Int. J. Environ. Res., 6(2):475-480, Spring 2012
ISSN: 1735-6865

Enzymatic Pre-Hydrolysis of high fat Content Dairy Wastewater as a Pretreatment for Anaerobic Digestion

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Received 19 Jan. 2011; Revised 20 Aug. 2011; Accepted 26 Nov. 2011

ABSTRACT: Enzymatic extract preparation from *Pseudomonas aeruginosa* KM110 under accession No. HQ730879 with lipase activity (0.3 U/ml), was used to perform enzymatic hydrolysis pretreatment of a synthetic dairy wastewater with 1000 mg/L total fat content. The pretreatment was optimized for 48 h hydrolysis time, at 45$^{\alpha\%}$ C with 10% v/v enzymatic extract. The biological treatment of synthetic dairy wastewater was investigated using a batch bioreactor. Both raw and prehydrolyzed wastewater was digested in a batch bioreactor. Enhanced anaerobic digestion efficiency compare to raw wastewater was achieved (chemical oxygen demand (COD), removal efficiency of 90% vs. 66% and biogas production of 4710 ml vs. 2330 ml after 13days). The results obtained in this study illustrated that the application of a pretreatment process to hydrolyze and dissolve fats may improve the biological degradation of fatty wastewaters, accelerating the process and reducing time of anaerobic digestion. Moreover, the pretreatment of wastewater from several sources is a new and promising application for lipases.

Key words: Wastewater, *Pseudomonas aeruginosa*, Anaerobic digestion, Enzymatic prehydrolysis

INTRODUCTION

Wastewaters from dairies (Cammarota *et al.,* 2001; Danalewich *et al.,* 1998; Jung *et al.,* 2002; Omil *et al.,* 2003) and slaughterhouses (Masse´ *et al.,* 2003) are rich in biodegradable organic molecules and nutrients and usually contain high levels of fats and proteins that have a low biodegradability coefficient. If not treated, they cause gross pollution of land and water with their high biochemical oxygen demand (BOD) and chemical oxygen demand (COD).

To alleviate the problem, aerobic and anaerobic treatments mainly are used, but in the last two decades anaerobic reactors have been increasingly used (Omil *et al.,* 2003). Application of anaerobic treatment is widespread in food and agro industries. However, there are some concerns about its capability to assimilate variable loads of oil and grease. The operational problems caused by oil and grease in up-flow anaerobic sludge blanket (UASB) reactors, such as sludge flotation, inhibitory and toxic effects of intermediate products were reported (Vidal *et al.,* 2000). These detrimental effects of milk fat on anaerobic treatment were also reported (Petruy *et al.,* 1997). These authors considered that the loss of process performance can be attributed to the low rate of fat hydrolysis in the anaerobic reactor. A large number of pretreatment systems are employed to remove oil and grease from these wastewaters prior to the main treatment process itself, which is generally of a biological nature. However, the cost of such reagents is high, the removal efficiency of dissolved and/or emulsified O&G is low and extremely problematic sludge is produced (Tano-Debrah *et al.,* 1999; Willey, 2001).

Application of a pretreatment process to hydrolyze and dissolve fats may improve the biological degradation of fatty wastewaters, accelerating the process and reducing time. Treatment of such wastewater from several origins is a new and promising actuation for lipases (Cammarota *et al.,* 2006). Also the effect of addition of lipase to a biological system treating restaurant wastewater has investigated (Dharmsthiti *et al.,* 1998). These authors reported that the lipid content was totally removed after 48h incubation of the wastewater with the enzyme

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(lipase). Utilization of enzymatic hydrolysis as a pre-treatment to the biological treatment of slaughterhouse wastewater was investigated by Masse´ *et al.,* (2001, 2003). The enzymatic hydrolysis promoted a slight increase on COD removal attained in an anaerobic sequencing batch reactor.

