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Biodegradation of Lipid-rich Waste Water by Combination of Microwave Irradiation and Lipase Immobilized on Chitosan

[1]N. Saifuddin and [2]K.H. Chua
[1]Department of Science, Chemistry Unit,
[2]Department of Civil Engineering, Environmental Unit,
College of Engineering, University of Tenaga Nasional,
Km 7, Jalan Puchong-Kajang, 43900 Serdang, Selangor, Malaysia

Abstract: Treatment of waste water containing fats and oils is not a new technology. Stringent standard for the discharge of waste into environment has driven researches to developed alternative processes such as enzymatic treatment for breaking down the oils and fats in waste water. Microwave radiation can be used as an alternative method for emulsion breaking. In this study a rapid and simple method will be discussed which provides the necessary enhancement of the enzyme via the immobilization for the subsequent use in treating oils and fats in waste water. This study used a combination of microwave irradiation for emulsion breaking and biodegradation of oil by enzymatic method. Results have shown that a very good separation of aqueous and oil phase was obtained after the emulsion sample was subjected to microwave irradiation at 900 W power output and irradiation time between 220-240 sec. Lipase enzyme was successfully immobilized using chitosan (cheap waste material from fish industry) hydrogel beads as a means to encapsulate proteins. The entrapment using an inotropic gelation technique provided a quick and effective method for producing spherical and rugged beads with desirable characteristics. In this study calcium ion enhance the reaction rate by up to 2.9 times at calcium concentration of 5 mM. The lipase in the optimized bead did not exhibit a substantial loss of activity after five consecutive runs, which indicates the re-usability of the entrapped enzyme.

Key words: Oily waste water, lipase, microwave irradiation, emulsion breaking, immobilization, chitosan

INTRODUCTION

Lipids (fats, oils and greases) are major organic matters in municipal and some industrial waste water and can cause severe environmental pollution. Waste water produced from edible oil refinery, slaughterhouse, wool scouring and dairy products industry contain a high (>100 mg L^{-1}) concentration of lipids. Oily waste water causes a lot of problem for treatment plants and discharge sewers. They are responsible for clogging sewer networks, unsettling water treatment plants and limiting the oxygen transfer to biomass due to build up of a lipidic film at the air/water interface (Wakelin and Forster, 1997; Lefebvre *et al*., 1998; Becker *et al*., 1999). If left untreated, this oil/waste water substance can severely hamper recovery capabilities of skimmers; reduce pumping volumes; and increase handling, oily waste disposal, segregation and storage problems.

Biodegradation of lipid-rich wastes, have been investigated (Borja *et al*., 1994; Borja and Banks, 1995; Starms and Elferink, 1997; Masse *et al*., 2001). Enzymatic treatment technique has gained more attention because of stringent environment regulations and clean and friendly application of enzymes (Jung *et al*., 2002; Gandhi, 1997). There are only a few reports on the potential usage of lipases in oily waste water treatment (Paiva *et al*., 2000). Lipases (EC 3.1.1.3) are hydrophobic proteins that catalyze cleavage of carboxyl ester bonds in tri-, di- and monoacylglycerols (the major constituents of animal, plant and microbial fats and oils) at the interface between an aqueous and an oil phase and have diverse application in biotechnology today (Sharma *et al*., 2001; Bornscheuer, 2001 and 2004).

Another alternative method that has been looked into is microwave radiation. It can be used as an alternative method for emulsion breaking (Klaila, 1983; Wolf, 1986). Other important studies and theoretical developments showed that microwave methods are able to destabilize water/oil emulsions (Lao *et al*., 1996; Fang and Lai, 1995). Once demulsified, the oil and water can be separated easily.

Corresponding Author: N. Saifuddin, Department of Science, Chemistry Unit, College of Engineering, University of Tenaga Nasional, Km 7, Jalan Puchong-Kajang, 43900 Serdang, Selangor, Malaysia

