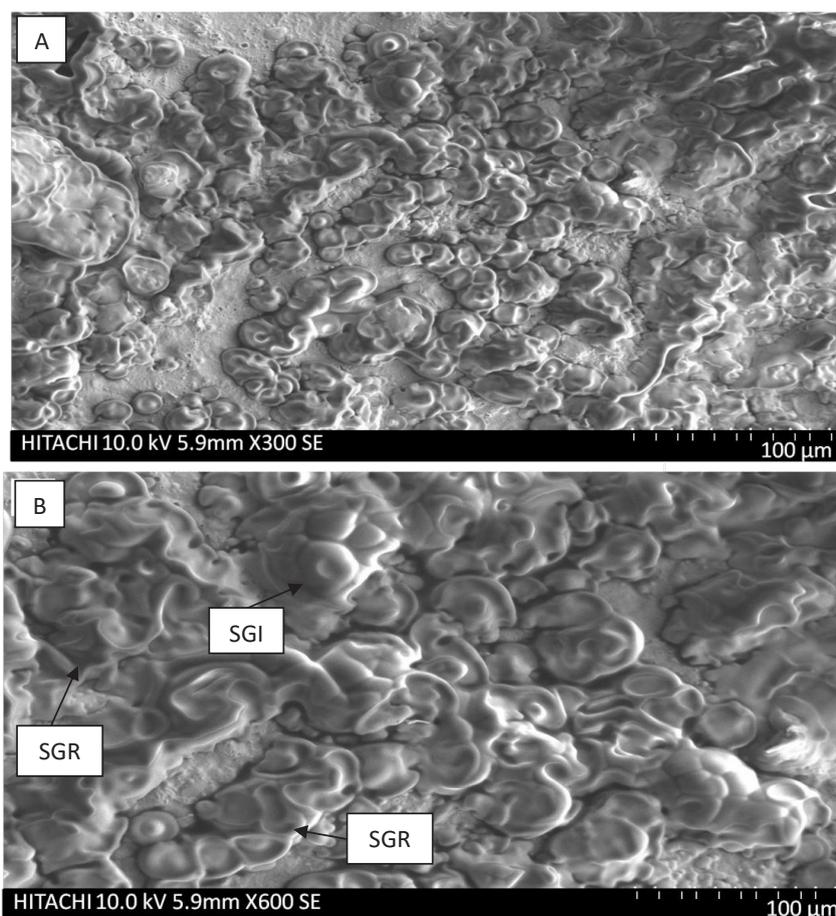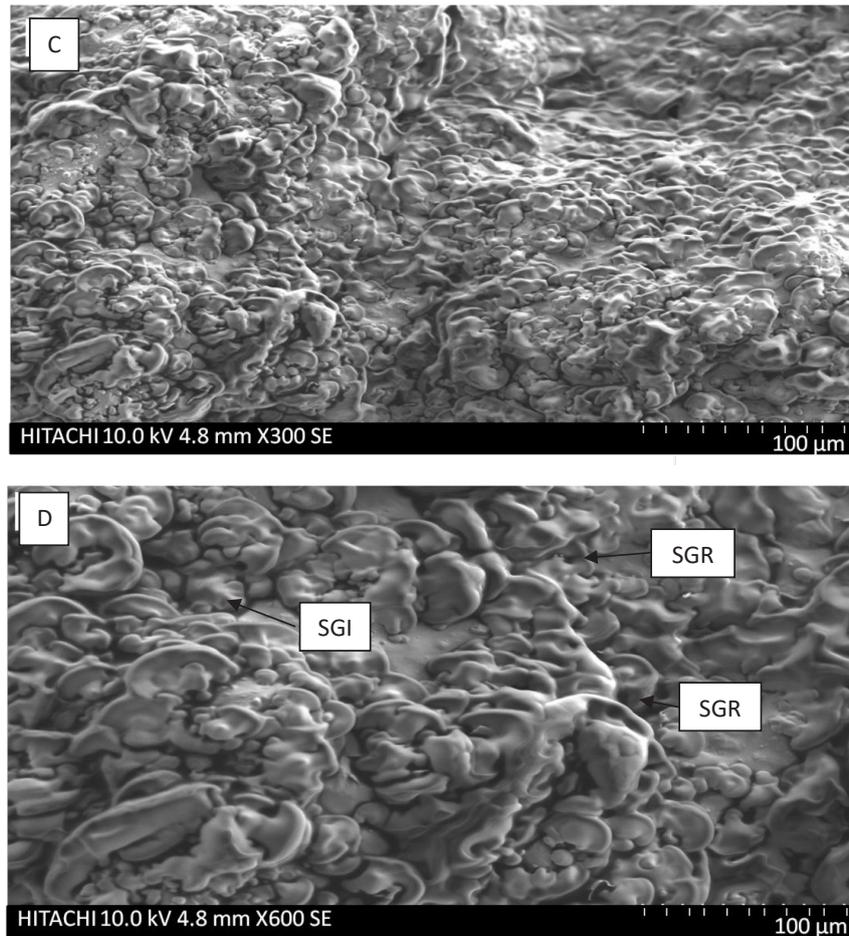
FIGURE 4. Scanning electron microscopy (SEM) images of (A) optimized residue sample of LC at 300× magnification; (B) optimized residue sample of LC at 600× magnification; (C) optimized residue sample of LD at 300× magnification; and (D) optimized residue sample at 600× magnification of LD. SGI: Starch granules intact, SGR: Starch granules ruptured

