Research Article

Received: 25 April 2011 Revised: 19 August 2011 Accepted: 29 August 2011 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.2740

Enhanced lipase production from *Aeromonas* sp. S1 using Sal deoiled seed cake as novel natural substrate for potential application in dairy wastewater treatment

Bayan A. Mahdi, Amrik Bhattacharya and Anshu Gupta*

Abstract

BACKGROUND: Sal (*Shorea robusta*) deoiled seed cake extract (SDOCE) was assessed for its suitability as a cheap natural substrate for lipase production under submerged fermentation. The bacterial isolate *Aeromonas* sp. S1 isolated from dairy industry was used for lipase production. Both the isolate and its lipase were shown to be potential tools for treatment of dairy wastewater containing higher organic load.

RESULTS: On substituting tributyrin with SDOCE, lipase production was enhanced 24-fold (195 U mL^{-1}) compared with the initial 8.13 U mL^{-1} lipase activity. Maximum lipase production was obtained at pH 8.0 and incubation temperature 30 °C. The lipase had pH and temperature optima of 10.0 and 55 °C, respectively. The isolate and its crude enzyme preparation were checked separately for applicability in dairy wastewater treatment. The isolate was able to reduce chemical oxygen demand (COD) by 93%, oil and grease (O&G) by 75%, and total suspended solids (TSS) by 47% after 96 h of treatment. Enzymatic preparation gave 86% reduction of COD after 12 h and 75 and 45% reduction of O&G and TSS, respectively, after 96 h of treatment.

CONCLUSION: Overall, the study shows the usefulness of Sal seed deoiled cake, a cheap agro-industrial by-product for the production of lipase. The isolate and its lipase can also be used effectively for the treatment of dairy wastewater.

Keywords: Sal seed cake; *Aeromonas* sp; lipase; dairy wastewater; wastewater treatment

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids at the water–lipid interface and the reverse reaction in non-aqueous media.[1] Due to their multifaceted properties, which find use in a wide array of industrial applications such as food technology, detergent, chemical industry and biomedical sciences, lipases have emerged as key enzymes in swiftly growing biotechnology.[2] Wastewater treatment for waste disposal is a new avenue in lipase biotechnology.[3] Lipases have been used successfully for hydrolysis/treatment of high lipid content wastewater originating from dairy and other food processing industries.[4,5]

Owing to the extensive industrial and environmental applications of lipases, efforts have been directed to explore the means to reduce lipase production costs through improving the yield. The use of either cost-free or low-cost agricultural by-products as raw material for lipase production can be one step to lower the overall enzyme cost.[6] In the recent past, many agro-industrial by-products such as wheat bran, rice bran, molasses, barley bran, maize meal, soybean meal, potato peel, coconut oil cake, etc. have been screened as low-cost solid substrates for microbial lipase production.[6,7] There are several reports describing production of various enzymes using oil cakes as substrate in solid-state fermentation (SSF), or as supplement to the production medium.[8] Oil cakes/oil meals, by-products obtained after oil extraction from the seeds, are ideally suited nutrient support, rendering both carbon and nitrogen sources, and reported to be good substrate for enzyme production using microbial species.[8]

Shroea robusta, commonly called Sal, is an important forest tree, found in abundance in central and parts of eastern and northern India. Sal seeds are an important forest by-product available from these forests, and are rich in fat and constitute about 69% symmetrical triglycerides, which makes them potentially useful for the food sector industries.[9] It forms the primary ingredient for a diverse range of products such as oil, soap, cocoa butter equivalent (CBE) in chocolate manufacturing and is also used for tanning purposes.[9] Sal seed is now identified as a potential source of biodiesel production.

At present about 1.50 million metric tons of Sal seeds are produced per year in India, which generates around 1.32 million

* *Correspondence to: Anshu Gupta, University School of Environment Management, Guru Gobind Singh Indraprastha University, Sector 16-C, Dwarka, New Delhi-110075, India. E-mail: anshurcy@yahoo.com*

University School of Environment Management, Guru Gobind Singh Indraprastha University, Sector 16-C, Dwarka, New Delhi-110075, India

metric tones of deoiled cake after oil extraction.[10] At present the cake is used for non-biological processes such as fuel for boiling in solvent extraction plants, as a sizing material in textile industries, coal briquettes and as cattle feed after standardization, as it is available at the cheaper cost of Rs. 4/- per kg.[9] Sal seed cake contains approximately 7.3% moisture, 11.7% protein, 2.7% fat, 13.5% crude fiber, 5.0% ash, 43.6% starch, 4.9% reducing sugars and 9.0% tannins.[11] This composition is appropriate for various bioprocesses and its meaningful utilization.