However, the use of soluble lipases has shown drawbacks such as high cost of the single use of the enzyme, contamination of the products by lipase presence in the product stream and thermal instability for soluble lipase in solution. With immobilization of the enzyme, improve in stability, reusability, continuous operation, the possibility of better control of reactions, easy separation from the product stream and hence more favorable economical factors can be expected (Frense *et al*., 1996; Tischer and Wedekind, 1999). Many different methods of enzyme immobilization are available (Paiva *et al*., 2000). The high cost of popular supports (silica-based carriers and synthetic polymers) causes many to search for a cheaper substitute such as $CaCO_3$ (Rosu *et al*., 1998), rice husk and rice straw (Tantrakulsiri *et al*, 1997) or chitin and chitosan (Martino *et al*., 1996; Felse and Panda, 1999). The derivative of chitin, chitosan, appears to be the most attractive since chitin is the second the most abundant biopolymer in nature next to cellulose (Bailey *et al*., 1999). This support offers several advantages as an enzyme immobilization carrier such as low biodegradability; low cost; ease of handling; high affinity for proteins and, above all, nontoxicity (Felse and Panda, 1999). Good results were obtained in a number of previous studies in which chitosan were used to immobilize lipase (Itoyama *et al*., 1994; Carneiro da Cunha *et al*., 1999).

Hence in this study a better system for removal of the oil from the waste water, which is a two step system, is proposed: first the oily waste water emulsion is separated by emulsion breaking using microwave irradiation and then the bioremediation of the oil layer using enzymatic treatment

The major aims of the study are (i) to study oily waste water from local restaurant for oil-water separation treatment by breaking the emulsion by means of coalescence using microwave radiation (ii) to develop simple and reliable immobilization method of enzyme (lipase) into chitosan beads whereby the selected methodology and the support must be able to provide features like large surface area, high enzyme activity, operational and thermal stability, better control of the reaction process and reusability and (iii) utilization of the immobilized enzyme for effective biodegradation treatment of oil from the oily waste water.

MATERIALS AND METHODS

Materials: Oily waste water samples (5 each) were taken (between January and February) from three restaurants (Chinese restaurant, Cafeteria at UNITEN and KFC fast food restaurant) all near the UNITEN campus in Bangi, Selangor, Malaysia.

Commercial *Candida rugosa* lipase (Type VII), Bovine Serum Albumin (BSA) and chitosan in flake form were purchased from Sigma Chemical Co. (St Louis, MO, United States). Palm oil was purchased at a local market. Solvents were standard laboratory grade and other reagents were purchased either from Aldrich Chemical Co. (Milwaukee, WI, USA) or Sigma Chemical Co. (St Louis, MO, USA). Domestic microwave oven with a volumetric capacity of 0.0235 m^3, with an effective output power of 930 W.

Methods

Preliminary studies on catalytic activity of soluble lipase to determine its optimal pH, temperature, mass loading and effect of calcium ions: In this study, experiments were conducted to determine the optimal temperature, pH and mass loading effect of calcium ions for the hydrolysis reaction of palm oil (as substrate) with soluble lipase from *Candida rugosa*. Effect of calcium ions on the hydrolytic activity of the lipase was also studied.

Hydrolytic activities of free lipase were assayed by the oil emulsion method proposed by Soares *et al.* (1999) with slight modification. In the assay used in this study the substrate was prepared by mixing a 1:1 ratio by volume of palm oil with gum Arabic solution (7% w/v). Gum Arabic was added in these experiments, because it is most commonly used for emulsifying oils and fats (Wang *et al*., 1988). The liberated fatty acids were titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of enzyme, which liberated 1 mmol of free fatty acid per min under the assay conditions. The density of palm oil used in these experiments was 2.7 g mL^{-1}.

pH optimization studies were conducted in 50 mL of 100 mM Trizma-HCl buffer of desired pH in the range of 4 to 8. The reaction mixture contained 5 mL palm oil (density 2.7 g mL^{-1}), 5 mL gum Arabic solution (7% w/v) and 0.8 mg lipase enzyme with slow agitation (100-130 rpm). Optimum temperature was investigated using the same composition of reaction mixture (as above) along with 50 mL of phosphate buffer pH 7.0 (optimum pH) in the range of 20-60°C. The studies for optimal enzyme concentration were carried out by varying enzyme concentration at optimal pH and temperature.

The effect of calcium ion concentration on enzymatic reaction was also studied. The studies was carried out at optimal pH, temperature and enzyme concentration with varying calcium ion concentration (0, 1, 2, 3, 5, 7 mM of $CaCl_2$).

In each of the above optimization studies, the reaction was stopped after 30 min using acetone-ethanol

mixture (1:1) and the fatty acid liberated were assayed by titrating with 25 mM KOH solution in presence of phenolphthalein as indicator. One unit of enzyme activity was defined as the amount of enzyme, which liberated 1 mM of free fatty acid per min. under assayed condition.