According to Mohd Thani et al. (2020b) that had observed the raw materials of LC and LD, there was no obvious difference on the surface of raw samples. They stated that the starch granules in the raw samples were still intact and embedded within the protein matrix. Hesso et al. (2015) explained, during preparation, when flour and water were mixed, a reticular structure of proteins and soluble solutes in the form of a matrix with embedded starch granules. The preparation formula may affect the gluten-starch network. However, after the EH (Figure 4), the starch granules seems to shrink which might indicate the gelatinization and the release of amylose from the granules. Rojas et al. (2000) explained that during enzymatic attack, the granules displayed as perforated granules due to the enzyme activity. Both LC and LD after EH showed changes on the starch granules but LD seems to have some starch granules that were still intact in its original form. In contrary, majority of starch granules within LC have all shrunk, which has been proven with higher total sugar yield. From SEM, the changes on the structure of the starch granules, can validate the previous findings where LC had higher total sugar yield than LD.

FTIR analysis was carried out to scrutinize the chemical composition for both LC and LD hydrolysate that obtain after EH. Figure 5 shows that the trends on the IR spectra of LC and LD hydrolysate were almost similar. However, in the two samples, several peaks exhibited different sizes.
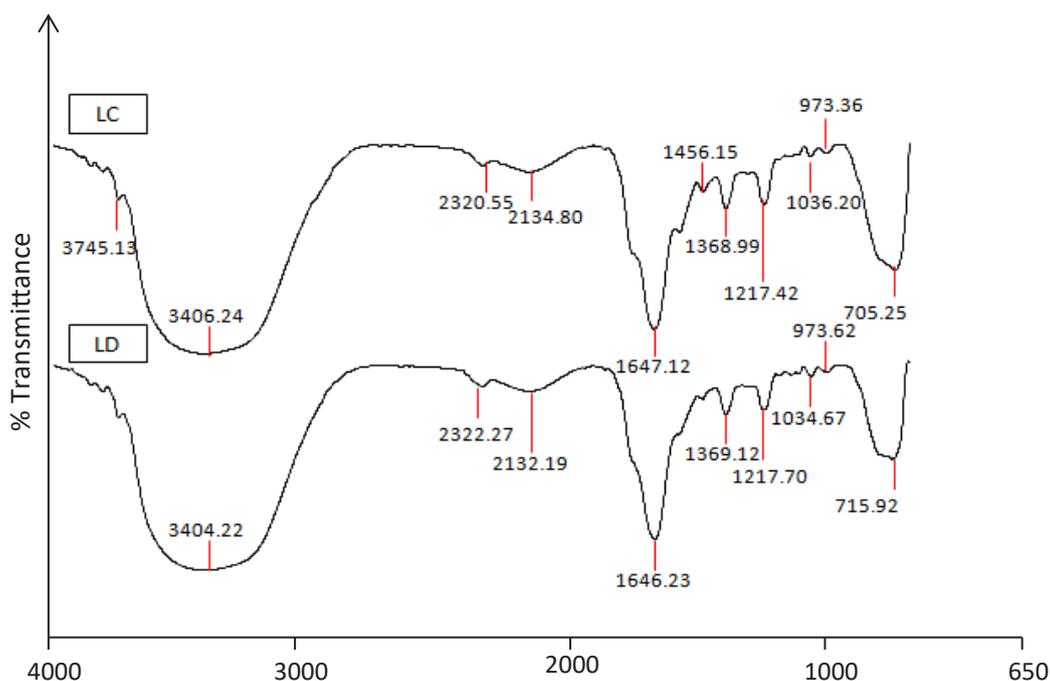
FIGURE 5. IR Spectra of hydrolysate from leftover croissants (LC) and leftover doughnuts (LD) after EH