Lipases (triacylglycerol hydrolases; E.C. 3.1.1.3) are hydrolases which catalyze the hydrolysis of carboxyl ester bonds present in acylglycerol with the consequent release of organic acids and glycerol. They are particularly important due to the fact that they specifically hydrolyze oils and greases, which are of great interest for different industrial applications, among them the treatment of industrial wastewaters containing high fat contents, such as dairy wastewaters (Sharma *et al.,* 2001). Utilization of a hybrid technology - enzymatic treatment associated with anaerobic biologic treatment - enables a reduction in hydraulic retention time and, consequently, in reactor volume, since it promotes hydrolysis of fats which cause problems of clogging of the sludge bed in anaerobic reactors of the UASB type (Masse´ *et al.,* 2001).

The aim of this study was to evaluate effect of enzymatic pretreatment for promoting hydrolysis of fatty wastewater in anaerobic reactor. In this study, lipase produced by *Pseudomonas aeruginosa* KM110 that is a lipase-producing strain was used. The bacteria were isolated from oil processing plant wastewater. At first, lipase was preliminarily characterized to determine its potential for fatty wastewater hydrolysis. Then enzymatic extract produced by *P. aeruginosa* KM110 with lipase activity (0.3 U/ml) was used to hydrolyze a synthetic dairy wastewater with 1000 mg/L total fat content prior to the biological anaerobic digestion.

MATERIALS & METHODS

The first stage in this study was isolation of a lipase producing bacteria from wastewater and then study of pH and thermal stability of lipase. *P. aeruginosa* was isolated from wastewater of an oil processing plant (Pegah industrial complex of Tehran, Iran). The culture medium for enzyme production was composed of (% w/v or v/v): peptone 0.2; NH4H2PO4 0.1; NaCl 0.25; $MgSO_4 \cdot 7H_2O$ 0.04; $CaCl_2.2H_2O$ 0.04; olive oil 2.0 (v/v); pH 7.0; inoculums density 5% (v/v); incubation time 24 h at 30ºC and 150rpm. Also, for measuring activity of lipase the raw enzyme used to assay was isolated from the culture broth following separation of cells and particles. After 24 hours of incubation, the culture medium was centrifuged at 10,000 rpm for 20 min at 4º C and the cell-free culture supernatant fluid was used as the source of extracellular enzyme. Lipase activity was determined using *p*-nitro phenol palmitate (*p*NPP) as substrate. The reaction mixture was composed of 700µl *p*NPP solution and 300µl of lipase solution. The *p*NPP solution was prepared by adding the solution A (0.001 g *p*NPP in 1ml isopropanal) into solution B (0.01 g gum arabic, 0.02 g Sodium deoxycholate, 50 µL Triton X-100 and 9mL of 50mM Tris-HCl buffer, pH 8) with stirring until all was dissolved. Subsequently, the absorbance measured at 410nm for the first 2 min of reaction. One unit (1U) was defined as that amount of enzyme was required to release 1µmol of *p*NPP per minute (ε: 1500l/mol cm) under the test conditions (Karadzic *et al.,* 2006).

The enzyme was normally stored at 4°C until used **.**The optimal temperature for activity was determined at different temperatures (30–70°C), at pH 8.0 for 10 min. For the determination of temperature stability, the reaction mixtures containing the enzyme in 50mM Tris–HCl buffer (pH 8.0) was incubated at different temperatures (37, 45, 50, 55, 65 and 70 °C) for 3h then immediately cooled. Residual activity was measured by the spectrophotometric assay. Optimal pH was measured on the first 2 minutes at 30°C in 50 mM buffer of pH values ranging from 5 to 11 (0.05M citrate-phosphate pH 5-7; 0.05M Tri s–HCl pH 8-9; 0; 0.05M Glysin – NaOH pH 11) containing substrate solutions of *p*NPP. The effect of pH on enzyme stability was analyzed by the spectrophotometric assay after pre-incubation of 300µL of enzyme solution for 1 h at 30 ζ%C, in 700µl of the above mentioned buffer solutions (pH 5–11).