There is no report on using Sal seed cake for enzyme production to the best of our knowledge. The present study aimed at developing an appropriate bioprocess for enhanced lipase production from isolate *Aeromonas* sp. S1, using cheaply available Sal seed cake, and potential application of the isolate and its lipase for treatment/hydrolysis of dairy wastewater.

EXPERIMENTAL

Materials

The media components were purchased from Hi Media Laboratories (Mumbai, India). The *p*-nitrophenyl palmitate (*p*NPP), substrate for lipase assay was obtained from Sigma Chemical Co. USA. All other chemicals used were of analytical grade. Sal (*Shorea robusta)* deoiled seed cake was obtained from local Sal oil extraction unit in Raipur, Chattisgarh, India and Mahua (*Madhuca* sp.) flowers were collected from local people of Jharkhand, India.

Screening, isolation and purification of microbes

For isolation of lipase-producing microbes, samples were collected from the local dairy industry, New Delhi, India. These samples were taken from sludge in the oil and grease chamber, and from soil enriched with dairy wastewater close to the dairy wastewater treatment plant. The samples (1 g) were suspended in 10 mL of sterilized distilled water and the resultant suspension was spread on nutrient agar plate. The plates were incubated for 24 h at 30 °C. Growing colonies were further purified by repeated streaking on nutrient agar plates. A total of four different isolates were successfully isolated from sludge samples while four different isolates were purified from soil samples.

Selection of lipase producers among isolated microbes

All eight isolates were checked for lipase production. The isolates were first spotted individually on tributyrin agar plates (pH 7.5), containing (g L^{-1}): peptone, 5; NaCl, 5; yeast extract, 1.5; beef extract, 1.5; agar, 15 and Tributyrin, 1% (v/v). Production of clear halo zones around the colonies confirmed lipase production by the isolates. The four isolates (S1, S6, S7 and S8) which showed a positive test on tributyrin plates were then checked for lipase production in liquid media.

Media and culture conditions for lipase production

Mother cultures were prepared by inoculating a loopful of stock cultures of the four selected isolates (S1, S6, S7 and S8) individually in nutrient medium (pH 7.5) containing (g L^{-1}): peptone, 5; NaCl, 5; yeast extract, 1.5; beef extract, 1.5 followed by incubation at 30 °C and 200 rpm in an orbital shaker. 300 µL of the above overnight grown mother cultures were used to inoculate 100 mL of the above medium containing 1% (v/v) tributyrin (pH 7.5) in 250 mL Erlenmeyer flasks individually. The flasks were incubated at 30 °C with constant shaking at 200 rpm in an orbital shaker (Brunswick, USA). Aliquots of various samples were withdrawn at different times and centrifuged at 13 684 *g* and 4 °C for 10 min to pellet down the cell mass. The supernatants thus obtained were used for lipase assay.

All four isolates were found to produce extra cellular lipase in liquid media. The best lipase-producing isolate (S1) was selected for further use. S1 was originally isolated from the sludge sample collected from the oil and grease chamber of the dairy wastewater treatment plant. Isolate S1 was maintained on nutrient agar slants at 4 °C and sub-cultured at 20-day intervals for further studies.

Identification of lipase-producing strain S1

Isolated and purified potential lipase-producing bacterium (isolate S1) was identified to be *Aeromonas* sp. from Microbial Type Culture Collection Facility (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and deposited at its National Facility with Accession No. MTCC 10 661.

Effect of various natural substrates on lipase production

Carbon sources in the form of substrate are known to be an important factor affecting yield and cost of enzyme production. Carbon substrate, tributyrin, was replaced with cheaper natural substrates Sal seed deoiled cake extract (SDOCE) and Mahua flower extract (MFE) to see their effects on lipase production by *Aeromonas* sp. S1.

