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Process Optimization of Waste Corn Oil Hydrolysis Using Extracellular Lipase of *Tritirachium oryzae* W5H in Oil-Aqueous Biphasic System

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Abstract

Increasing demands for efficient management of waste cooking oil were currently brought up to prevent sewer system blockage, reducing wastewater treatment cost and to overcome many environmental associated drawbacks. Among the several suggested approaches, ecofriendly bioconversion and biodegradation of waste oil using microbes and their enzymes were proposed and preferred. Therefore, in the current project, process optimization of domestically collected waste corn oil hydrolysis was performed by investigating the most relevant operational parameters. Maximum degree of hydrolysis of 96.8% with enzyme stability of 92% residual activity was achieved at the optimized operational parameters of pH 5.0, 33^{o}C, 300 rpm mixing speed in the presence of 4.6 g oil and 368.2 enzyme unit per g oil in 1:5 volume ratio biphasic reaction mixture. The reusability of the biocatalyst in further hydrolysis experiments under the previously described optimum conditions showed reduced results of approximately 62% hydrolysis after 12 hr reaction time due to enzyme instability. Previous studies regarding extracellular lipase production by the species *Tritirachium oryzae* W5H were not available in the literature, which makes this report the first reporting extracellular lipase production and application in waste oil hydrolysis. The recent results supported the employment of the extracellular lipase from the indigenous fungi *Tritirachium oryzae* W5H for the bioremediation of used cooking oil.

Keywords: *Tritirachium oryzae* W5H, Indigenous fungi, Extracellular lipase, Waste corn oil hydrolysis.

1. Introduction

Edible and non-edible vegetable oils are natural hydrocarbons of plant origin used tremendously in human life for cooking, as basic ingredients in processed foods and personal care products, in soaps and detergents manufacturing, and in many other commercial products. Despite of their importance in human life, the bulk emission of waste cooking oil originated from both domestic and industrial human activities has become a major concern of municipalities worldwide (Husain *et al*., 2014). Many serious environmental issues arise due to the impetuous and irresponsible practice of direct discharge of waste cooking oil into the sewer system, which causes the blockage of sewer pipes and finally leads to an increase of wastewater treatment cost (Lopes *et al*., 2019). Therefore, pretreatment, recycling, down cycling and valorization of waste cooking oil are important as to prevent sewer blockage and to reduce their environmental impacts (He *et al*., 2017; Husain *et al*., 2014). Furthermore, the International Fat, Oil and Grease (FOG) management programs has stressed considering waste oil as valuable and renewable resources rather than simply a waste (Wallace *et al*., 2017).

From the environmental point of view, the management of used oil through biotechnological processes is generally more preferable compared to the physical and chemical approaches. This ecofriendly bioconversion and bioremediation of waste cooking oil using the intracellular and extracellular activities of microorganisms or their enzymatic systems have been reported. For instance, high quality biodiesel and derived fuel were produced through enzymatic activities using food grease trap and low-quality oils feedstocks under milled conditions by immobilized lipases from *Candida antarctica*, *Thermomyces lanuginosa* and *Mucor miehei* (Pinotti *et al*., 2018; Chang *et al*., 2018; Vescovi *et al*., 2016). Furthermore, the microbial bioconversion of waste cooking oils supplemented to the growth media of *Yarrowia lipolytica* W29, *Aspergillus* and *Penicillium* strains enhanced lipase enzyme and lipid-rich biomasses production (Lopes *et al*., 2019; Papanikolaou *et al*., 2011). The bioconversion of residual soybean oil and palm oil-based waste cooking oil into the close analog of plastics, polyhydroxyalkanoates (PHAs), by *Cupriavidus necator* strains was also reported (Nascimento *et al*., 2018; Kamilah *et al*., 2018). Other valuable biomaterials such as rhamnolipid, biosurfactant, biolubricants, carotene and methane were also obtained through the microbial bioconversion of other waste cooking oils (Ozdal *et al*., 2017; Chowdhury *et al*., 2013; Nanou *et al*., 2017; Liu *et al*., 2018).