The broadest peak detected was a hydroxyl group (-OH), between 3000 cm$^{-1}$ and 4000 cm$^{-1}$. This finding was expected as the EH involved a portion of water molecules, thus there is no significant difference in this region between both samples. Polysaccharides, which consists of C-C and C-O stretching vibration 950 - 1200 cm$^{-1}$, while band between 1200 - 1500 cm$^{-1}$ are indicating the C-H deformation vibrations and C-OH bending vibrations (Ge et al. 2009; Huang & Zhang 2009; Xu et al. 2009). Sugar monomers, such as sucrose, glucose, and fructose lie in the band 900-1500 cm$^{-1}$ region (Anjos et al. 2015). From Figure 5, the peaks in this region were strong (intensified in terms of its width and height) for LC as compared to LD. This supports the previous findings that LC has a higher total sugar yield than LD.

EH treatment on LC and LD might produce a by-product (hydroxymethylfurfural (HMF)) due to sugar degradation. Therefore, in order to determine the possible HMF produced during EH, both hydrolysate samples (LC and LD) were analyzed using HPLC. It was found that HMF content in both hydrolysate samples was relatively low, where LC has slightly higher HMF content than LD, 0.043±0.0334 mg/g and 0.023±0.0124 mg/g, respectively. The distinction of HMF contents might be due to different process conditions, as for LC (840

U/g substrate, 49 °C and pH 3) and for LD (1176 U/g substrate, 47 °C and pH 3). The factor that might affect HMF formation during EH is the carbohydrate content of LC and LD, where the LC had a higher carbohydrate content (52.42±0.29%) than the LD (46.59±0.23%). Thus, more sugar molecules were available to be hydrolysed and further degraded into HMF and/or other by-products.

CONCLUSION

This study has proven that sugar can be recovered from LC and LD through enzymatic hydrolysis. Among the process conditions studied, enzyme concentration has the greatest effect on LC and LD . The initial carbohydrate percentage within the sample prior to EH also influenced the yield of sugar recovered, as LC has higher total sugar yield than LD, regardless of the process conditions. From RSM, it was found the optimum process condition for LC and LD were; 840 U/g substrate, 49 °C and pH 3 with sugar yield of 574.21±0.74 mg/g and 1176 U/g substrate, 47 °C and pH 3.19 with total sugar yield of 460.53±0.74 mg/g, respectively. Further characterization on the sugar recovered from optimal process conditions, had shown that from morphological analysis of the residue, enzymatic hydrolysis has an impact on the

structure of the starch granules, where the granules have shrunken indicating the occurrence of gelatinization process. While from FTIR analysis, it was found that the sugar recovered not only contained monosaccharides, but also oligosaccharides. The HMF content of LC has outnumbered LD due to the initial content of carbohydrate in LC. Thus, through enzymatic hydrolysis process, bakery leftovers can be potentially utilized for sugar recovery. Also, the findings from this work can facilitate for scaling up of the enzymatic hydrolysis process.

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