The second stage was preparation of synthetic wastewater. The synthetic wastewater was prepared using 2 g/L of skim milk in tap water in addition to an appropriate amount of fat from the flotation unit of a dairy industry. The amount of fat incorporated into the wastewater in order to reach approximately, 1000 mg/L of fat, varied. The incorporation of fat to the aqueous phase was performed using a mechanical impeller, which produced stable emulsions. The wastewater prepared was further sent to a conditioning stage or to the hydrolysis treatment step (Leal *et al.,* 2006).

And the third stage was enzymatic wastewater hydrolysis. The hydrolysis was performed at three different temperatures (30, 37 and 45°C) for wastewater containing 1000 mg /L total fat content with 10 and 20% v/v enzymatic extract of 0.3 $U.mL^{-1}$ lipase activity. In experiments performed to establish the most adequate hydrolysis conditions, the reaction lasted 48 h. The reaction progress was assessed through the determination of free fatty acids by titration with 0.05 M NaOH solution. The last stage was anaerobic digestion of pre-hydrolysed wastewater. A flow diagram of the experimental set-up is shown in Fig. 1. The batch reactor was a glass cylinder with a diameter of 10 cm and a height of 15 cm (working volume 1 L). Effluent samples were drawn from the bottom of the reactor using a sampling port. Prior to the experiments, 200 mL (20% (v/v) inoculation) mixture of the enriched cultures (methanogens and acetogens) with defined M/A was used to seed the batch reactor. For starting up the

bioreactor, the ratio of methanogens to acetogens (M/A) was taken as the relative amount of their VSS concentrations. An electrical heating tape (heating capacity: 40 W/m) was attached to the outside surface of the reactor and a temperature probe was connected to the transmitter. The temperature of the reactor was set to the mesophilic (37±1°C) condition. To ensure efficient transfer of the intermediates and to release gas bubbles trapped in the medium mixing was performed with an intensity of 50 rpm, duration of 5 min per each 10 h, using a magnetic stirrer. The produced biogas was vented out the top of the bioreactor through a connecting pipe and was collected by the water-displacement method. During this period, samples were taken for pH measurements and COD assays. A comparison of COD removal efficiency allowed us to evaluation of the effect of wastewater enzymatic pretreatment on the efficiency of the anaerobic digestion. COD was determined according to the standard methods (APHA, 1992). The pH was measured using the Metrohm 620 pH meter (made in Germany).

RESULTS & DISCUSSION

The effect of pH on the activity of lipase was determined in four different buffers covering the range of pH 3.0 to 12.0. The most enzyme activity was monitored at pH 6.0 and 9.0 (Fig .2), but retained over 65% of its activity at pH 8.0 (Fig. 3). Interestingly, other *Pseudomonas* sp. lipases designated as alkaline, e.g., *P. fluorescents* HU380 (Kojima *et al.,* 2003), *P. fluorescents* 2D (Makhzoum, 1996) have lower pH optima of 8.5, 8.5, respectively. *P. aeruginosa* KM110 lipase was stable between pH 7.0 and 10.0, but the stability was low at acidic pH. The remarkable stability of *P. aeruginosa* KM110 lipase in this range has proved it to be a potential alkaline lipase similar the others.

The optimum activity of the enzyme was observed at 30°C and 45°C (Fig. 4). Assessment of the thermo stability of lipase was performed by measuring the residual activity at various times, following incubation at different temperatures. As for the stability of the enzyme (Fig. 5), 80% activity remained after 3 h of storage at 45°C and 70% at 37°C. At higher temperatures, the stability of the enzyme was lower; ie 40% activity remained after 3h at 65°C. The stability of the lipase decreased sharply after 1 h of incubation at high temperatures (Fig. 5). This confirms that *P. aeruginosa* KM110 lipase is a mesophilic enzyme. The *P. aeruginosa* MB 5001 lipase requires an optimum temperature of 55°C for activity (Chartrain *et al.,* 1993) ,but other *Pseudomonas* lipases, such as those from *P. fluorescens* 2D (Makhzoum *et al.,* 1996) , *P. fluorescens* HU380 (Chartrain *et al.,* 1993) and *P. fragi* (Mencher *et al.,* 1967) were found to be optimally active at 35–45°C. *P. aeruginosa* lipases seem to be more thermos table than others from this genus.

