Pretreatment of Coconut Mill Effluent Using Celite-Immobilized Hydrolytic Enzyme Preparation from *Staphylococcus pasteuri* and Its Impact on Anaerobic Digestion

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Biological treatment of oil and grease (O&G)-containing industrial effluents has long been a challenging issue. Practically feasible avenues to bring down their O&G load and enhance treatability are desired. In one such endeavour, the partially purified lipase from Staphylococcus pasteuri *COM-4A was immobilized on celite carrier and applied for the enzymatic hydrolysis of unsterilized coconut oil mill effluent. In batch hydrolysis experiments, optimum conditions of 1% (w/v) immobilized lipase beads, one in four effluent dilution, and a contact time of 30 h resulted in 46% and 24% increase in volatile fatty acids and long-chain fatty acids and a concomitant 52% and 32% decrease in O&G and chemical oxygen demand (COD) levels, respectively. Batch anaerobic biodegradation trials with this prehydrolyzed effluent showed 89%, 91%, and 90% decrease in COD, proteins, and reducing sugars, respectively. These results were validated in a hybrid stirred tank—upflow anaerobic sludge blanket reactor. Average COD and O&G reductions effected by the hybrid reactor were found to be 89% and 88%, whereas that by the control reactor without enzymatic hydrolysis were only 60% and 47%, respectively. A maximum of 0.86 L methane gas was generated by the hybrid reactor per gram of VS added. Hence, this celite-immobilized crude lipase, sourced from a native laboratory isolate, seems to be a workable alternative to commercial enzyme preparations for the management of lipid-rich industrial effluents.* © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 31:1249–1258, 2015
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Introduction

Oil mill effluents are characterized by high biochemical oxygen demand (BOD), chemical oxygen demand (COD), and an especially high oil and grease (O&G) content, parameters which hinder conventional biological treatment of such effluents. The oil layer interferes with oxygen transfer in aerobic activated sludge systems and also encourages the growth of filamentous microorganisms, resulting in bulking sludge.[1] Sludge characteristics are adversely affected in anaerobic processes as well. This has serious impact on the process efficiency, owing to slow growth rate of microorganisms and low biomass production in anaerobic systems.[2] Moreover, lipid hydrolysis occurring during the treatment process can release glycerol and long-chain fatty acids (LCFA) that are inhibitory to microorganisms in the biological treatment unit.

Clogging of sewer systems, unpleasant odors, unsightly foam, and scum layers also occur, thus necessitating pretreatment to tackle the O&G contents in wastewater. Removal using dissolved air floatation and grease traps before release into the main biological treatment unit are often practised. However, these measures are ineffective in removing emulsified oil, and the cost is also high.[3] Hence, an alternative strategy of enzymatic hydrolysis could be applied to bring about lipid hydrolysis and improve biological treatment efficiency. Lipase-producing microbial strains find promising applications in this arena.[4]

Lipases (triacylglycerol acylhydrolases, EC. 3.1.1.3) are serine hydrolases that catalyze the hydrolysis of esters formed from glycerol and LCFA. Such ester hydrolysis is catalyzed in the aqueous environment. In nonaqueous environments, the reverse reactions of esterification, interesterification and transesterification occur. Lipases possess chemo-, regio-, and enantio-selectivities, finding widespread applications in leather, paper, textile, pharmaceutical, food, and detergent industries.[5]

The enzyme's relevance in the pretreatment of O&G-laden wastewaters has also been investigated. However, many such studies have reported the use of lipolytic microorganisms as whole cell biocatalysts, whereas others have relied upon commercially procured lipase preparations for such applications. Fungal and yeast lipases have been more extensively researched than bacterial lipases.[6–8] Enzymes directly obtained from laboratory-isolated wild-type bacterial strains have not been much applied in wastewater treatment. Our study makes use of one such novel source, the partially purified lipase from *Staphylococcus pasteuri* COM-4A (GenBank Accession No. KJ020928). The strain was previously isolated

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in our laboratory and tested for oil biodegradation.[9] This preparation exhibited multiple hydrolytic enzyme activities and its lipase, protease, and amylase activities were quantified to be 234, 128, and 85 U/mL, respectively. This multienzyme extract, predominantly comprising of lipase, was immobilized by physical adsorption onto celite carrier and applied for the pretreatment of coconut oil mill effluent. The efficacy of enzymatic hydrolysis was tested by comparing the anaerobic digestibility of prehydrolyzed and raw effluents.

Materials and Methods

Chemicals and samples

Celite R-630 for lipase immobilization and *p*-nitrophenyl palmitate (p-NPP) for the lipase assay were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and procured from Hi-Media, India. Raw effluent was collected from Shakthi Oil Mills located in Coimbatore District, Tamil Nadu, India, and stored at −4°C until further analysis. Acclimatized seed sludge for the anaerobic reactor was also sourced from the same unit.

Lipase immobilization

Immobilization was performed with the partially purified *S. pasteuri* COM-4A lipase, and the method used was modified from Weetall.[10] The celite beads were reacted with 1% solution of 3-aminopropyltriethoxysilane in acetone (v/v), dried at 110°C overnight, and further reacted with 2.5% glutaraldehyde in pH 7 phosphate buffer. 500 mg of lyophilized lipase was dissolved in 10 mL of 0.1 M sodium phosphate buffer (pH 9.0) and mixed with 5 g Celite under low stirring for 2 h at room temperature. The lipase adsorbed on the support was filtered and washed three times with the same buffer. The free and immobilized lipases were designated as FL and IL, respectively.