Immobilization processes

Immobilization of Lipase on Chitosan: Lipase-loaded hydrogel beads were manufactured according to a modified method of Bodmeier *et al.* (1989), using sodium tripolyphosphate (TPP) as the gelling counterion. The chitosan dispersion was prepared by dissolving 150 mg in 8 mL of 1% v/v acetic acid. The enzyme solution was made by dissolving 15.0 mg of *Candida rugosa* lipase (EC 3.1.1.3) in 2 mL of 1% v/v acetic acid and then the two solutions were mixed. The beads were formed by dropping the 10 mL of bubble-free mixture through a disposable plastic syringe with a 22-gauge needle at a speed of about 70 mL h^{-1} into the gelling counterion medium (20 mL of a gently agitated solution of 0.5% w/v TPP, prepared in 0.05 M Tris HCl buffer pH 7.0). The beads were cured in the TPP solution for 75 min and then washed twice using 3 mL of a pH 7.0 Tris HCl buffer. The decanted solutions and washes were collected for further study. Freshly made or microwave dried beads were used for further studies. Microwave drying was found to be efficient and faster compared to air drying, oven drying, ethanol extraction or even freeze drying which usually took more than 18 hr. Drying was done using domestic microwave oven at low power (temperature 40°C) for 2 min. The drying process was repeated for further 2 min cycles, if the beads were not completely dried.

Determination of the encapsulation efficiency by protein assay: The decanted 20 mL of TPP solution and the two 3 mL washings were analyzed for protein content using the modified micro bicinchoninic acid (BCA) protein assay (Stoscheck, 1990). The amount of bound protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction and the amount of protein in the filtrate and in the washing solution.

For the assay samples containing 0.2 to 50 μg protein in 0.5 mL were prepared. 0.5 mL of the working solution [Working solution: 1 volume reagent C was mixed with 25 volumes reagent B, followed by addition of 26 volumes reagent A to the C/B mixture; Reagent A: 8 g sodium carbonate monohydrate, 1.6 g sodium tartrate, brought to 100 mL with distilled water, pH was adjusted to 11.25; Reagent B: 4 g BCA in 100 mL distilled water; Reagent C: 0.4 g copper sulfate pentahydrate in 10 mL water] was added to each sample and mix thoroughly. Mixture was incubated at 60°C for 1 h. The samples were cooled to room temperature. Different concentrations of 0.01-0.04 mg mL^{-1} of Candida rugosa lipase were used as standards. The enzyme lost in these decanted solutions and washes were quantified by measuring the absorbance at 562 nm (Beckman DU 7400 UV-Vis spectrophotometer). The entrapment efficiency percentage was obtained by calculating the percentage of the lipase entrapped, based on the initial 15.0 mg mass of lipase present in each chitosan dispersion (batch).

Determination of lipase enzymatic activity of the immobilized enzyme: Analyses of the hydrolytic activities was carried out on soluble lipase solution and immobilized preparation to determine the coupling yield (η%) according to the following expression:

$$\eta\% = \frac{\text{Overall activity of the immobilized enzyme}}{\text{Overall activity of the initial enzyme solution loaded}} \times 100\%$$

The enzymatic activity of free and immobilized lipase were assayed by the oil emulsion method proposed by Soares *et al.* (1999) with slight modification. In the assay used in this study the substrate emulsion mixture was prepared by mixing 50 mL of palm oil with 50 mL of gum Arabic solution (7% w/v) and 5 mM $CaCl_2$. Gum Arabic was added in these experiments, because it is most commonly used for emulsifying oils and fats (Wang *et al.*, 1988). The enzymatic determination was carried out the procedure involves hydrolysis of triglycerides from palm oil into fatty acids, diglycerides and to a negligible extent, monoglycerides and glycerol.

The reaction mixture containing 5 mL of the substrate emulsion mixture, 2 mL of 100 mM sodium phosphate buffer (pH 7.00) and either free lipase (1 mL, 7.5 mg mL^{-1}) or immobilized lipase (8.0 mg samples of freshly prepared beads) was incubated for 10 min at 37°C. The reaction was stopped by the addition of 10 mL of acetone-ethanol solution (1:1). The amount of fatty acids formed in 30 min, under the specific conditions of the test, is a measure of lipase activity in the sample. The liberated fatty acids were titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of enzyme, which liberated 1 mmol of free fatty acid per min under the assay conditions.