Initially, the progress of the enzymatic hydrolysis was carried out at two different temperatures (35 and 45 °C). The production of free fatty acids increased almost linearly with time during 48h hydrolysis reaction for two tested conditions (Fig.6). But the hydrolysis progressed significantly in shorter time at 45 °C compare to 35 °C ($P<0.05$).Thus the temperature of 45 °C was selected to perform the hydrolysis step.

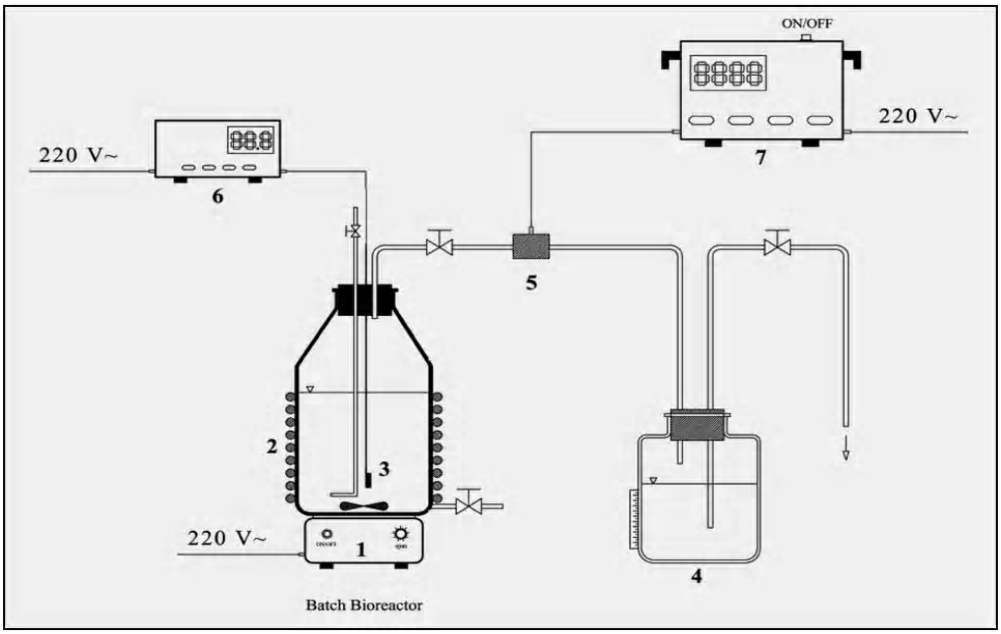

Fig. 1. Schematic flow diagram of experimental set-up: (1) magnetic stirrer, (2) electrical heating tape, (3) temperature probe, (4) biogas collector vessel, (5) methane sensor, (6) temperature controller, (7) methane sensor transmitter

Additionally, the concentration of enzymatic extract (10 or 20% v/v) was tested. As shown in Fig. 7, there is an increase in concentration of free acids in two experiments. Doubling the concentration of enzyme extract resulted in an increase in free acid contents in the medium, but the excessive use of raw enzyme extract would make this process economically unfeasible on larger scales. For practical and operational reasons, the time of reaction selected for the hydrolysis process, as pretreatment to the batch bioreactors, was 48 h. Thus, the hydrolyzed wastewater fed to the batch bioreactors was obtained after 48 h of reaction at 45 °C. Based on the results obtained in this preliminary phase, wastewater hydrolysis was carried out under the following conditions: 45ºC, 10% v/v enzyme extract (0.3 U.m/L), without agitation and a hydrolysis time of 48 hours.