Activity assay

Spectrophotometric assay using p-NPP was adopted, and the procedure was modified from Winkler and Stuckmann.[11] Accordingly, the substrate solution was prepared by adding solution A (30 mg of p-NPP in 10 mL of isopropanol) into solution B (0.1 g acacia gum and 100 mL phosphate buffer, pH 9). The reaction mixture containing 1.0 mL of substrate solution and 0.3 mL of lipase was incubated at 50°C for 15 min. 50 μL of Triton X-100 was added at the end of the reaction time, to obtain a clear solution. The absorbance was measured in a Spectrophotometer (Shimadzu, UV-1800) at 410 nm, against an enzyme-free blank. Molar extinction coefficient of 0.0146 $\mu moles^{-1}\ cm^{-1}$ was used. One unit of lipase activity is defined as micromoles of *p*-nitrophenol released per minute under the assay conditions. All activity assays were performed in triplicate, and the values represent the mean.

Immobilization efficiency

The efficiency of the immobilization process was evaluated by determining the enzyme activity (U/g support), specific activity (U/mg protein), protein loading (%), and activity yield (%) of the IL preparation.[12]

PH and temperature stability of IL

For testing the pH stability, the IL was incubated in buffers of varying pH values ranging from 3.0 to 10.0. The thermal stability was determined through activity assays performed after incubating the enzyme preparation at temperatures ranging from 30°C to 80°C. The enzyme assays were performed under optimum conditions, and residual activities were calculated. The data were compared with FL.

Application of IL in effluent treatment

Effluent Collection and Characterization. The effluent sample was collected from a coconut oil mill that generates around 50 m^3/day of effluent. It originates from the production process and also from cleaning operations (equipment and floor cleaning). It was subjected to physical, chemical, and biological characterization. Although studies with olive mill effluents are rampant,[13,14] there are no data available on coconut mill effluent. Considering the extensive usage of coconut oil in the region, this effluent was chosen, aiming to reveal useful strategies for its management.

Preliminary Batch Studies—Enzymatic Hydrolysis. The batch treatability studies were carried out in 1-L Erlenmeyer flasks containing 250 mL of unsterilized effluent and varying amounts of IL. The pH of the flasks was adjusted to 9.0 ± 0.2 and incubated in an orbital shaker (120 rpm) set at 50°C, optimum pH and temperature for lipase activity. COD, O&G, LCFA, and volatile fatty acids (VFA) were the parameters estimated. The conditions for the batch hydrolysis experiments were optimized. To start with, different dilutions of the effluent were tested (1:1–1:5). The optimum dosage of IL to be added to the effluent was checked by varying the amount from 0.5 to 3.0 g. The optimum incubation time was arrived at by monitoring the change in pollution parameters on a 6-hourly basis for up to 36 h. The effect of existing effluent microflora on the process was also ascertained by comparing the results of unsterilized and sterilized (autoclaved at 121°C, 20 min) effluents. One flask without the enzyme served as control in all cases. All experiments were duplicated, and the values represent the mean.

Repeated Use of IL. The immobilized lipase preparation was reused in effluent O&G hydrolysis for several cycles, and the percentage of retained activity was measured every time. After each use, the enzyme was separated from the reaction medium, washed with phosphate buffer in order to remove traces of retained substrate or product, and dried at room temperature, before starting the next cycle using fresh substrate.

Anaerobic Biodegradability Tests. The tests were performed based on the methods of Pereira et al.[15] and Mendes et al.,[16] with a few modifications. One-liter flasks containing 250 mL of prehydrolyzed effluent were flushed with N_2 gas and sealed. A 10% inoculum in the form of sludge from an upflow anaerobic sludge blanket (UASB) reactor treating oil mill effluent and containing acclimatized microbial communities was added to the flasks. This study was performed using effluent samples with varying free fatty acids (FFA) content (T_1–T_5), and the efficiency of enzymatic pretreatment was judged based on the extent of organic load removal and biogas production. Unhydrolyzed effluent was taken as control in one of the flasks (C_1). Unhydrolyzed effluent and 1 g of IL were taken in another flask (C_2) to assess the effects of simultaneous hydrolysis and anaerobic digestion. All flasks

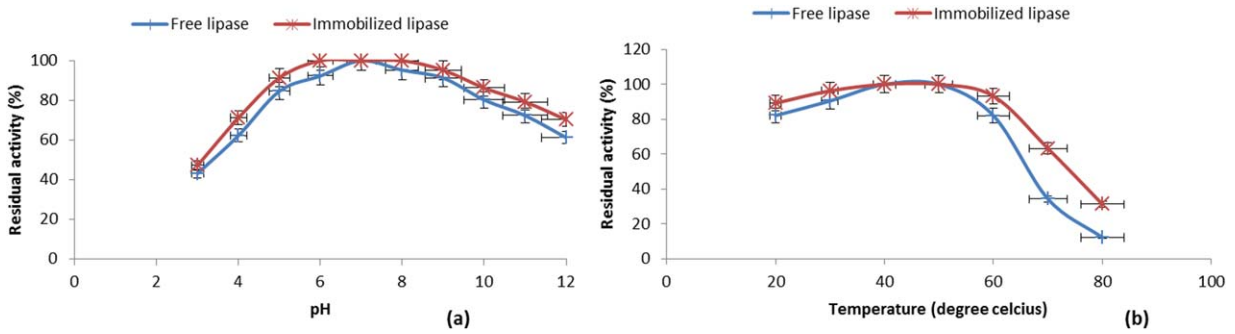

Figure 1. pH and temperature stability profiles of free and immobilized *Staphylococcus pasteuri* COM-4A lipase.

were incubated at 35°C ± 1°C for a period of 10 days. They were connected to inverted Duran flasks containing 0.5% w/v NaOH for biogas measurement.

Reactor Studies. A hybrid reactor system was designed for conducting enzymatic hydrolysis and anaerobic treatment of the effluent based on the configurations specified by Jeganathan et al.[17] and Leal et al.,[18] with relevant modifications. Accordingly, the effluent was pretreated in a 1.8-L bench-scale stirred tank reactor made of Plexiglas and containing 5 g of IL. This pretreated effluent was then passed into a 10-L UASB reactor made of PVC and seeded with sludge from a large-scale reactor treating oil mill effluent. The reactors were operated for 70 days and upon attainment of pseudostate-conditions, the flow rates were doubled, and reactor performance at higher organic loading rates was assessed. A control system comprising of only the UASB reactor was also operated for comparing the results. The methane gas generated was measured using MTL GIR6000, Biogas Analyzer.

