Preliminary investigations into the utilization of lipase cross-linked enzyme aggregates (CLEA) in the hydrolysis of lipid-rich wastewater

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Research Article

Keywords: Lipase, *Rhizopus oryzae*, cross-linked enzyme aggregates, wastewater treatment

Posted Date: February 1st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1318116/v1>

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Abstract

This study utilized *Rhizopus oryzae* lipase (*RoL*) as a potential biocatalyst in the hydrolysis of oil-contaminated wastewater. Immobilization of the *RoL* occurred via the preparation of cross-linked enzyme aggregates of the *RoL* using ammonium sulphate (50% w/v) and glutaraldehyde (0.125% v/v) as the precipitant and cross-linker respectively. Characterization of the *RoL*-CLEA was carried out using scanning electron microscopy. Prior to the enzymatic treatment of the wastewater, the lipid concentration and the chemical oxygen demand levels were determined. The *RoL*-CLEA could be reused up to five cycles and its catalysis increased free fatty acid levels in the wastewater by 84.8% and 142.1% at 0.5% and 1% (w/v) CLEA respectively. There was an increase in chemical oxygen demand (COD) removal by 7.6% and 29.5% from the oil-contaminated wastewater when 0.5% (w/v) and 1% (w/v) of the immobilized biocatalyst was used. In conclusion, the *RoL* CLEA is a unique biocatalyst in the treatment of oil-contaminated wastewater. Hence, there is the possibility of utilizing this biocatalyst for the large-scale remediation of oil-contaminated water bodies.

Introduction

The hydrolysis of triglycerides to free fatty acids and glycerol is catalyzed by lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) ^[1]. Besides hydrolysis, lipases catalyze various synthetic reactions, such as transesterification, in low water activity media ^[2].

It is widely known that severe environmental pollution can result from oil-contaminated wastewater. The diffusion of oxygen from air into water is prevented by the formation of oil film on water surfaces, causing the death of numerous aquatic lives ^[3]. Also, particles in wastewater, especially aggregates formed by oil droplets, can also cause blockage in drainage lines ^[4]. Besides the aforementioned environmental problems, there are also operational problems, which may arise from the non-treatment of wastewater with high fat contents, such as granular biomass flotation ^[5], fat scum layers forming at the surface of the reactor as well as fats solidifying at lower temperatures leading to clogging and unpleasant odour ^[6].

Employment of specific enzymes such as lipases has gained wide attention due to the clean and friendly nature of enzyme applications, as well as the firm environmental regulations ^[3]. Previous studies have shown the feasibility of enzymatic treatment of lipid-rich wastewater which aids in increasing its anaerobic biodegradability ^[5].

Free enzymes can only be utilized once in solutions because they are generally soluble and unstable. They are also susceptible to and frequently inactivated by various environmental conditions including inhibitors, ionic strength and pH ^[7]. Most importantly, they are too expensive to be used in wastewater treatment. These challenges can be overcome by immobilization which has its unique benefits.

Immobilization confers certain advantages on the enzymes such as increased stability at different temperature, pH and ionic strength, as well as recyclability from the reaction mixture^[8]. Different methods have been employed for lipase immobilization over the years, including cross-linking, adsorption, multipoint covalent attachment and physical entrapment^[9], with each having its advantages, disadvantages and peculiarities. The cross-linking of enzyme molecules' physical aggregates results in the formation of cross-linked enzyme aggregates (CLEAs)^[10]. Some advantages over carrier-bound immobilized enzyme is the important savings on the cost of the manufactured superstructure due to the lack of support, the simplicity and broad applicability of the technique^[11] and the reality that no previous purification of the enzyme is required^[10, 12]. The study aimed to immobilize *Rhizopus oryzae* lipase by cross-linking and evaluate the application of the immobilized enzyme in wastewater hydrolysis.

Materials And Methods

2.1. Materials

p-nitrophenyl butyrate (pNPB), ammonium sulfate, p-nitrophenol, glutaraldehyde, tritonX-100, oleic acid, isopropanol, toluene, sodium dihydrogen phosphate, disodium hydrogen phosphate and methyl heptadecanoate were purchased from Sigma-Aldrich (St. Louis, USA). Pierce™ BCA (bicinchoninic acid) protein assay kit was obtained from Thermo Fisher Scientific™ (Massachusetts, USA). All the chemicals and reagents used were of analytical grade.