As mentioned, both raw and pre-hydrolyzed wastewaters were digested in a batch bioreactor. Effects of the enzymatic pretreament on the COD removal efficiency and accumulated biogas production are summarized in Fig. 8. Based on results, after 13 days, the COD removal efficiency (90%) and biogas production (4710 ml) of pre-hydrolyzed effluent were greater than that of raw effluent (66% and 2330 ml, respectively) containing the same initial fat content. Similar results were obtained for the pre-hydrolysis of a synthetic dairy wastewater containing 200, 600 and 1000 mg/l oil and grease, using an enzyme preparation obtained through solid-state fermentation, presenting pronounced lipase activity (Leal *et al.*, 2006). According to these authors, the benefits of the hydrolysis step became evident with the highest concentration (1000 mg/l), COD removals averaged 90% in the UASB reactor fed with the hydrolyzed effluent and 82% in the control reactor and the biogas content produced in both reactors was almost the same. Moreover, in another study removal efficiencies of 19% and 80% for raw and hydrolyzed dairy wastewater with 1200mg/L fat

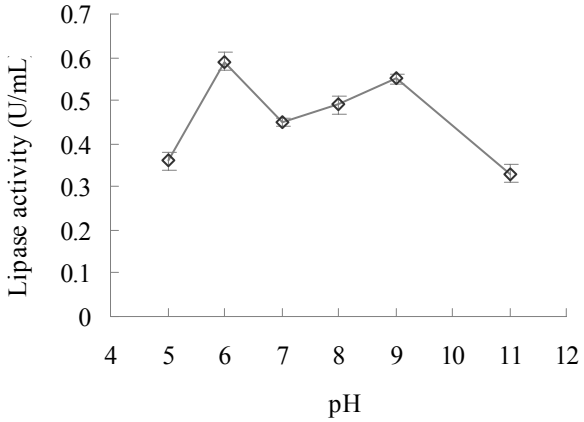

Fig. 2. Effects of pH on lipase activity. Enzymatic activity was measured according to a standard protocol with *p*NPP as the substrate

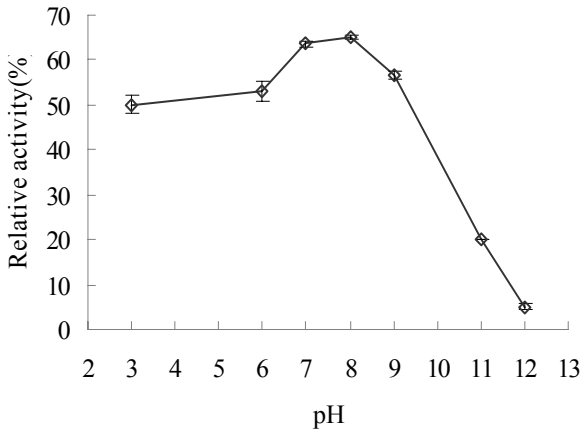

Fig. 3. Stability of lipase at different pH . Residual activity was measured by a standard assay method

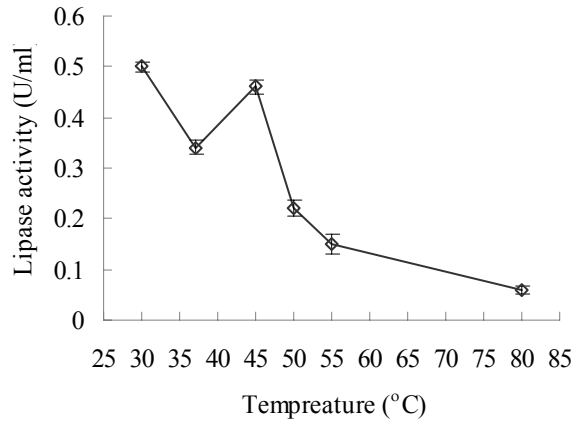

Fig.4. Effects of temperature on lipase activity. Enzymatic activity was measured according to a standard protocol with *p*NPP as the substrate