The UASB reactor was chosen for anaerobic digestion because it is the most widely and successfully used high-rate anaerobic technology for treating several types of wastewater. The success of the reactor can be attributed to its capability to retain a high concentration of sludge and efficient solid–liquid–gas phase separation. In fact, one of the main benefits of the UASB concept is that it selects for microorganisms with the best settling properties. The reactor is also best suited for operation in tropical countries like India, where the ambient temperature throughout the year is high, at around 25°C–35°C. Further, the technology is simple in construction and operation, and hence low in cost.

Analytical methods. Analysis of total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), BOD, COD, and O&G were carried out as per the protocols of Standard Methods for the Examination of Water and Wastewater.[19] LCFA and VFA in the sample were measured using a gas chromatograph (Shimadzu, Japan) equipped with FID detector (split/splitless injection system with auto-sampler). An analytical column of fused silica type (0.25 mm × 3 m, Agilent, India) was used. The detector temperature was set at 280°C. The column temperature was initially set at 150°C for 1 min, then increased to 225°C at the rate of 10°C per min, and maintained at that temperature for 20 min. Hydrogen was used as carrier gas (average linear velocity 30 mL/min). Fatty acid distribution was also estimated, and the peaks of the respective fatty acid methyl esters were identified using Sigma standards.[20] Acidity and saponification values of the effluent were determined based on the modified protocols of Conceição et al.[21] Protein was assayed using Folin–Ciocalteu reagent,[22] and reducing sugars were determined using 3,5-dinitrosalicylic acid reagent.[23] Glycerol was determined according to the methodology proposed by Coks and Rede.[24] Effluent color was determined according to the CPPA Standard Method.[25]

Results and Discussion

Immobilization efficiency

The immobilized lipase preparation exhibited an activity of 254.43 U/g of support and a specific activity of 51.19 U/mg of protein. A protein loading of 83.14% onto the carrier and an activity yield of 91.38% were achieved. These findings suggest that enzyme immobilization by adsorption onto the celite carrier is an efficient immobilization method with good enzyme retention as well as activity of the bound enzyme. A *Burkholderia* lipase immobilized similarly on celite carrier had displayed an enzyme activity of 273.5 U/g celite, and the lipase loading was around 62.9 mg/g of carrier used.[26]

PH and temperature stabilities of IL

Concentration of H^+ and OH^- ions in the medium modify the enzyme's activity as a result of changes in three-dimensional structure that occur in close proximity to the active site.[27] In our studies, IL exhibited remarkable stability, with more than 90% residual activity in the pH range of 5–9. It also displayed better stability in the pH range of 10–12, upon comparison with FL (Figure 1a). Immobilized *Burkholderia cepacia* lipase had been observed to be most stable in the pH range of 6–9.[28]

Thermal stability is an important requirement for the enzyme to function as a biocatalyst at elevated temperatures. Although FL showed good stability in the range of 30°C–50°C, the IL exhibited appreciable activity retention upon exposure to a higher temperature of 70°C (Figure 1b). The immobilized preparation could, thus, resist temperature-induced conformational change and subsequent activity loss. Upon incubation for 24 h at 50°C, the free *Pseudomonas gessardii* lipase had retained an activity of 52%, whereas its immobilized counterpart had displayed a residual activity of 79%.[29]

Application of IL in effluent treatment

Preliminary Batch studies—Enzymatic Hydrolysis. The physicochemical and biological characteristics of the raw effluent are listed in Table 1. Batch hydrolysis experiments

were carried out using IL, and their effects on key effluent parameters were assessed. The studies showed hikes in VFA and LCFA contents and a fall in O&G contents, because of ester hydrolysis catalyzed by the IL. A moderate decrease in the overall COD load was also witnessed owing to the action of hydrolytic enzymes on the effluent organic matter.

Initially, the effects of effluent dilution were studied, and a maximum of 29% COD and 45% O&G removal were achieved at 1:4 dilution. It also caused a simultaneous 38% VFA and 21% LCFA increase in the sample (Figure 2a). Because of the high organic load of the raw effluent, its dilution has resulted in better hydrolysis efficiency.

Table 1. Initial Characteristics of the Coconut Oil Mill Effluent

Parameter	Value
TS (mg/L)	7,853
TSS (mg/L)	4,582
TDS (mg/L)	3,271
TCOD (mg/L)	9,573
SCOD (mg/L)	4,586
BOD (mg/L)	6,970
O&G (mg/L)	3,582
Proteins (mg/L)	4,510
Reducing sugars (mg/L)	875
Glycerol (mg/L)	582
VFA (mg/L)	2,253
LCFA (mg/L)	697
Color (CU)	9,432
pH	6.2
Temperature (°C)	25
Density (g/cm^3)	1.03
Microbial load	
Bacteria (CFU/mL)	48×10^6
Fungi (CFU/mL)	31×10^3

TS, total solids; TSS, total suspended solids; TDS, total dissolved solids; TCOD, total chemical oxygen demand; SCOD, soluble chemical oxygen demand; BOD, biochemical oxygen demand; O&G, oil & grease; VFA, volatile fatty acids; LCFA, long chain fatty acids.