2.2. Preparation of Cross-linked Enzyme Aggregates (CLEAs) of RoL-ZAC3

Crude and purified RoL-ZAC3^[13] were prepared as previously reported and immobilized by cross linking with glutaraldehyde to form CLEAs, which is a simple and robust immobilization method to provide highly stable enzymes with superior retention of activity. Ammonium sulfate was progressively added to the enzyme solution to reach 50% saturation, and the mixture was stirred on a magnetic stirrer at 4 °C for 30 min before glutaraldehyde was added in a dropwise manner to reach a final concentration of 0.125% (v/v)^[10, 14–15]. The mixture was again subjected to magnetic stirring at 4 °C for 24 h and centrifuged at 4000 rpm. The recovered pellets were washed with phosphate buffer (50 mM, pH 8), dried and stored at 4 °C^[16]. The activity of the CLEA was determined by the modified copper soap colorimetric assay method^[16] which estimates the amount of fatty acids released from the hydrolysis of triglycerides. The substrate consisted of 1 ml of emulsion of olive oil–isopropanol (1:10 v/v) and 1 ml of phosphate buffer (50 mM, pH 8) which was pre-incubated for 5 min at 37 °C. Then, 0.1 mg of CLEA was added to initiate the reaction. The reaction was stopped with 1 ml of toluene after 15 min and the upper organic phase containing the free fatty acids was separated. To this phase, 1 ml of 100 mM copper acetate was added and the absorbance was read at 710nm and oleic acid standard curve was used to estimate the fatty acid concentration. After each cycle of catalysis, the CLEA was washed and reused to catalyze another round of olive oil hydrolysis. The CLEA was also characterized by obtaining a scanning electron micrograph of the CLEA and its size was determined.

$$\text{CLEAActivity(U/g)} = \frac{\text{Abs} \times V}{m \times M \times t}$$

where Abs = Absorbance at 710nm

V = Volume of fatty acid solution

m = slope of oleic acid standard curve

M = mass of CLEA

t = reaction time

$$\text{ActivityRecovery(\%)} = \frac{\text{totalactivityofCLEA(U)}}{\text{totalfreeenzymeactivityusedforCLEAproduction(U)}} \times 100$$

2.3. Waste Water Characterization

2.3.1. Lipid Content Determination

The determination of the lipid content of the treated and pre-treated wastewater was done according to the method of Prasad and Manjunath ^[17]. Diluted HCl (1:1) was used to acidify 250 ml of wastewater sample to pH 2.0. Thirty milliliters of 1,1,2-trichlorotrifluoroethane was repeatedly used to extract the lipids in the wastewater. This was done until the solvent phase was clear and there was no oil layer shown in the aqueous phase. The solvent extracts were combined and dried by evaporating at 70°C. The amount of lipid present in the sample was indicated by the dry weight obtained following evaporation.

2.3.2. Determination of Chemical Oxygen Demand (COD)

The wastewater sample (100 ml) was digested overnight using 10ml of 0.1N potassium dichromate and 20ml of concentrated sulphuric acid. After 24 hours of digestion, 100 ml of distilled water and 5 ml of orthophosphoric were added to the mixture. The blank was obtained by using distilled water in place of the sample. The sample mixture was then titrated against 0.1N ferrous ammonium sulphate with a few drops of 5% barium diphenylamine sulphonate as the indicator ^[18, 19].

2.4. Enzymatic Wastewater Hydrolysis

The hydrolysis reactions were carried out in 250 ml flasks containing 50 ml of raw wastewater (from vegetable oil factory). The wastewater pH was adjusted to 8, followed by the addition of the required lipase amount (0.5 or 1% w/v of the immobilized CLEA) and sodium ions (0.1% w/v) as additive for the lipase activity. Reactions were carried out at 40 °C at a constant speed of 150 rpm in a shaker for 4 h. An aliquot of sample (1 ml) was withdrawn from the reaction and transferred to a new tube. Toluene (1 ml) was added to the sample to extract the fatty acid and to inactivate the enzyme. This was followed by the addition of 1 ml of 100 mM copper acetate in order to determine the concentration of free fatty acids

released. Absorbance was read at 710nm and fatty acid concentration was estimated from the oleic acid standard curve. The free fatty acids produced during the hydrolysis reaction were used to calculate the percentage hydrolysis.