Waste water characterization

Characteristics of oily waste water: The following parameters, pH, oil content, BOD (Biochemical Oxygen Demand), TS (Total Solids) and VSS (Volatile Suspended

Solids) were determined for the oily waste water, in order to study the characteristic of the waste water.

Oil content was determined according to the standard method. The waste water sample was heated in a boiling water bath at 70°C for about 30 min to release the oils from the water and dirty matter. The oil was then extracted from the waste water by n-hexane in sepratory funnel. The oil was then quantified by collecting the n-hexane fraction, evaporating the hexane on a water bath and drying the oil to a constant weight in an air-oven at 105°C. This extraction was duplicated several times until there was no more oil present in the waste water sample.

TS, SS and VSS were carried out according to Standard Methods (Clesceri *et al*., 1989). BOD values were determined as described by Helrich (1990). BOD bottles containing 300 mL of properly diluted samples, which had been previously air-blown for 10 min, were incubated in the dark at 20°C for 5 days prior to the determination of dissolved oxygen using the azide method (Helrich, 1990).

Microwave irradiation of oily waste water: Two hundred milliliter of oily waste water samples was used initially to see the effect of demulsification by microwave irradiation. The waste water was exposed to microwave irradiation at output power of 700 W for 30, 60, 80, 120 and 240 sec. Acid was also added to the waste water to see if there was any improvement in the demulsification process. Hydrochloric acid (6 M HCl) of varying amounts (4, 8, 12, 16 and 20 mL) was used for a series of five sample of waste water, respectively, giving a final concentration of 0.12, 0.24, 0.36, 0.48 and 0.6 M acid.

Water recovery is the amount of aqueous phase that coalesces and separates from the emulsion after the radiation treatment. For determining the in initial amount of water, the Karl-Fisher titration method was used (U.S. EPA NEIC Method-9000, 1991). Initial amount of water in the samples was denoted as Vi. After heating the sample by the predetermined exposure time t, (30, 60, 80, 120, 240 sec), the coalesced water volume V_s, was extracted by periods of 2, 5, 10, 25 and 60 min. These periods are accounted as sedimentation time, t_s. For each t_s a water separation percentage was be calculated as below:

$$\% \text{ water separation} = \frac{V_s}{V_i} \times 100\%$$

For each exposure time t_s, percentage of water separation (% water separation) was plotted against sedimentation time, t_s. After separation the oil content obtained from the waste water was determined by the method mentioned earlier.

Enzymatic treatment of oil for oily waste water

Hydrolysis of oil from the waste water using soluble lipase: The oil separated from the waste water above was collected in a container for enzymatic treatment with soluble lipase. The substrate was prepared by mixing 50 mL of the above oil obtained from waste water with 50 mL of gum Arabic solution (7% w/v) and 5 mM $CaCl_2$. The hydrolysis of oil in waste water was carried out using soluble lipase according to the method described earlier except that the substrate was the oily portion of the waste water instead of palm oil emulsion. It has already been found that the optimum lipase loading was 0.7 mg g^{-1} oil when palm oil was used as substrate.

Hydrolysis of oil in the waste water using immobilized lipase: The oily portion of the waste water whose oil content had been determined was subjected to hydrolysis using immobilized lipase according to the method described earlier except that the substrate was the oil obtained from waste water instead of palm oil emulsion.

Multiple use of the immobilized lipase: Operational stability: Immobilization of enzymes provides an attractive opportunity for multiple use of the same enzymes. For this reason, the use of immobilized enzymes is now firmly established as an effective and economically favorable approach in industry. The purpose of this study was to assess the multiple use of the encapsulated lipase by measuring the enzymatic activity of lipase-loaded chitosan hydrogel beads, as well as the enzyme lost, in five sequential reactions.

The operational stability of the immobilized system was assayed by using 0.5 g of immobilized lipase (dry weight) in palm oil hydrolysis in successive batches. At the end of each batch, the immobilized lipase was removed from the reaction medium and washed with hexane to remove any substrate or product retained in the matrix. One hour later (length of time required for evaporation of the solvent), the immobilized lipase was introduced into a fresh medium. Activities were estimated at the end of each cycle and expressed as μmol/min/mg of catalyst.