When the contact time of the effluent with the IL was varied, an increase in contact time resulted in enhanced enzymatic hydrolysis, and 30 h was concluded to be the optimum contact time, as there was only negligible change in the parameters upon further incubation. Over 30% COD and 50% O&G removal were achieved within this time duration. The percent increase in VFA and LCFA amounted to 46% and 24%, respectively (Figure 2b). In an experiment that investigated the effect of enzymatic hydrolysis on the anaerobic digestion of synthetic dairy wastewater, a contact period of 14 h had been found to be optimum for hydrolysis.[18]

The optimum dosage of IL was investigated, and 2.5 g proved to be the right amount for 250 mL of the effluent treated (1% w/v). It resulted in 37% COD and 52% O&G reduction, along with 41% VFA and 28% LCFA increase in the sample (Figure 2c). A maximum 78% fat hydrolysis had been achieved in dairy wastewater using lipase as biocatalyst, when coupled with ultrasound irradiation.[30]

When the hydrolysis kinetics of sterilized, unsterilized, and control samples were compared, the enzyme-free controls, whether sterilized or otherwise, showed only marginal changes in pollution parameters. In addition to the total COD (TCOD), soluble COD (SCOD) was also estimated in this study, to monitor any increase in SCOD that may occur due to the conversion of lipids into soluble products of hydrolysis. There were only marginal variations between the results obtained with sterilized and unsterilized samples. The unsterilized sample showed slightly higher reductions in TCOD and O&G, which could be attributed to the degradative activities of native effluent microflora. However, the corresponding increase obtained in SCOD, VFA, and LCFA was lower in the unsterilized effluent (Figure 2d). This paradox could be the result of soluble products of hydrolysis being used up partly for microbial metabolism. A study in

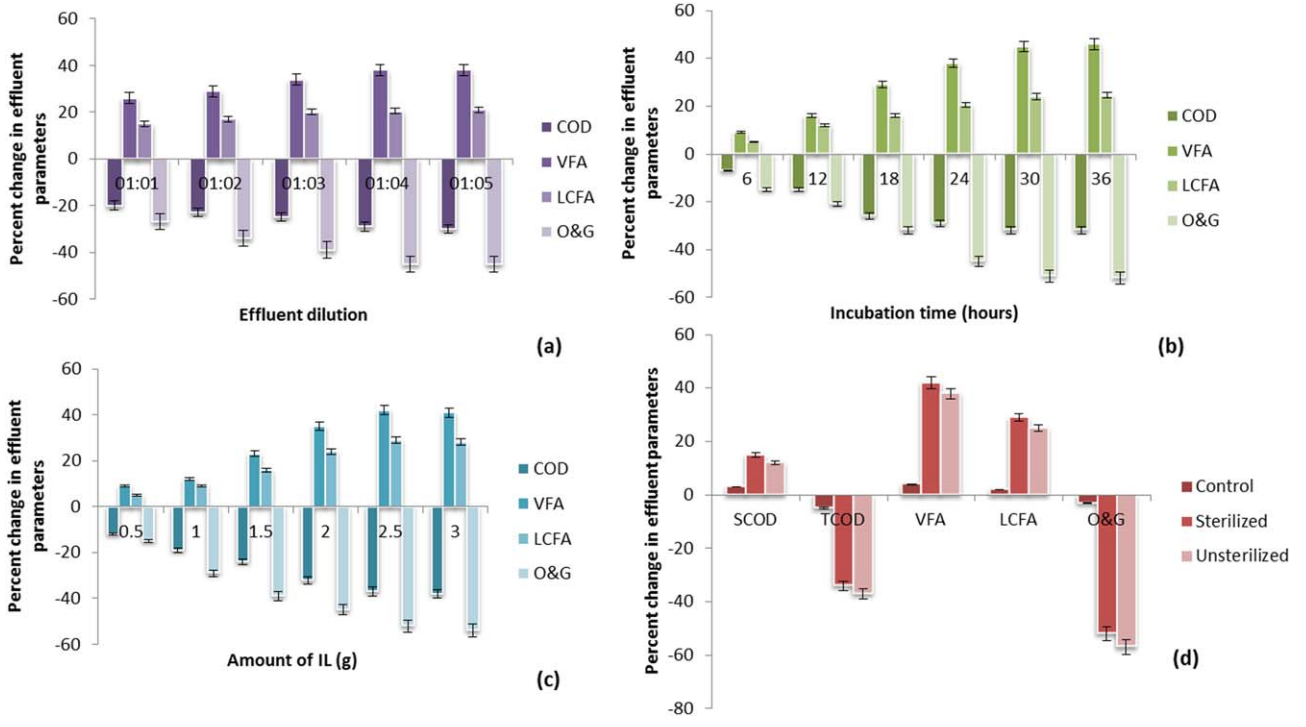

Figure 2. Effects of various factors on hydrolysis efficiency: (a) effluent dilution, (b) incubation time, (c) amount of immobilized lipase, and (d) effluent sterilization.

Table 2. VFA Distribution Patterns of Control, Sterilized, and Unsterilized Samples

	Percent Increase			
VFA	Control (Unsterilized)	Control (Sterilized)	Sample (Sterilized)	Sample (Unsterilized)
Acetic acid	8 ± 0.2	9 ± 0.13	36 ± 0.34	29 ± 0.24
Propionic acid	12 ± 0.08	10 ± 0.03	24 ± 0.09	18 ± 0.36
Butyric acid	6 ± 0.15	4 ± 0.26	42 ± 0.06	35 ± 0.07
Valeric acid	9 ± 0.25	9 ± 0.01	32 ± 0.15	27 ± 0.26

which the effects of enzymatic hydrolysis on fat particles in slaughterhouse wastewater was studied, SCOD had not significantly increased and had not served as a good indicator of lipolysis.[31] It could be concluded from our results that sterilization did not have any significant impact on the outcome, in terms of reduction in pollution parameters. Hence, further experiments were performed with unsterilized effluent, because it is not also practically feasible to carry out routine sterilization of effluent in a treatment plant. The study was done in order to establish that the added enzymatic preparation was largely responsible for the observed results and not the metabolic activities of indigenous effluent microflora. Because the observed variations between the sterilized and unsterilized effluents were always within 5% (Figure 2d), it could be postulated that the effluent microflora, especially in the absence of nutrient supplementation, did not influence the process to any significant extent.