However, lipolytic hydrolysis of waste cooking oil is the main principle reaction in all aforementioned microbial and enzymatic bioprocesses. Lipases (EC 3.1.1.3) are

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biocatalysts that catalyze the hydrolysis of triacylglycerols into free fatty acids and glycerol (Preczeski *et al*., 2018). Subsequently, the free fatty acids and glycerol were utilized either by microbial or in enzymatic production of lubricants, biodiesel, lactic acid, ethanol and biosurfactants (Dobson *et al.*, 2012; Nicol *et al*., 2012; Chowdhury *et al*., 2016; Vescovi *et al*., 2016; Xu *et al*., 2016; Yuwa-amornpitak and Chookietwatana, 2018). Similar to other bioprocesses, successful lipolytic hydrolysis of waste cooking oil is influenced by biocatalyst availability and other operational parameters such as temperature, pH, aqueous phase to oil phase ratio, oil loading and mixing speed (Jamie *et al*., 2017). Therefore, in the current project, process optimization of waste corn oil hydrolysis was investigated using the extracellular lipase produced by the indigenous fungal strain *Tritirachium oryzae* W5H.

2. Materials and Methods

2.1. Fungal strain and enzyme production

The investigated lipolytic fungus strain, *Tritirachium oryzae* W5H, was recovered from contaminated indigenous soil samples obtained from olive oil mills in Al-Karak province, south of Jordan. The fungal strain was identified using internal transcribed spacer (ITS) sequencing (GENWIZ, USA). The ITS gene sequence was registered in NCBI database and MK028996 accession number was obtained. Extracellular lipase enzyme by *Tritirachium oryzae* W5H was produced under the optimized conditions of 0.5% (w/v) glycerol, 1.0% (w/v) peptone, 0.6% (w/v) olive oil and 0.5% (w/v) NaCl. The sterilized media (pH 6.0) were inoculated with 1.0 mL of 1.63 $x10^6$ spore suspension and incubated at 35°C with 150 rpm agitation speed (Incu-Shaker 10L, Benchmark, Germany) for 96 hr (unpublished results).

2.2. Process optimization of waste corn oil hydrolysis in oil-aqueous biphasic system

The optimization of bioprocess parameters of waste corn oil hydrolysis was performed using one parameter per time approach. The studied operational parameters include aqueous phase pH (4-6), reaction temperature (33-37^{o}C), mixing speed (100-400 rpm), waste corn oil amount (9.2, 6.9 and 4.6 g), hydrophobic organic solvents (cyclohexane, n-hexane and isooctane), oil to aqueous phase volume ratio (1:7.5, 1:5.0 and 1:2.5 v:v) and amount of enzyme loading (184.1, 276.1, and 368.2 enzyme unit per g oil). In addition, aqueous phase recovery percentage and biocatalysts reusability were also determined. The hydrolysis reaction of waste corn oil was conducted in oil-aqueous biphasic system. The waste corn oil was domestically-collected after two to three times frying activities.

The initial experiment of the biphasic system composed of 20 mL of crude enzyme filtrate (184.1 EU/g oil), citrate buffer solution (pH 5.0), distilled water (DW) and 9.2 g of waste corn oil incubated at 35^{o}C with 200 rpm mixing speed. In all experiments, the final concentration of citrate buffer in the aqueous phase and the oil to aqueous phase volume ratio were maintained as 50 mM and 1:5, respectively unless otherwise stated. All experiments were conducted as 12 hr time profile in 100 mL conical flask using multi-position magnetic stirrer (Super-Nuova Multi-Place, Thermo Scientific, USA) under controlled temperature (Incu-Shaker 10L, Benchmark, Germany). Sample aliquots were withdrawn every 2 hr of the reaction time and analyzed for degree of hydrolysis and lipase stability.

Hydrolysis degree (%) of waste corn oil was measured using the titration method of the reaction mixture against 0.2 M KOH solution. Residual activity of lipase enzyme through the course of the reaction was evaluated and compared to the original activity at zero time. The composition of the blank experiment was formulated and incubated as the test experiment with the exception that the crude enzyme filtrate was replaced with thermally deactivated enzyme filtrate (DeAngelis *et al*., 2007).