Results And Discussion

Cross-linking the lipase with glutaraldehyde was a form of immobilization to create stable cross-links (Schiff bases) between the aldehyde group of the glutaraldehyde and the lipase amino group ^[15, 20]. The scanning electron micrograph of the cross-linked lipase (*RoL-ZAC3-CLEA*) showed that it is an amorphous structure with many cavities and a high surface area, an indication that the cross-linked enzyme molecules are easily accessible to the substrates in order for reactions to proceed (Fig. 1).

Both crude and purified lipases were cross-linked and aggregated and both were active in catalyzing the hydrolysis of olive oil to oleic acid. Though the purified *CLEA* gave higher activity per cycle compared to the crude, it lost activity completely after the fifth cycle. The crude did not show as much activity as the purified, however it was still active up to the eighth cycle (Fig. 2A & 2B). The fact that the crude enzyme could be used directly makes the procedure an economically viable one. The crude also showed full activity recovery consistent all through the eight cycles (Fig. 2C). The loss of activity observed as the number of cycles progressed may be due to leaching of the enzyme molecules during the washing steps ^[20]. Reports in literature have it that *CLEAs* present higher stability than free un-immobilized enzymes because of the rigidity conferred on the enzyme structure by the cross-linking. The immobilization also confers on the enzyme conformation protection from distortion that may arise from extremes of pH and/or temperature. Thus, the enzyme is less prone to activity loss even in tough environments ^[15]. In terms of the parameters assessed following enzymatic hydrolysis of lipid-rich wastewater with *RoL-ZAC3-CLEA*, an increase in free fatty acids and a decrease in lipid content and chemical oxygen demand (COD) were observed after incubating for 4 h. Increasing the *CLEA* amount from 0.5–1% w/v increased the free fatty acid levels from 102.4 to 134.1 μmol , which corresponds to an increment of 84.8% and 142.1% respectively compared to the control (Table 2). Untreated lipid-rich wastewaters normally have high concentrations of lipid and chemical oxygen demand levels ^[21]. Decreased COD is an indication of decreased organic content. This decrease in COD, tells the quantity of oxidizable/organic pollutants in wastewater expressed as the mass of consumed oxygen over the solution volume ^[22]. There was an increase in COD removal by 7.6% and 29.5% from the wastewater when 0.5% (w/v) and 1% (w/v) *RoL-ZAC3-CLEA* were used respectively (Table 3). The efficiency of the enzymatic degradation was verified by comparing with the control (which was wastewater not pretreated with enzyme). Oily wastewater treatment helps to improve its biodegradability and reduce its toxicity ^[23]. Prasad and Manjunath ^[17] obtained a 22% increase in the COD removal from swine and bovine meat industry wastewater following treatment with bacterial lipase. The successful utilization of *RoL-ZAC3-CLEA* in the degradation of lipid-rich wastewater makes the immobilized enzyme a promising alternative for this application.

Conclusion

Cross-linked RoL-ZAC3 at 0.5% and 1% w/v hydrolyzed lipid-rich wastewater, decreasing the total lipid by 4.5% and 18% respectively and improving other wastewater properties such as free fatty acids and COD. The immobilized enzyme showed potential in wastewater degradation that might be exploited on a large scale remediation of oil-contaminated wastewater.

Declarations

Declaration of interests

The authors declare no potential conflict of interests

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Table 1: Wastewater Characterization

Parameter	Value
pH	5.9
Initial COD (mg/ml)	23.7
Lipids (mg/ml)	2.0
Free fatty acids (μmol)	55.4

Table 2: Wastewater degradation with *RoL-ZAC3* at two different concentrations

<i>RoL-ZAC3</i> (% w/v)	Wastewater	Free fatty acid (μmol)	Lipids (mg/ml)	COD (mg/ml)
0	Control, C	55.4 ± 3.2	2.0 ± 0.1	23.7 ± 1.5
0.5	Wastewater, W1	102.4 ± 9.5	1.9 ± 0.1	21.9 ± 0.8
1	Wastewater, W2	134.1 ± 3.3	1.7 ± 0.2	16.7 ± 3.0

Table 3: Summary of wastewater degradation parameters

ROZAC3-CLEA (%w/v)	% Free Fatty acids	% Lipid Hydrolysis	% COD Removal
0.5	84.8	4.5	7.6
1	142.1	18.0	29.5

Figures

Figure 1

Scanning electron micrograph of the *RoL*-ZAC3-CLEA.

Figure 2

Activity of crude and purified CLEA. B. Total activity of crude and purified CLEA. C. Activity recovery (%) of crude and purified CLEA.