RESULTS AND DISCUSSION

Preliminary studies on factors affecting the hydrolysis reaction rate of soluble lipase from *Candida rugosa*: Enzymatic activities are strongly dependent on pH and temperature. Figure 1 indicates that the lipase from *C. rugosa* maintains better activity in acidic range with optimal pH at 7.0. Lipase activity dropped significantly when pH was above 7.0. The temperature dependence determined at optimum pH of 7.0 showed that the enzyme

Table 1: Effect of calcium ions on lipase catalytic activity

Concentration of $CaCl_2$ (mM)	Enzyme catalytic activity (μmol/min/mg lipase)
0	2.34
1	3.15
2	5.21
3	5.81
5	6.75
7	5.73

The reaction mixture contained 50 mL of 25 mM phosphate buffer pH 7.0 containing 5 mL palm oil, 5 mL gum Arabic solution (7% w/v), 0.8 mg of lipase enzyme and desired amount of calcium ions (0, 1, 2, 3, 5, 7 mM $CaCl_2$) at the temperature of 40°C for 30 min with slow agitation

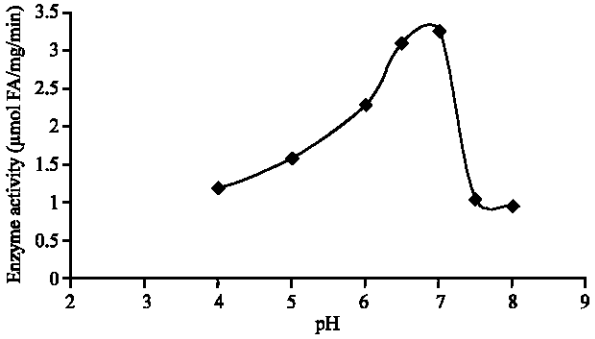

Fig. 1: Enzyme activity as a function of pH. Fifty milliliter Trizma-HCl buffer of desired pH containing 5 mL palm oil, 5 mL gum Arabic solution (7% w/v) and 0.8 mg lipase enzyme at 37°C with slow agitation (100-130 rpm). Reaction was stopped after 30 min by the addition of 10 mL of acetone-ethanol solution (1:1). Fatty acid liberated was assayed

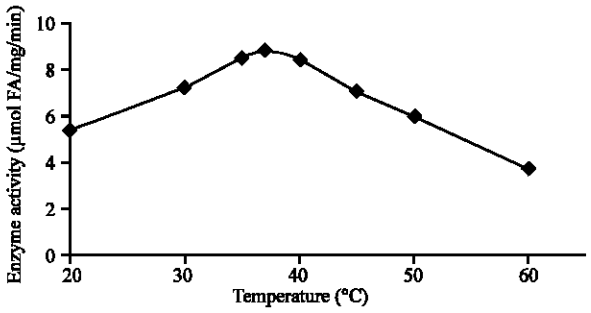

Fig. 2: Enzyme activity as a function of temperature. Fifty milliliter phosphate buffer pH 7.0 containing 5 mL palm oil, 5 mL gum Arabic solution (7% w/v) and 0.8 mg lipase enzyme at desired temperature for 30 min with slow agitation. Reaction was stopped after 30 min. by the addition of 10 mL of acetone-ethanol solution (1:1). Fatty acid liberated was assayed

activity was higher between 30-40°C, peaking at 40°C as shown in Fig. 2. At above 40°C, the lipase activity dropped sharply. It is well known that most protein

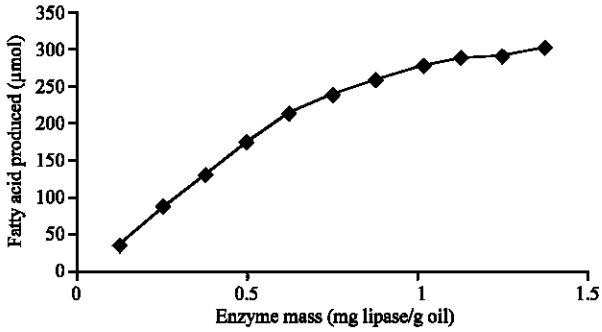

Fig. 3: Fatty acids produced as a function of lipase load. Fifty milliliter of 25 mM phosphate buffer pH 7.0 containing 5 mL palm oil, 5 mL gum Arabic solution (7% w/v) and varying amount of lipase enzyme at the temperature of 40°C (optimum temperature) for 30 min with slow agitation. Reaction was stopped after 30 min as mentioned above reaction was stopped after 30 min by the addition of 10 mL of acetone-ethanol solution (1:1). Fatty acid liberated was assayed