2.3. Enzyme recovery and reusability

The aqueous phase containing the applied crude enzyme in the biphasic reaction mixture was separated from the oil phase using separation funnel. The recovery percentage of the separated aqueous phase was calculated from the ratio between the recovered volume and the original volume applied multiplied by 100%. The possible reusability of the recovered biocatalysts was measured by applying the recovered aqueous solution in the second hydrolysis experiment. The obtained degrees of hydrolysis from the second run were expressed as time profile and compared with the results of the degrees of hydrolysis of the initial experiment.

2.4. Enzyme stability

Enzyme stability throughout the course of the reaction was assessed by measuring the remaining enzyme activity for every 2 hr. Enzyme stability was expressed as residual activity (%) calculated from the ratio between the enzyme activity obtained throughout the course of the reaction and the original activity at zero time multiplied by 100%. Lipase activity was determined using copper soap colorimetry method as described by Kwon and Rhee (1986). The intensity absorbance of the recovered green color of isooctane phase containing the liberated free fatty acids-copper complex was measured at 715 nm (SPUV-19, SCO TECH, Germany) and compared to oleic acid as standard curve. One unit (U) of lipase enzyme activity was defined as: the amount of lipase enzyme that releases 1.0 µmol of free fatty acid per min under specific assay conditions.

2.5. Degree of waste corn oil hydrolysis

Hydrolysis degree of waste corn oil was obtained through titration of 2.0 mL sample aliquots of the hydrolysis reaction mixture by 0.2 M potassium hydroxide using digital titration system (Digitrate, Jencons, UK). To each sample, 15 mL of diethyl ether-ethanol mixture (1:1 v:v) containing phenolphthalein as pH indicator was added. Acid value (AV) was calculated using the following equation:

$$AV = \frac{56.1\ X\ CKOH\ X\ Vtitra}{m}$$

where CKOH is the concentration of KOH (M), Vtitra is the amount of titration (mL) and m is the amount of oil sample (g). Hydrolysis degree (X%) was calculated using the following equation:

$$X\% = \left(\frac{AV}{SV}\right) X\ 100\%$$

The saponification value of 92.3 ± 0.52 for waste corn oil was determined experimentally according to the AOAC Official Method 920.160 (1989).

2.6. Statistical analysis

Results were expressed as the mean value of three independent determinations. Standard deviations for each of the experimental results were indicated by error bars in the figures and were calculated using Microsoft Excel software (2010). Student *t* test was performed using SPSS version 16.0.1. Significant differences from the experiment with the highest degree of hydrolysis were designated in figures as $*$ ($p \leq 0.05$).

3. Results

3.1. Extracellular lipase production by Tritirachium oryzae W5H

Extracellular lipase production by *T. oryzae* W5H was carried out using the optimized enzyme production medium. Maximum lipase production of 84.7 ± 0.63 U/mL was obtained under the improved production conditions and medium composition. The extracellular crude enzyme filtrate used in the subsequent waste corn oil hydrolysis experiments was obtained through filtration.

3.2. Process optimization of waste corn oil hydrolysis in biphasic oil-aqueous system

3.2.1. Effect of pH

The influence of the aqueous phase pH on the extracellular lipase catalytic activity and stability was illustrated in Figure 1. It was observed that the highest hydrolysis percentage of 42.5% of waste corn oil occurred at the most stable pH of 5.0 suggesting the hydrolytic activity relatively depending on the enzyme stability. A slight deviation from the optimum pH by 1.0 unit led to significant reduction in oil hydrolysis noted as 35.8 and 37.2% at pH 4.0 and 6.0, respectively.