Preparation of Sal deoiled cake extract (SDOCE) and Mahua flowers extract (MFE)

To 10 g of ground deoiled Sal seed cake and Mahua flowers, 100 mL of distilled water was added to make individual slurries. After boiling for 10 min, the slurries were filtered through muslin cloth and filtrates thus obtained were used as substrate extracts. 3% (v/v) of each extract was added to the above nutrient media to study the effects of different nutrient supplements on lipase production. Maximum lipase production was obtained with SDOCE after 24 h fermentation. Henceforth, this time period and nutrient media containing SDOCE were used for further optimization studies.

Effects of culture conditions on lipase production

Fermentation was carried out in nutrient media containing SDOCE as supplement nutrient source or substrate to study the effects of various nutritional and physical factors on lipase production by *Aeromonas* sp. S1. Different concentrations of SDOCE (1%, 3%, 5% and 10%; v/v), initial pH of the medium (5, 7, 8, 9 and 10), incubation temperature (15 °C, 25 °C, 30 °C, 35 °C and 40 °C), carbon sources (mustard oil, 1% (v/v); coconut oil, 1% (v/v); maltose, 1% (w/v) and dextrose, 1% (w/v)) and nitrogen sources (sodium nitrate, 1%; ammonium chloride, 1% and ammonium sulphate, 1% (w/v)) were the parameters used for optimization studies. All the conditions were varied, one at time, in the above media and their individual effects on lipase production were monitored. After growth, cultures were harvested by centrifugation at 13 684 *g* and 4 °C for 10 min. Lipase activity was determined in cell free supernatants. Unless otherwise mentioned, lipase production was carried out by inoculating nutrient media with 3% (v/v) of SDOCE extract (pH adjusted to 8.0) with 300 µL of *Aeromonas* sp. S1 mother culture followed by incubation at 30 °C and 200 rpm. After 24 h of growth, the samples were centrifuged and the supernatant thus obtained was used as crude enzyme preparation.

Lipase assay

Lipase activity in the cell free supernatant was determined as described by Mahanta *et al*.,[12] using *p*NPP as the substrate. Briefly, 200 µL of cell free culture supernatant (crude lipase preparation) were added to 1.8 mL of solution containing 0.15 mol L^{-1} NaCl and 0.5% Triton X-100 in 0.1 mol L^{-1} Tris–HCl buffer (pH 8.0). 20 µL of substrate (50 mmol L^{-1} *p*NPP in acetonitrile) was added to the reaction mixture and incubated at 37 °C for 30 min. The amount of liberated *p*-nitrophenol (*p*NP) was recorded at 400 nm (UV-visible spectrophotometer, HACH). One unit is defined as the amount of enzyme liberating 1 nmol of *p*NP per minute under standard assay conditions.

Enzymatic characteristics

The effect of pH on crude lipase was studied by assaying the enzyme at different pH values in the range of pH 4.0–11.0, using *p*NPP as the substrate.

To determine the optimum temperature, the activity of lipase was measured at various temperatures (25–75 °C) using *p*NPP as the substrate. The thermal stability was studied by incubating the enzyme at 55 °C. Aliquots were withdrawn at different time intervals and the residual activities determined at assay temperature.

Preliminary treatment/hydrolysis of dairy wastewater

Collection and characterization of dairy wastewater sample

Fresh inlet dairy wastewater (raw) was collected from local dairy industry located at New Delhi, India. The wastewater sample was characterized for the following parameters; pH, temperature, COD (chemical oxygen demand), BOD (biological oxygen demand), TSS (total suspended solids), O&G (oil and grease), total nitrogen, total phosphorus and total coliforms. The pH and temperature of the sample was analyzed on the spot. For BOD estimation, wastewater samples were collected in BOD bottles (non-reactive borosilicate glass bottles of 300 mL capacity). BOD and COD were determined on the same day, after bringing the sample to the laboratory. Whereas, analysis of other physico-chemical and biological parameters was started as soon as possible after collection to avoid unpredictable changes in water sample. The O&G content was determined using a partition gravimetric method.[13] Other parameters were estimated according to standard APHA method.[14]

The rest of the sample was stored in cold room (4 °C) for further studies.

Microbial treatment

For the treatment of dairy wastewater samples, a microbial sample was prepared by inoculating the loopful of stock-culture of *Aeromonas* sp. S1 in a 250 mL Erlenmeyer flask containing 100 mL of nutrient media. The flask was then incubated overnight at 30 °C and 200 rpm in an orbital shaker.