denaturation begins to occur at 45°C and becomes more severe at 55°C. Experiments were also done at fixed substrate concentration (10% v/v) using varying enzyme concentration to see the effect of enzyme amounts on the hydrolysis rate. Figure 3 shows the amount of fatty acid released as a function of lipase concentration. It can be seen that fatty acid production reaches maximum at lipase concentration of 0.7 mg g^{-1} oil and begins to drop when the enzyme load was increased above 0.7 mg g^{-1} oil.

Generally, calcium ion was reported to be an important enhancer in various enzymes (Fu *et al*., 1995; Xia *et al*., 1996). In this study calcium ion enhance the reaction rate by up to 2.9 times at calcium concentration of 5 mM (Table 1). It has been suggested that calcium ion may remove free fatty acids produced during hydrolysis reaction, as insoluble calcium soaps, thereby permitting hydrolysis to continue (Jaeger *et al*., 1975). The polarization of calcium ions is greater than sodium ions, therefore calcium ions are more prone to interact with lipase than sodium ions (Xia *et al*., 1996).

Enzyme immobilization processes: When the chitosan dispersion in acetic acid was dropped into a TPP solution, gelled spheres formed instantaneously by electrostatic interaction between positively charged chitosan and negatively charged TPP. Approximately 220-240 beads were produced in each batch. The beads were dried using microwave at low power output (45°C) and the dried beads weighed about 90 mg per batch. The lipase used in this study was a fungal lipase, an excellent enzyme due to

Table 2: Adsorption recovery, coupling yield and catalytic activity of *Candida rugosa* lipase immobilized in the chitosan beads

Lipase forms	Bound protein (%)	Coupling yield (%)	Substrate	Amount of lipase/g oil	Hydrolytic activity (μmol fatty acid/mg lipase/g oil/min)
Soluble lipase solution (6.5 mg)	-	-	10 mL Palm oil	0.7 mg g^{-1} oil	0.611
Immobilized (lipase entrapped in chitosan beads)	79	42.0	10 mL Palm oil	0.7 mg g^{-1} oil	0.323

Lipase loading: 147 units/150 mg chitosan; Protein loading: 15 mg/150 mg chitosan; 10 mL of palm oil = 9.3 g; 50 mg of dried chitosan beads with immobilized lipase was used (0.13 mg lipase/mg beads)

Table 3: Characteristics of oily waste water

	Chinese restaurant	Cafeteria at UNITEN	KFC fast food
No. of samples	5	5	5
pH	6.6-8.8	6.4-7.9	6.9-8.9
BOD, mg L^{-1} (Biological Oxygen Demand)	1644-21340	1120-17330	1440-19560
Oil and Fat, mg L^{-1}	380-8020	185-4125	360-8500
TS, mg L^{-1} (Total Solid)	235-3750	205-2960	205-3040
SS, mg L^{-1} (Suspended Solid)	216-1320	157-2780	180-3230
VSS, mg L^{-1} (Volatile Suspended Solid)	160-1100	60-1450	107-2036

its pH stability, substrate specificity and thermal stability over a wide range than possible for other lipases (Bickerstaff, 1995). Lipases have been successfully used to catalyze the hydrolysis of triglycerides for the production of fatty acids, a reaction that takes place in the degradation of oil in treatment of oily waste water.

Entrapment efficiency: Even if a hydrogel matrix can be achieved, it is also important to examine the ability of the polymer or the resultant matrix to efficiently retain the enzyme during the various steps of the manufacturing processes and hence a low entrapment efficiency would limit the use of such a process. Entrapment efficiency was calculated by determining the loss of the lipase (protein content assay) during the entire manufacturing processes. A minimum loss of lipase during the curing process would lead to a high loading efficiency of the beads.

It was found that the amount of protein lost in the washing process was about 21% which gives an entrapment of enzyme in the chitosan beads of about 79% at pH 7.0 (Table 2). This was not surprising considering the literature claim that microbial lipases have shown a profound stability and maximum enzymatic activity at a pH of 6.5-7.0 (Arpigny and Jaeger, 1999). By preparing the beads at a near neutral pH, the lipase is more likely to be in its fully functional conformation even if it has associated in some way with the hydrogel matrix material. Based on 79% entrapment efficiency, the 90 mg of dried beads produced per batch (initial protein loading per batch is 15 mg/150 mg chitosan) would have protein content (protein bound) in the chitosan beads as 11.85 mg protein or 0.13 mg protein per mg dried chitosan beads.