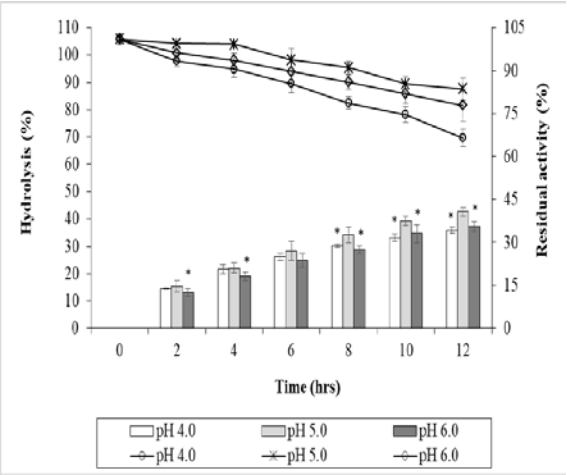

Figure 1. Effect of aqueous phase pH on lipase enzyme activity in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

3.2.2. Effect of temperature

As demonstrated in Figure 2, comparable degrees of hydrolysis with non-significant differences of 42.7 and 43.1% were obtained when the hydrolysis experiments were conducted at 33 and 35°C, respectively. Meanwhile, at elevated temperature of 37°C, less enzyme activity together with lesser enzyme stability were detected. The results clearly indicated the direct impact of operating temperature on enzyme stability and hence enzyme activity in oil hydrolysis. Although a higher degree of hydrolysis was obtained at 35°C, the following experiments were conducted at 33°C taking into consideration the highest enzyme stability of 91.7% residual activity which is 10% higher compared to the enzyme stability at 35°C.

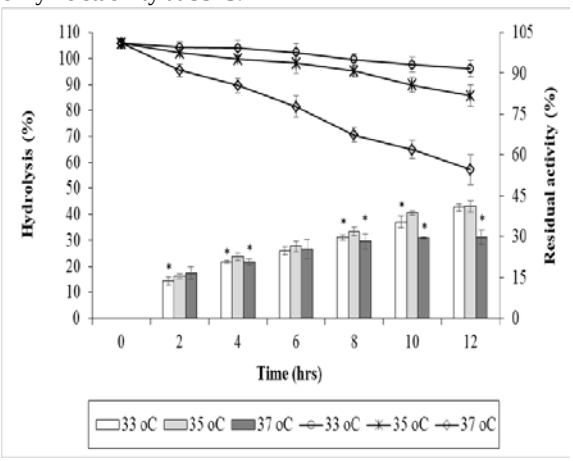

Figure 2. Effect of incubation temperature on extracellular lipase activity in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

3.2.3. Effect of mixing speed

An investigation into the impacts of 100, 200, 300 and 400 rpm mixing speeds of the reaction mixture was conducted in order to determine their effects on waste corn oil hydrolysis process (Figure 3). The results clearly indicated that a maximum degree of hydrolysis of 48.3% was obtained at 300 rpm mixing speed. At lower or higher rotation speeds, significantly less corn oil hydrolysis was obtained. However, enzyme stability was not affected by the mechanical shearing as comparable residual activities were obtained throughout all the investigated mixing speeds.

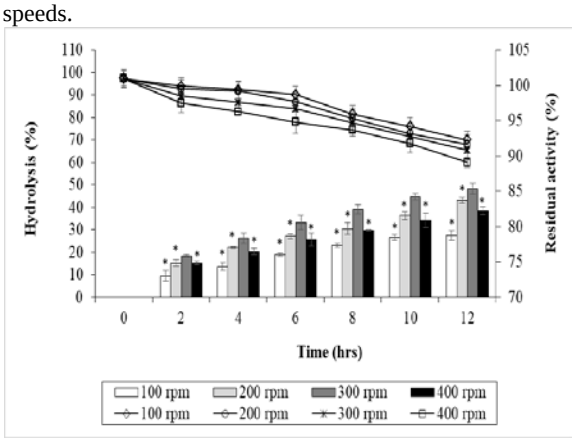

Figure 3. Effect of mixing speed (rpm) on extracellular lipase activity in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

3.2.4. Effect of waste corn oil amount

In this experiment, the effect of waste corn oil amount on degree of hydrolysis was evaluated. From the results

presented in Figure 4, the waste corn oil amounts 9.2, 6.9 and 4.6 g showed significantly linear improvements in the degree of oil hydrolysis when the substrate concentration was lowered. Maximum degree of hydrolysis was obtained when 4.6 g waste corn oil was applied into the reaction mixture with 72.8 hydrolysis percentage recorded after 12 hr. Obviously, enzyme stability was not a critical determinant in this experiment as similar stability results with more than 91% residual activities were attained throughout the 12 hr reaction time.