For the treatment, 5% (v/v) of this overnight grown microbial culture was added to a 2 L Erlenmeyer flask containing 500 mL of raw wastewater. Before inoculation, pH of raw wastewater was adjusted to 9.0, using 0.1N NaOH. After inoculation, the flask was incubated at 37 °C and 200 rpm for 96 h.

Enzymatic treatment

For the enzymatic treatment of dairy wastewater, *Aeromonas* sp. S1 lipase was used. Production of lipase was carried out under optimized conditions: nutrient media containing, 3% (v/v) SDOCE; initial pH, 8.0; inoculum size, 300 µL and incubation temperature, 30 °C. The sample was withdrawn after 24 h of fermentation and centrifuged at 13 684 *g* and 4 °C for 10 min. This cell free supernatant was used as crude enzymatic preparation for dairy wastewater treatment.

The pH of raw wastewater was adjusted to 9.0, using 0.1N NaOH before treatment. 5% (v/v) of the above-described crude lipase preparation (cell free) was added to 500 mL of raw wastewater (in a 2 L Erlenmeyer flask). The treatment was carried out at 37 °C and 200 rpm for 4 days.

During treatment, all samples were withdrawn periodically including zero day samples for estimation of COD, O&G and TSS changes. A control flask without microbial (*Aeromonas* sp. S1) inoculation/enzyme preparation was also kept under similar conditions.

Each experiment in to test the effects of culture conditions and enzymatic characteristics were done at least twice and the differences in the individual results in each set of experiments were less than 5%.

RESULTS AND DISCUSSION

The primary objective of the work was to use the SDOCE as a substitute for tributyrin and inducer/carbon source for cost-effective production of lipase under submerged fermentation.

The other major objective was to find possible potential applications of this lipase in wastewater treatment originating from food processing industries, since the utilization of commercial enzymes for treatment/prehydrolysis is expensive and requires high enzyme yield for treatment.[15] Thus use of cheaper and easily available SDOCE as natural inducer for enzyme production can reduce the cost of enzyme production and hence treatment cost.

Isolation, purification and identification of potential lipase producing microbes

Eight different isolates (S1–S8) were isolated from samples collected from local dairy industry. During screening for lipase production, four isolates (S1, S6, S7 and S8) showed a positive test on tributyrin plates.

These four isolates (S1, S6, S7 and S8) were then checked for lipase production in nutrient media containing 1% (v/v) tributyrin as inducer. Figure 1 shows the time course of production of lipase by the different isolates in nutrient media containing 1% (v/v) tributyrin. Lipase activity was found to be maximum (16.8 U mL^{-1}) in isolate S1 on the third day of fermentation. Thereafter, enzyme production started decreasing. The second highest enzyme-producing isolate was found to be S6, with 4.2 U mL^{-1} of enzymatic activity on the third day of fermentation. Isolates S7 and S8 exhibited lower lipase activities of less than 4 U mL^{-1}.

The isolate S1 showing maximum lipase production was selected as the potential isolate for further studies. Isolate S1 was identified morphologically (based on physiological and biochemical characteristics) as *Aeromonas* sp. from Microbial Type Culture Collection Facility (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and deposited at its National Facility with accession number MTCC 10 661.

Effects of various natural substrates on lipase production

In a recent review paper, Treichel *et al*.[1] reported that the combination of both synthetic medium (defined compounds)

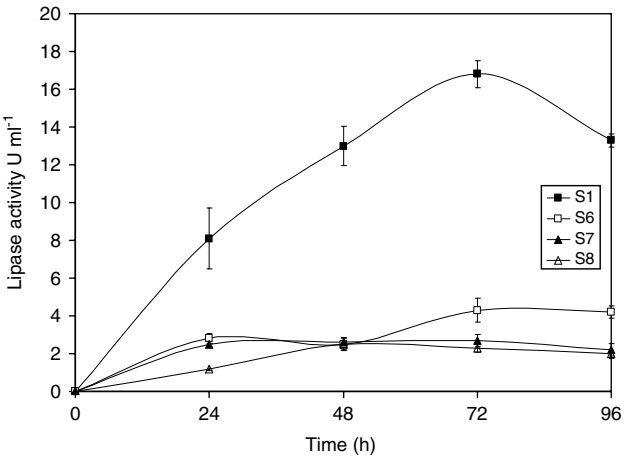

Figure 1. Time course of lipase production by different isolated microbial strains in nutrient medium containing 1% (v/v) tributyrin. The experiment was done in triplicate. Error bars show the percentage error.

and agro-industrial residues can be used for lipase production. In view of this, the combination of nutrient media with agro-industrial/forest by-products for enhanced lipase production was attempted in the present study. Lipase production in poor and simple media like mineral salt medium containing only SDOCE or MFE was also tested in the present study using *Aeromonas* sp. S1. Since no microbial growth and enzyme production was observed in the above simple media, further studies were undertaken with nutrient media.