These results have been obtained with partially purified IL. High specific activity of pure lipase preparation might give it an edge in catalyzing O&G hydrolysis. However, the moderate reduction in effluent organic load achieved during this hydrolytic pretreatment stage might not have been possible, because the carbohydrates and proteins present in the effluent and contributing to the COD load would have remained unaltered. Moreover, the extent of purity depends on the intended use of the enzymatic preparation. High-purity enzyme preparations come under high value, low volume products, and their applications in large-scale wastewater treatment would not be practically feasible or economically justifiable, even though bench-scale laboratory trials might appear promising.

It is important to document the changes occurring in individual components of VFA during the enzymatic hydrolysis. Both autoclaved as well as nonautoclaved controls showed only trivial increase in the VFA analyzed, whereas the enzyme-treated samples revealed substantial increase in VFA contents. Pronounced differences between sterilized and unsterilized samples were not observed, although the sterilized sample showed slightly higher increase in all fatty acids (Table 2). These studies confirm the release of VFA due to lipid hydrolysis and their partial utilization by microbial communities in the unsterilized effluent.

At the end of the hydrolysis experiments, the flasks were removed from the shaker and left undisturbed for the layers to separate. Visual observation showed three distinct layers—an uppermost frothy layer comprising of O&G, a predominant clear liquid layer in the middle with some dissolved solids, and a bottommost layer containing effluent settleable solids and the IL (Figure 3). The control flask showed a visibly higher volume of floats, which is due to the absence of O&G hydrolysis. Upon comparison of the sterilized and unsterilized flasks, the sterilized flask had more volume of clear liquid and fewer solids, probably due to the thermal effect of autoclaving.

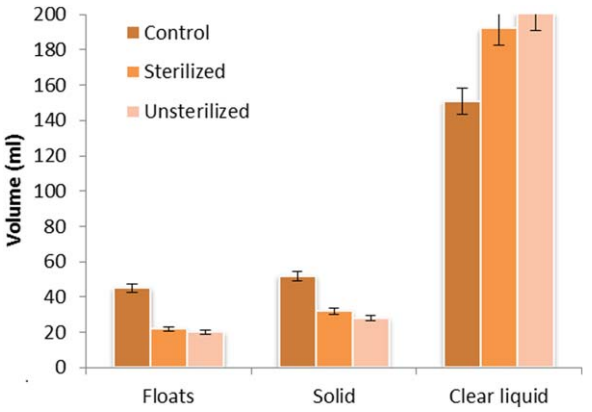

Figure 3. Separation of layers in control, sterilized, and unsterilized samples.

Reusability is a key requirement for immobilized enzymes, so as to make them more attractive for commercial applications.[32] In repeatability tests, the IL was able to retain appreciable hydrolytic activity (> 60%) for up to seven cycles, and the activity dropped drastically after the eighth cycle (Figure 4). This may be attributed to enzyme leakage or its inhibition by retained substrate or product molecules. *Candida antarctica* lipase immobilized by adsorption cross-linking methods had shown 62.1% activity retention after 10 repetitive uses for olive oil hydrolysis.[8]

The proportion of IL beads that could be recovered after the hydrolysis experiments and the degree to which they retain their enzyme activity are significant aspects that influence the economic viability of the process. These parameters were calculated after filtering, washing, and drying the IL at the end of the experiments. 69% beads recovery, 68% activity recovery, and 47% total activity recovery were obtained (Table 3).

Anaerobic Biodegradability Tests. Anaerobic digestion offers several advantages over aerobic treatment processes in terms of energy generation, high organic load that could be applied to the system, and low production of waste biosolids.[33] Initial characteristics of the effluent samples used in this study are listed in Table 4. C represents the raw effluent sample not subjected to enzymatic prehydrolysis. T_1–T_5 are samples hydrolyzed for different time periods (6–30 h) and contained increasing levels of glycerol and FFA but decreasing lipid levels. Decreasing protein contents were also witnessed. From the data given in Table 4, it could be seen that the protein content of the unhydrolyzed effluent sample (C) is 1127 mg/L. After enzymatic hydrolysis for different time durations, the protein contents of the samples have been progressively reduced, reaching 754 mg/L in T_5. This shows the presence of protease activity and such protein hydrolysis contributes toward reducing the effluent organic load. In

earlier studies too, hydrolytic enzymes had been proven to play an important role in organic matter degradation.[34]

The samples were subjected to anaerobic digestion, and the percent reductions observed in COD, reducing sugars, and proteins at the end of the 10-day digestion period are listed in Table 5.

The unhydrolyzed raw effluent (C_1) showed only 42% reduction in COD after the digestion process, because of obstacles posed by O&G contents on the anaerobic system. C_2, in which hydrolysis and digestion occurred together, revealed only a slightly better 54% COD reduction. Release of glycerol and LCFA during lipid hydrolysis might have been inhibitory to the sludge microbial communities and reduced the process efficiency. However, the prehydrolyzed samples showed superior COD removal, with a maximum of 89% occurring in T_5. This shows that the anaerobic microbial communities present in the sludge were able to tackle the organic load present in the hydrolyzed samples. When a mixed bacterial culture comprising of *Pseudomonas aeruginosa* LP602, *Bacillus* sp. B304, and *Acinetobacter calcoaceticus* LP009 was used in the treatment of lipid-rich wastewater, the BOD and lipid values had been reduced from ~3,500 and 20,000 mg/L to <20 mg/L within 12 days.[35]

A 38% reduction in protein content was seen in case of raw effluent and 49% in case of simultaneous hydrolysis and anaerobic digestion. Protein removal achieved during digestion of the prehydrolyzed samples varied from 56% to 91%, with the maximally hydrolyzed sample showing highest removal. The hydrolysis of proteins into low-molecular-weight peptides and amino acids might have facilitated their easy assimilation by the sludge microflora. This is the advantage conferred by proteases in the preparation. Although lipases facilitate substantial reduction in O&G load of the effluent, multiple hydrolytic enzyme activities are required to bring down the BOD and COD load of the effluent significantly. Contrary to our observation, in another study involving dairy wastewater, it had been reported that the extent of enzymatic hydrolysis did not influence protein removal during anaerobic digestion.[17]