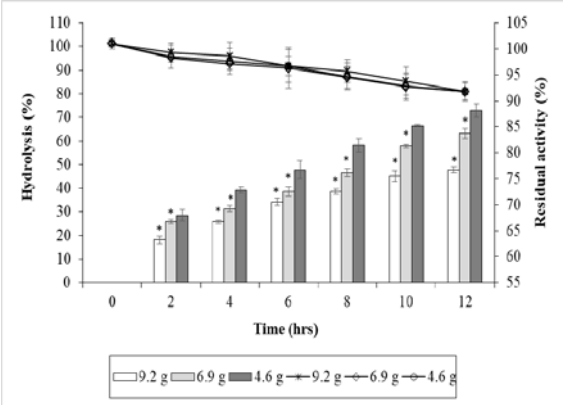

Figure 4. Effect of waste corn amount on extracellular lipase activity in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

3.2.5. Effect of organic solvents

Hydrophobic organic solvents of cyclohexane, n-hexane and isooctane were tested for their effect on the hydrolysis of waste corn oil by the crude lipase of *T. oryzae* W5H (Figure 5). All the tested water immiscible organic solvents have negative effects on the enzyme stability and therefore result in significantly lower waste corn oil hydrolysis percentage as compared with the control. Cyclohexane showed the lowest enzyme stability and hydrolysis results followed by n-hexane and finally isooctane. Therefore, the following experiments were conducted without the addition of any organic solvents to the oil phase.

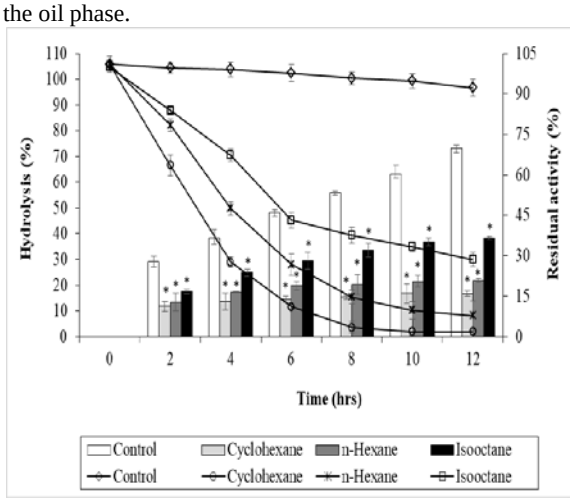

Figure 5. Effect of immiscible organic solvents on extracellular lipase activity in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

3.2.6. Effect of oil to aqueous phase volume ratio

The time profiles in Figure 6 illustrated the influence of the varied volume ratios between the two phases of the reaction mixture on waste corn oil hydrolysis degree. The maximum degree of hydrolysis of 72.9% was recorded when the volume ratio of the two phases was maintained as 1:5 (v:v). On the other hand, a significant reduction in oil degree of hydrolysis results was observed at lower and higher investigated volume ratios with the lowest recorded as 37.2% when the volume ratio was adjusted to 1:2.5 (v:v). It was also noted that the residual enzyme activity remains constant between 89-91% throughout the entire time profile, suggesting the negligible effect of the studied parameter on enzyme stability.

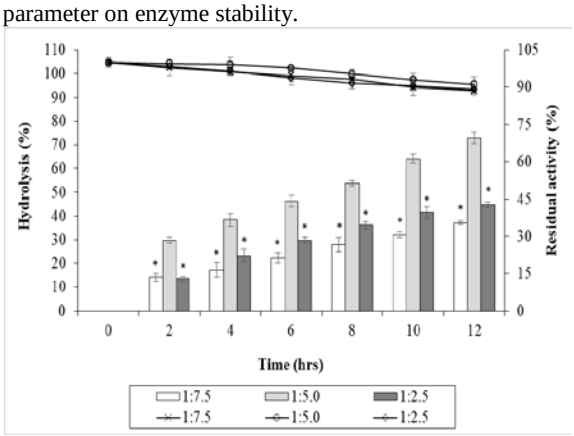

Figure 6. Effect of oil to aqueous phase volume ratios on extracellular lipase activity in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