The time course of lipase production by *Aeromonas* sp. S1 in the presence of different substrates is presented in Fig. 2(a). The results show that lipase activity was maximum for SDOCE as supplement substrate. Enzyme production was highest (107 U mL^{-1}) at 24 h of fermentation and decreased thereafter. MFE was also found to produce good enzyme production after 24 h (27 U mL^{-1}), but less than SDOCE. For SDOCE and MFE, lipase production was enhanced 13-fold (107 U mL^{-1}) and 3-fold (27 U mL^{-1}), respectively, compared with the initial 8.13 U mL^{-1} in the presence of tributyrin at 24 h. This shows that cheap SDOCE could efficiently substitute tributyrin as supplement substrate to work as inducer and carbon source for enhanced and cost-effective lipase production.

There was minimal production of lipase (4.2 and 13.8 U mL^{-1} in 24 and 72 h respectively) in media without any additional substrates i.e. nutrient media only. However the addition of SDOCE increased production to 107 U mL^{-1} after 24 h of fermentation. This shows that more lipase can be produced rapidly with supplementation of natural substrates. Control tests were also done in which enzyme preparations were first boiled at 100 °C for 10 min and then the usual assay was performed using pNPP as substrate. No lipase activity was detected in the control sets (data not shown). Since maximum lipase production was observed in SDOCE after 24 h fermentation, henceforth nutrient media with 3% (v/v) SDOCE as basal media and an incubation time of 24 h was used for further optimization studies. Lotrakul and Dharmsthiti[16] reported that addition of soybean meal (by-product of the vegetable oil industry) and whey (a dairy industrial waste) to a defined media increased lipase production 10-fold (450 U mL^{-1}) over that in nutrient media (40 U mL^{-1}), using *Aeromonas sorbia* LP004 as lipase producer. Similarly Rajagopalan and Krishnan[17] have shown that addition of sugarcane bagasse hydrolysate SBH

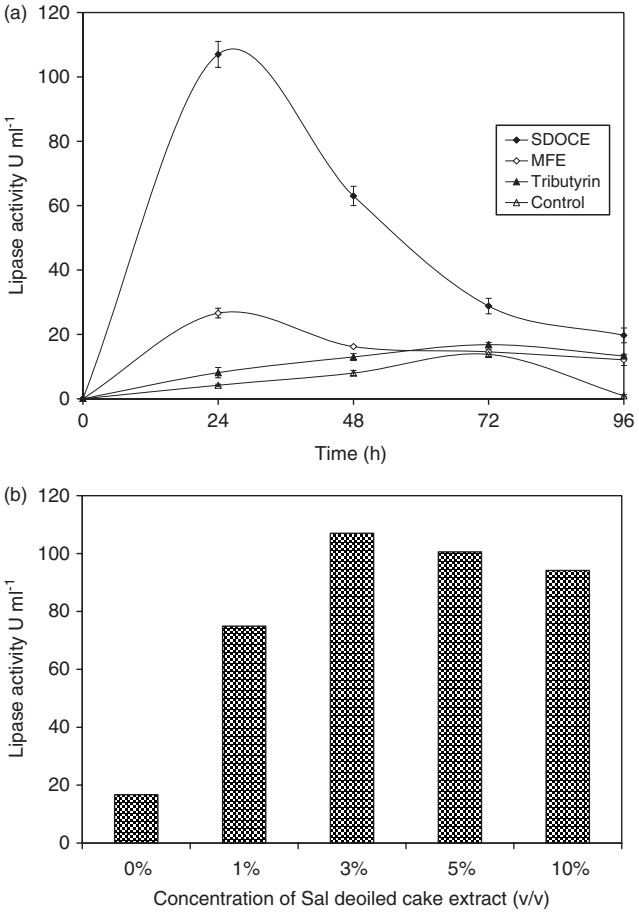

Figure 2. (a) Effect of various substrates on lipase production by *Aeromonas* sp. S1. Nutrient media was supplemented with substrates SDOCE (3%), MFE (3%) and tributyrin (1%). Control was without any additive. Fermentation was carried out at 30 °C and 200 rpm. Samples were withdrawn at 24 h intervals for determination of lipase activity. The experiment was done in triplicate. Error bars show the percentage error. (b) Effect of different concentrations of SDOCE on lipase production by *Aeromonas* sp. S1. Fermentation was carried out in nutrient media with varying concentrations of SDOCE as described in text.