Enzymatic activity of immobilized lipase: Catalytic property of the immobilized lipase was obtained by performing hydrolysis reaction on palm oil (as substrate). Comparison was done using free lipase instead of immobilized lipase. Table 3 shows that the enzymatic activity of the entrapped lipase in the chitosan bead is about 0. 323 μmol/mg lipase/g oil/min.

The characteristics of the oily waste water: Oily waste water was characterized in terms of oil and fats content, BOD, pH, TS, SS and VSS, based on the analysis of a total of 5 samples from three restaurants near the UNITEN campus. The results are listed in Table 3. As expected the oil and fat content were very high and the BOD varied from 1120-21000 mg L^{-1}. Amongst these three waste water samples, the Chinese Restaurant waste water was selected randomly as the waste water resource for further studies.

Microwave irradiation of oily waste water-Demulsification process: Microwave irradiation is able to destabilize water/oil emulsions by two means. First by increasing temperature, which reduces the continuous phase viscosity and breaks the outer film of the drops, thus allowing for coalescence and second by rearranging the electrical charge distribution of the water molecules while rotating them and moving ions around the drops. These two actions combined result in the breaking of the emulsion without adding any chemical agent (Lao *et al*., 1996; Fang and Lai, 1995). Once demulsified, the oil and water can be separated easily. Microwave power output and irradiation time have effects on the demulsification process (Fig. 4). There was no aqueous phase separation observed with a 240 W power output even after 300 sec irradiation. With power output of around 900 W, a critical irradiation time greater than 30 sec is necessary to give any significant phase separation. Demulsification rate increased with the irradiation time. However, irradiation time greater than 300 sec suffered from boiling over. Therefore, 900 W power output and irradiation time ranging from 30 to 240 sec were used for all subsequent experimental runs. The maximum exposure time, which gives the highest water separation percentage, is between 220-240 sec. Beyond this time the waste water solution would over boil.

Table 4: Enzyme activities for different lipase forms on oil from oily wastewater

Lipase forms	Substrate	Lipase amount (mg)	Enzyme activity (μmol fatty acid/mg lipase/g oil/min).	Residual Activity (%)
Soluble (taken from Table 2 above)	10 mL Palm oil	6.5	0.611	100.0
Soluble	10 mL oil from oily waste water	6.5	0.515	84.3
Immobilized	10 mL oil from oily waste water	6.5	0.238	39.0

Oil and fat content in the oily waste water = 4655 mg L^{-1} waste water 10 mL oil from the waste water = 9.21 g 50 mg of dried chitosan beads with immobilized lipase was used (0.13 mg lipase/mg beads)

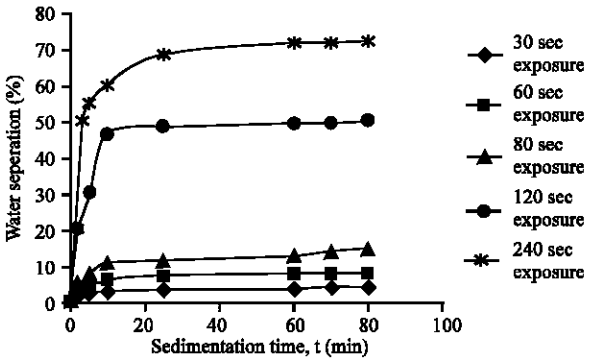

Fig. 4: Water separation (%) calculated at various sedimentation time interval for several exposure times, for oily waste water. Oily waste water volume = 200 mL. The experiment was carried out at output power of 700 W and was exposed to microwave radiation for 30 to 240 sec