3.2.7. Effect of enzyme loading on waste corn oil hydrolysis

As depicted in Figure 7, the increment in enzyme loading per gram oil showed a significant improvement in oil degree of hydrolysis. The maximum degree of hydrolysis of 96.8% was achieved when the highest enzyme loading of 368.2 EU per g oil was applied, indicating the importance of biocatalyst availability in oil hydrolysis applications. The accumulative effect of various parameters on waste corn oil hydrolysis showed a significant improvement in the degree of hydrolysis from 42.5% to 96.8% after optimization. The results of the enzyme stability analysis showed that 90-92% residual activity could be maintained by applying the optimized operational parameters, which is preferable in such kinds of enzymatic applications.

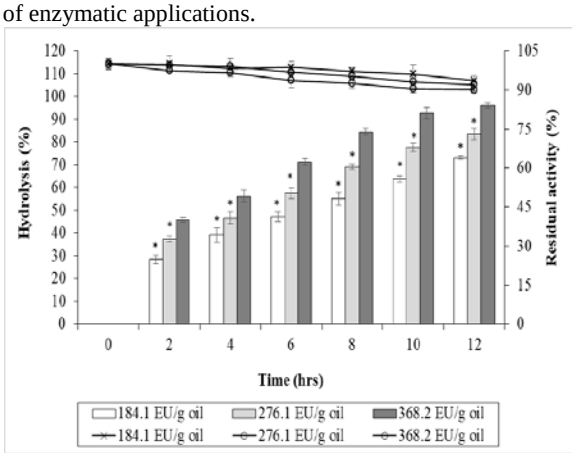

Figure 7. Effect of enzyme loading on extracellular lipase activity in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

3.3. Recovery and reusability

A total of 20.3 mL aqueous phase representing 80.1% of the original aqueous phase applied in the first run was recovered. The recovered volume of the aqueous phase was topped up to 25 mL using used aqueous phase collected from other flasks. The results in Figure 8 showed that when the recovered biocatalysts were used in second hydrolysis experiment, the enzyme gave significantly much lower hydrolysis results compared to the fresh biocatalysts. Such reduced results can be explained through the enzyme stability analysis as the enzyme gradually lost most of its activity throughout the course of the reaction reaching 23.1% residual activity after 12 hrs reaction time. Meanwhile, throughout the first 12 hrs in the first run the enzyme maintained 92.1% of its original activity.

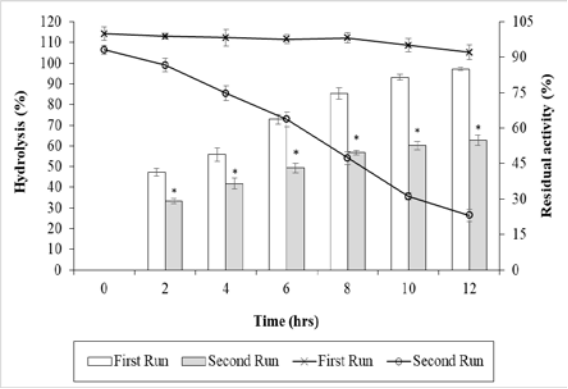

Figure 8. Enzyme reusability in waste corn oil hydrolysis. Columns represent the oil hydrolysis results while lines represent enzyme stability results.

4. Discussion

From the environmental point of view, enzyme mediated oil biodegradation processes are more preferable than other conventional methods (Goswami *et al*., 2013). The involvement of microorganisms and their associated lipolytic enzymes in the bioremediation of lipidic contaminated wastewater represent a promising opportunity in wastewater treatment (Hu *et al*., 2018). Higher waste oil hydrolysis can be achieved under improved process parameters that support higher enzyme activity and maintain its stability at longer reaction times (Kanmani *et al*., 2015; Goswami *et al*., 2009). Therefore, in the current study the effects of several operational parameters on waste corn oil hydrolysis by *T. oryzae* W5H extracellular lipase were evaluated.