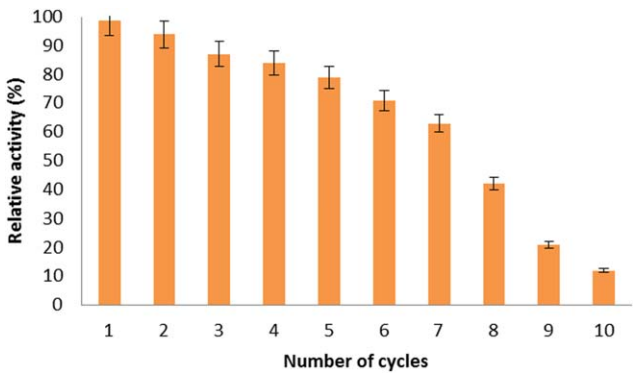

Figure 4. Reusability of IL in effluent hydrolysis.

A good reduction in the amount of reducing sugars was also obtained. Unhydrolyzed sample showed a 48% reduction in reducing sugars, which shot up to 56% when hydrolysis and digestion occurred parallely. The extent of removal in prehydrolyzed samples fluctuated from 69%–96%, depending upon the degree of enzymatic hydrolysis, yet again confirming the positive impact that prehydrolysis has on the performance of anaerobic systems. Previously, a maximum of 85% reduction in reducing sugars had been achieved in olive mill effluent using a consortium of yeast strains.[36]

Poor settling sludge leading to flotation and biosolids loss to the effluent were observed in C_1 and C_2. This loss of acclimatized microorganisms, especially considering their slow growth under anaerobic conditions, must have affected the removal efficiencies in these flasks. In our study, all results showed a positive correlation between the extent of enzymatic hydrolysis and the digestion efficiency.

Biogas generation during the anaerobic digestion was also monitored. Yields obtained from hydrolyzed samples were found to be in the range of 205–347 mL, with maximum production being obtained in flask C_5. This represents a 2.36- and 1.90-fold increase upon comparison with C_1 and C_2, respectively. Although lipid hydrolysis occurred in C_2, the conversion of glycerol and LCFA to short-chain VFA by the anaerobic biomass might have curbed biogas generation in this flask. Kinetic profiles of biogas generation over the 10-day period showed a linear increase initially, which slowed down and reached saturation eventually (Figure 5). Near maximum levels were reached around 6–9 days, depending upon the extents of hydrolysis in the samples.

Reactor Studies. The control and hybrid reactors were operated for a period of 70 d, with the flow rates doubled after the 35th day. Their O&G removal, COD removal, and methane gas production were assessed during this entire duration. The operating conditions of the reactors are summarized in Table 6, and the reactor set-up is diagramatically illustrated in Figure 6.

Day-to-day fluctuations in the influent and effluent COD (mg/L) levels of the reactors are illustrated in Figure 7a. The average COD levels of the influent were 2,171 and 2,989 for Phases I and II, respectively. Analysis of effluent from the control UASB showed its average COD levels to be 875 and 1,221 for Phases I and II, respectively. The COD levels of effluent from the hybrid UASB were much lower at 239 and 330, for the two phases. When expressed as percentage, the COD removal of the control reactor was 60%, whereas that of the hybrid reactor was found to be 89%. Fluctuations in the effluent COD level were also far lesser in the hybrid reactor. These results establish unequivocally that the organic load removal of the hybrid reactor is superior to that of the control reactor, even during the higher loading rate in phase II. The COD degradation efficiency had been reported to be 42.9% during the first 12 h, and reaching 97.6% after 72 h in an open activated sludge bioreactor system employing whole-cell *Yarrowia lipolytica* expressing fungal lipases.[37]

Table 3. Extent of IL Beads and Activity Recovery After the Hydrolysis Experiment

Weight of Beads (g)			Enzyme Activity (U/g)			
Initial	Final	Beads Recovery (%)	Initial	Final	Activity Recovery (%)	Total Activity Recovery (%)
2.5	1.73	69	218	148	68	47

Temporal variations in influent and effluent O&G (mg/L) levels are shown in Figure 7b, from which it could be seen that the average influent O&G concentrations were 894 and 1,251 for Phases I and II, respectively. After anaerobic digestion, the O&G levels in the effluent were found to be 455 (phase I) and 692 (phase II), on an average, for the control reactor. This represents 49% and 45% removal for the two phases. On the contrary, average effluent O&G levels for the hybrid reactor were 93 and 175, representing percent removals of 90% and 86%, for the two phases. Thus, the removal efficiency has been nearly doubled in the hybrid reactor. In a study with synthetic oily wastewater, approximately 48% of the O&G had been hydrolyzed by chitosan-immobilized lipase from *Aspergillus niger*. In addition, about 47% reduction in COD was estimated.[38] Our study has effected far better removals and that too, with actual wastewater.

Table 4. Composition of the Oil Mill Effluent Prehydrolyzed with IL

Sample No.	Parameters (mg/L)			
	Glycerol	FFA	Lipids	Proteins
C	145 (100%)	750 (100%)	895 (100%)	1127 (100%)
T_1	167 (115%)	852 (114%)	802 (90%)	1053 (93%)
T_2	189 (130%)	965 (127%)	713 (80%)	912 (81%)
T_3	203 (140%)	1035 (138%)	608 (68%)	843 (75%)
T_4	213 (147%)	1112 (148%)	535 (60%)	798 (71%)
T_5	221 (152%)	1235 (164%)	445 (49%)	754 (67%)

Table 5. Percent Reduction in Pollution Parameters After the Anaerobic Digestion

Sample No.	Percent Reduction in Parameters			Biogas Production (mL)
	COD	Proteins	Reducing Sugars	
C_1	42	38	48	147
C_2	54	49	56	183
T_1	62	56	69	205
T_2	69	69	76	236
T_3	74	76	84	287
T_4	82	83	92	325
T_5	89	91	96	347