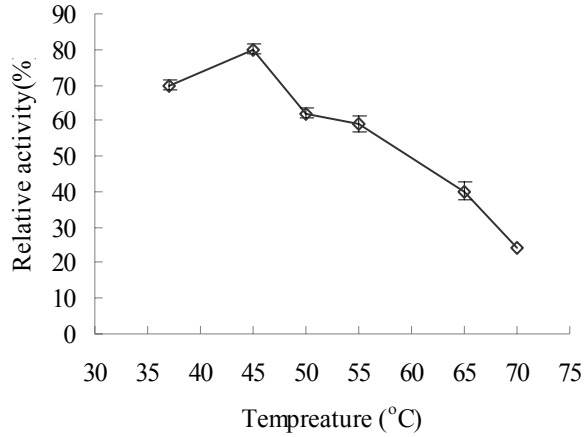

Fig. 5. Thermal stability of lipase. Residual enzyme activity was measured under standard enzyme test conditions

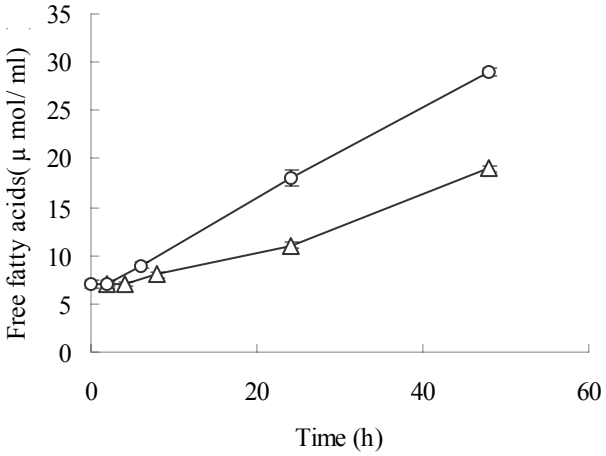

Fig. 6. Evolution of the production of free acids throughout the hydrolysis time at 35 (Δ) and 45°C (°)

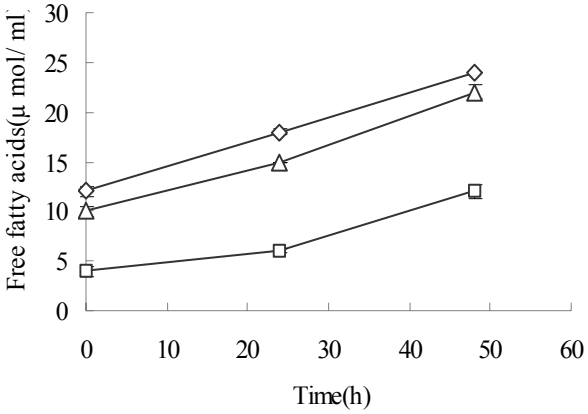

Fig.7. Evolution of the production of free acids throughout the hydrolysis time. (□) control, (Δ) 10% v/v enzymatic extract (✧) 20% v/v enzymatic extract