When investigated, the pH of the aqueous phase showed a direct influence on the enzyme catalytic activity and stability. pH 5.0 was observed as the optimum pH when the enzyme was most active and stable. The lower activity and stability results observed at other investigated pH values were most probably due to the influence of pH on the ionization state of the enzyme causing progressive reduction in the enzyme activity and stability (Goswami *et al*., 2009). The enhanced degree of hydrolysis of waste oil observed at 33 and 35^{o}C can be accredited to the preferable catalytic activity and substrate solubility at the aforementioned temperatures (Goswami and Basu, 2012). At an elevated temperature of 37^{o}C, the reduction in hydrolysis rate and enzyme stability might be due to the effect of temperature on the ionization state of catalytically important amino acids in the active site and the state of the reaction emulsion interface (Gupta *et al*., 2012; Maidina *et al*., 2008).

The linear enhancement in waste corn oil hydrolysis with the increment of mixing speed that peaked at 300 rpm can be explained by the continuous formation of smaller oil droplets that leads to interfacial contact enhancement between the enzyme and substrate (Jamie *et al*., 2017). Lower enzyme activity obtained at higher mixing speed of 400 rpm was most properly due to the desorption of the enzyme from the interface (Al-Zuhair *et al*., 2008) rather than the deactivation of the enzyme by the mechanical shear stress (Raspe *et al*., 2013), which was proven by the enzyme stability results. The highest degradation rate at lower substrate concentration (4.6 g oil) was expected due to the presence of proportional substrate molecules to access the active sites of the available enzymes at the interface (Cavalcanti-Oliveira *et al*., 2011). On the other hand, the depleted hydrolysis results at higher substrate concentrations can be explained by the saturation of the active sites with the excess substrate molecules and not enzyme intolerance toward higher substrate concentrations as indicated by enzyme stability findings (Serri *et al*., 2008).

Although they are not preferable additives (Jamie *et al*., 2017), hydrophobic organic solvents are routinely included in oil hydrolysis processes to promote the reaction by reducing substrate viscosity by forming finer oil droplets with better interfacial area (Raspe *et al*., 2013). However, this was not the case in the current work as the addition of the three hydrophobic organic solvents resulted in less degrees of oil hydrolysis compared to the control reaction due to the interfacial tension destabilizing the molecular forces of the enzyme (Kumar *et al*., 2016; Al-limoun *et al*., 2015). The order of the catalytic performance and the stability of the enzyme in presence of organic solvents were in correlation with the partitioning coefficient (log p) of the tested solvents with the highest stability, and hence activity, obtained in the presence of the most hydrophobic solvent, isooctane (Priyanka *et al*., 2019; Al-limoun *et al*., 2019).

As the hydrolysis action of the lipase enzyme occurs at the interface of the emulsion, the increase in the aqueous phase volume up to 1:5 (v/v) ratio improved waste corn oil degradation due to the increase in the interfacial area and substrate availability at the interface. Meanwhile, excess amount of aqueous phase reduced enzyme activity due to the competition of water molecules with the substrate for the active site of the enzyme (Nguyen *et al*., 2018). The elevated improvement in the degree of hydrolysis with the increase in enzyme concentration can be accredited to the accumulative adsorption of enzyme molecules monolayer at the interface of the reaction-reaching saturation point (Jamie *et al*., 2017; Santos *et al*., 2015). Finally, the unfortunate reusability results of the biocatalyst in relation to the initial run can be explained by the instability of the enzyme in prolonged repetitive application.

5. Conclusion

The influences of the reaction parameters on waste corn oil hydrolysis by *T. oryzae* W5H extracellular crude lipase were explored using stepwise evaluation approach. The

stepwise improvement of waste corn oil hydrolysis resulted in 96-97% hydrolysis with residual activity of 92.1%. Unfortunately, the reuse of the recovered enzyme in second hydrolysis experiment resulted in much less hydrolysis percentage due to the instability of the enzyme in longer operation time. The current results indicated the potential use of the extracellular lipase enzyme produced by *T. oryzae* W5H in the bioremediation of used cooking oil under either a natural or an experimental environment.

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