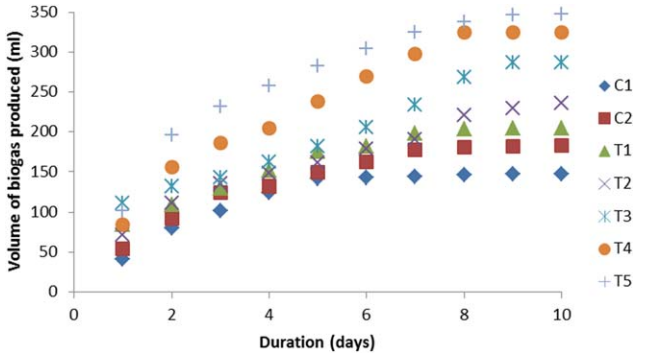

Figure 5. Biogas generation from anaerobic digestion of effluent samples.

Methane gas generation (L/g VS added) patterns in the hybrid and control reactors are presented in Figure 7c. The average yields obtained from the control reactor during Phases I and II were measured to be 0.43 and 0.63, respectively. The yields obtained from the hybrid reactor were found to be 0.73 and 0.86, for the two phases. These data mean 41% and 27% higher methane yields from the hybrid reactor during Phases I and II, respectively. The ultimate methane yield from fruits waste has been reported to range from 0.18 to 0.732 L/g VS added.[39] The amount of methane gas generated from an anaerobic reactor is an important factor that determines the economic viability of the process. Anaerobic systems operated at low organic loading rates are known to have a proportionately low methane production. Similarly, slower conversion of the organic load can result in reduced methane generation, and the gas cannot be effectively used as an energy source by the treatment plant. The rate of lipid hydrolysis determines the subsequent biodegradation rate, and a slow hydrolysis occurring in the control reactor is thought to have adversely affected the biodegradation rate.

Formation and maintenance of the sludge granules is the most important operational problem in UASB reactors, and effluent characteristics influence sludge granulation. Excessive O&G contents in the raw wastewater caused foaming in the control reactor beyond 10 days of operation. An average of 0.21 ± 0.07 L of foam was skimmed off from the reactor on a weekly basis. The foam contained 178 ± 0.23 mg/L of COD and 83 ± 0.16 mg/L of O&G. Sludge degranulation, flotation, and biosolids loss from the control reactor were also witnessed. Such problems were not encountered in the hybrid reactor. Foaming was minimal and did not require removal. The sludge granules were of the right density and resisted wash out from the reactor. Such stably maintained seed sludge is postulated to have been the prime contributing factor to a superior performance of the hybrid reactor.

Performance of Unit I of the hybrid reactor that contained the IL was also satisfactory to a large extent. Breakage of the lipase-immobilized celite carrier due to collisions upon stirring was minimal (<5%), and replacement was not required. Celite, thus, proves to be a robust support material for this application.

Economic aspects

The process has sound economic viability primarily based on the fact that an indigenous enzymatic preparation has been used. Multi-step purification procedures with low yields

Table 6. Operating Conditions of the Control and Hybrid Reactors for Effluent Treatment

Parameter	Hybrid Reactor Unit I		Hybrid Reactor Unit II		Control Reactor	
	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
Duration of operation (days)	35	35	35	35	35	35
Flow rate (L/day)	3	6	3	6	5	10
HRT (day)	0.7	0.35	2	1	2	1
OLR (kg/m^3/day)	4.15 ± 0.14	7.93 ± 0.18	1.83 ± 0.23	3.61 ± 0.07	1.83 ± 0.23	3.61 ± 0.07
O&G LR (kg/m^3/day)	1.95 ± 0.07	4.85 ± 0.91	0.7 ± 0.28	2.2 ± 0.14	1.1 ± 0.28	3.15 ± 0.07

HRT, hydraulic retention time; OLR, organic loading rate; O&G LR, oil & grease loading rate.

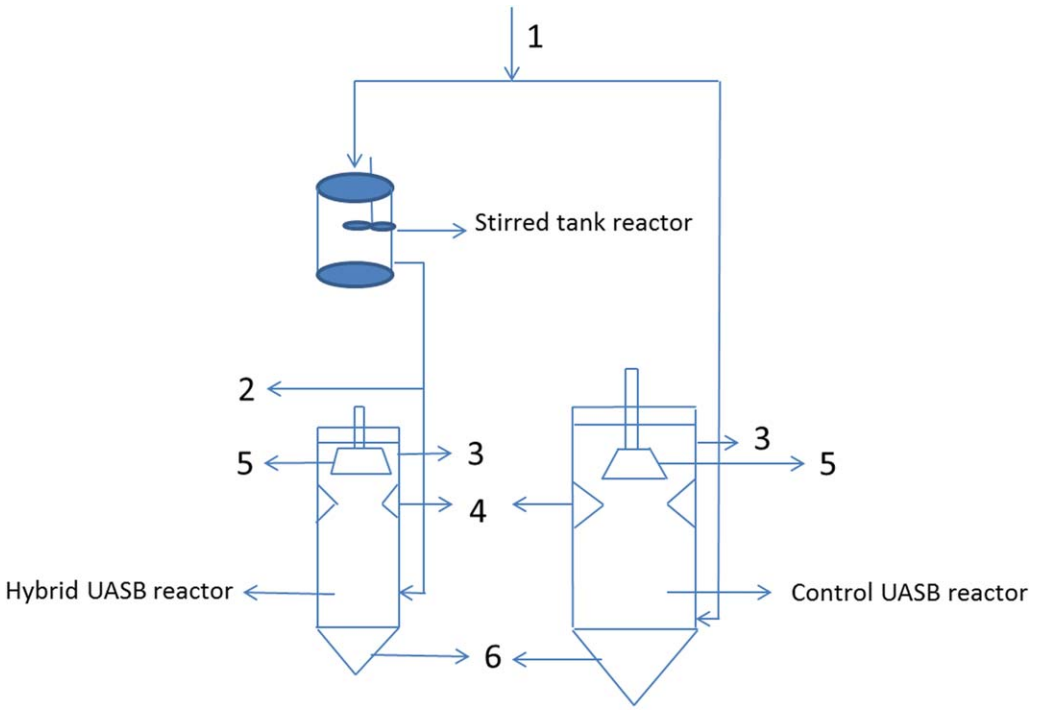

Figure 6. Diagrammatic representation of the control and hybrid UASB reactors.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