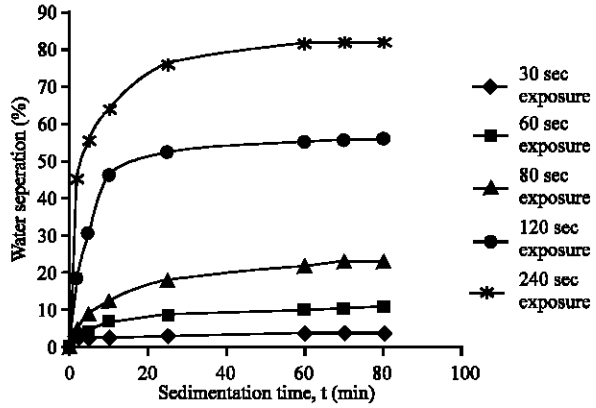

Fig. 5: Water separation (%) calculated at various sedimentation time interval for several exposure times, for oily waste water with HCl acid (0.36 M final concentration) added. Oily waste water volume = 200 mL. The waste water contained 0.36 M HCl and the experiment was carried out at output power of 700 W. The sample was exposed to microwave irradiation for 30 to 240 sec

Figure 5 shows the effect of 0.36 M (final concentration) on the demulsification process of oily waste water. It was observed that solutions with different amount of acids do affect the demulsification process. Separation efficiency and demulsification rate increased with the acid concentration. The addition of acid can convert fatty acids from water soluble salts to non-water-soluble free acids. Ionic migration is thought to be responsible for this effect (Strauss and Trainor, 1995; Chan and Chen, 2002). Due to the effects of both dipole rotation and ionic conduction, acidified emulsions will have higher dissipation factor and thus higher microwave heating effect. A solution with high dissipation factor gives good microwave absorption capability and thus shows high microwave heating effect. This was clearly observed in the experiments with emulsions added with acid up to a final concentration of 0.48 M. In this range of acid concentration the separation efficiency and demulsification rate increased with increasing acid concentration. However at acid concentrations greater than 0.5 M the dependence was different. The separation efficiency and demulsification rate decreased with acid concentrations higher than 0.5 M. The limitation of dipole rotation of water molecules due to salting effect, especially in the concentrated range, is thought to be responsible for this reverse effect (Chan and Chen, 2002; Larhed and Hallberg, 2001)

Microwave treatment also brings about additional advantages such as the destroying of some water-borne bacteria by heating and removal of some of the water-soluble inhibitors in the waste water as the oil is separated from the water layer. This is important since enzymes are sensitive to inhibition by heavy metals and microbes.

Hydrolysis of oil in the waste water: Table 4 compares the fatty acids production when oily waste water treated with microwave irradiation, untreated oily waste water and palm oil were used separately as substrate under identical conditions. It clearly shows that hydrolysis of palm oil proceeds more rapidly than the hydrolysis of oils from the waste water. It indicates that there are some the substances present in the oil from the waste water, which inhibits the lipase by about 15%.

Repeated use of the immobilized lipase: Commercially available lipases can be expensive and methods to extend their active life have been investigated and developed. Immobilization, in particular, offers the advantage of facilitating enzyme recovery for re-use. Three sets of

70 beads each were involved in five consecutive enzymatic reactions. A negligible loss of activity could be measured after five consecutive runs, which indicates the re-usability of the entrapped enzyme. Dried hydrogel beads retained activities exceeding 70% of their initial activity after five cycles. This retention in activity is much better that reported for other immobilized lipases that retained only 45% of their initial activity after five cycles (Carneiro-da-Cunha *et al*., 1999; Tischer and Kasche, 1999).

CONCLUSIONS

The study consisted of breaking the simplest of the emulsions in terms of content, in order to obtain free oil/fat from the oily waste water. The sample emulsions underwent a domestic microwave radiating process at several exposure times. A very good separation of aqueous and oil phase was obtained when the sample was irradiated at 900 W for a duration of 220 sec. This study shows the successful attempt in using chitosan hydrogel beads as a means to encapsulate lipase enzyme (proteins). The entrapment using an ionotropic gelation technique provided a quick and effective method for producing spherical and rugged beads with desirable characteristics. The lipase in the optimized bead did not exhibit a substantial loss of activity after five consecutive runs, which indicates the re-usability of the entrapped enzyme. The current study covers only the improvement in lipase activity via immobilization and the use of microwave to break the oil-water emulsion. More comprehensive studies are required in the following areas:

Further theoretical modeling for lipase reaction kinetics may be required to explore the mechanism of hydrolysis.

Further detailed studies are needed for external and internal diffusion effects to the immobilized lipase system.

More detailed investigations are required to evaluate the effects of microwave heating on breaking of oil-water interfacial and effects of other additives to enhance the process.

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