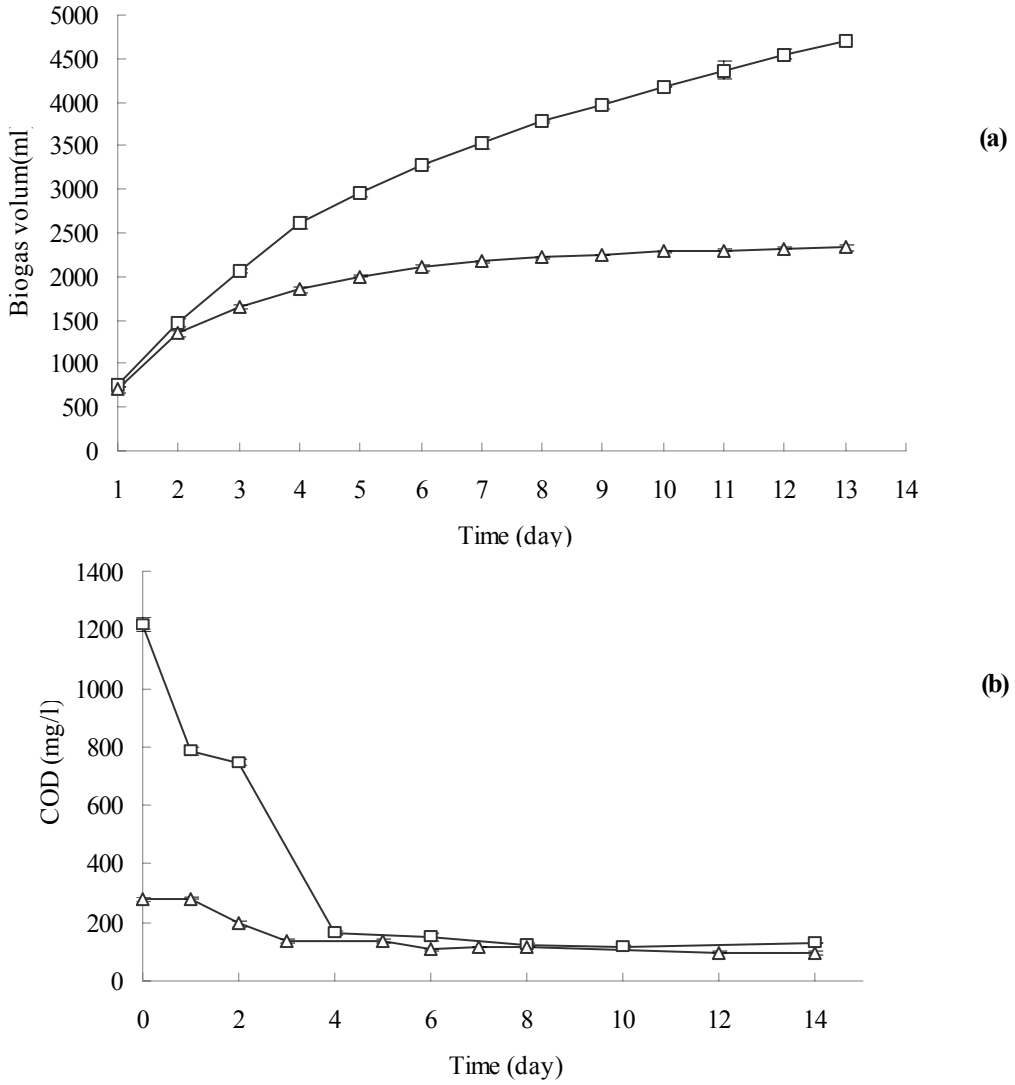

Fig. 8. Evolution of biogas production (a) and COD decline (b) in tests with raw wastewater and with hydrolyzed dairy wastewater containing 1000 mg/L total fat content. (Δ)Raw wastewater without pre-hydrolysis and (□) wastewater pre- hydrolyzed

content reported, respectively (Leal *et al.*, 2002). No significant difference between the pH of the bioreactorÒ s influent (7.5± 0.6) and effluent (7.7± 0.4) was observed. The results clearly demonstrate a positive effect of a pre-hydrolysis stage on fat content in the wastewater as evidenced by enhanced COD removal efficiency and increased biogas production (Fig. 8).

CONCLUSION

The results obtained in this study showed that potential alkaline lipase produced by *P.aeruginosa* KM110 is an attractive agent for the hydrolysis of oily wastewater such as dairy wastewater. Furthermore, the batch bioreactor fed with pre-hydrolyzed wastewater proved to be more efficient in the COD removal (90%) compared to raw effluent (66%). Biogas production also improved (4710 ml vs. 2330 ml raw effluent) after 13 days. Thus, it is apparent that the use of hybrid technology (enzymatic-biological treatment) can serve an alternative for treatment of high fat containing wastewater.

ACKNOWLEDGEMENT

We thank the department of Biology of Alzahra University and Biotechnology Group of Tarbiat Modares University, Tehran, Iran for their support of this paper.

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