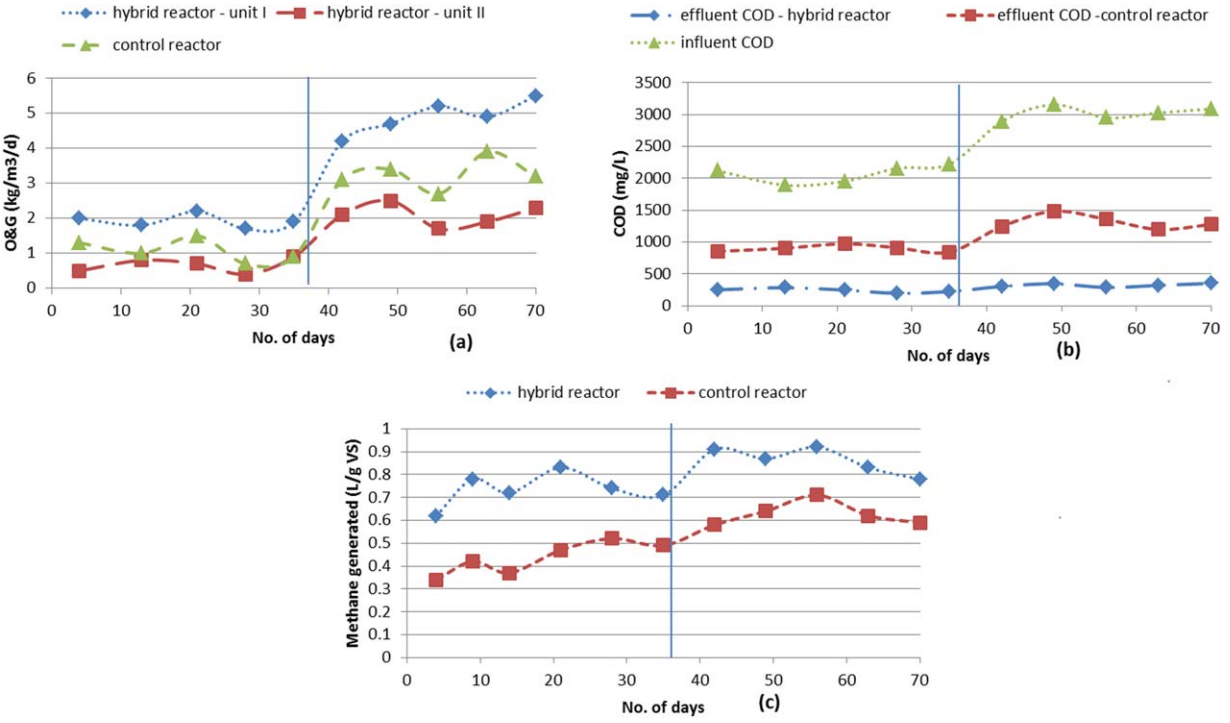

Figure 7. (a) Variations in influent and effluent COD levels. (b) Variations in influent and effluent O&G levels. (c) Methane gas generation in control and hybrid reactors.

can make such preparations also cost prohibitive. Because partially purified enzyme has been used here, such costs have been circumvented. It also offered the additional advantages of protease and amylase activities, which would have been excluded from pure lipase preparations.

Laboratory-sourced crude lipase preparation was calculated to be around 10 times cheaper than purified lipase (based on downstream processing steps and yields obtained) and 30 times cheaper than commercially available pure lipase (Sigma Aldrich – 62279, immobilized lipase from *Pseudomonas cepacia* was taken as reference). Apart from this cost associated with the enzyme, its recyclability, reactor capital, and operational costs, effluent flow rate and hydraulic retention time (HRT) were taken into account for arriving at the final cost estimate per cubic meter of effluent, as per the Indian market price. All cost estimates were made in Indian Rupees and finally converted to US Dollars. The final cost was calculated to be around $\$4/m^3$ of effluent treated. However, the projected cost with purified lipase preparation was a far higher $\$43/m^3$. Using commercial enzyme preparations worked out to a minimum of $\$120/m^3$. Hence, the immobilized crude lipase preparation makes the process economically attractive.

Conclusion

The use of hydrolytic enzymes for the pretreatment of lipid-rich industrial effluents results in better performance of biological treatment units receiving such effluents. For this application, *S. pasteuri* can be considered to be a novel source, and the use of IL from this organism adds to the novelty of the work. Cost analysis has shown the process to be affordable. VFA and LCFA contents of the hydrolyzed effluent have been significantly increased, and the O&G contents have come down appreciably. Batch anaerobic biodegradation trials with such prehydrolyzed effluent have resulted in good organic load removal and biogas production. Reactor system has also been constructed, and the results obtained in preliminary studies have been corroborated. The hybrid UASB reactor has shown good COD and O&G removal even under higher organic loading rates in Phase II operation, and the resulting effluent had minimal fluctuations in these parameters. The hybrid reactor has faced negligible foaming and biosolids loss when compared with the control reactor, factors which contributed to its enhanced performance. The use of an anaerobic reactor that permits energy generation from the organic load consumed is an added advantage. These findings ascertain that the immobilized *S. pasteuri* lipase is conducive for enzymatic prehydrolysis of coconut mill effluent, enhancing its biodegradation prospects.

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