(1% reducing sugar (w/v)) to the nutrient medium supported maximum α-amylase production of 67.4 U mL^{-1} by *Bacillus subtilis* under submerged fermentation.

Since maximum lipase production was observed in SDOCE, among the various substrates tested, the effect of concentration of SDOCE on lipase production was investigated. Figure 2(b) shows that SDOCE enhanced lipase production, with an optimum at 3% (v/v); SDOCE concentration beyond this led to a slight decrease in lipase production.

According to Gupta *et al.*,[2] lipases are considered to be inducible enzymes thus their production is strongly influenced by carbon sources such as triglycerides, sugars, sugar alcohol, polysaccharides, whey, casamino acids and other complex sources. SDOCE does not contain significant amounts of residual oil but has a major amount of total carbohydrates in the form of starch polysaccharides,[11] which might have played an important role in enhanced lipase production by *Aeromonas* sp. S1. Immanuel *et al.*[18] and Gunashekaran *et al.*[19] have also reported enhanced lipase production in the presence of starch using *Serratia rubidaea* and *Citrobacter freundii* IIT-BT L139 respectively.

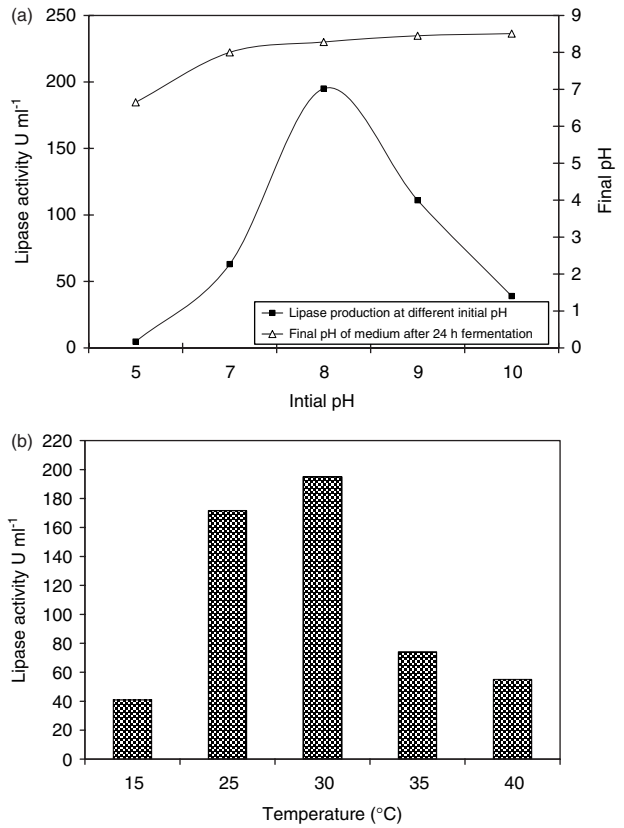

Figure 3. (a) Change in initial pH of fermenting media and effect of pH on lipase production by *Aeromonas* sp. S1. Fermentation was carried out in nutrient media supplemented with 3% (v/v) of SDOCE at different pH for 24 h as described in the methods section. (b) Effect of different incubation temperature on lipase production by *Aeromonas* sp. S1. Fermentation was carried out in nutrient media supplemented with 3% (v/v) of SDOCE at pH 8.0, 200 rpm and various incubation temperatures for 24 h.

Effect of pH on lipase production

To check the influence of pH on lipase production in the presence of SDOCE, fermentation was carried out at different initial pH values of the medium. Lipase production was found to be greatly affected by pH variation as shown in Fig. 3(a). Maximum enzyme activity was observed at pH 8.0 (195 U mL^{-1}). Very poor enzyme activities were observed at pH 5.0 (4.7 U mL^{-1}), 7.0 (63 U mL^{-1}) and 10.0 (39 U mL^{-1}), while at pH 9.0 good lipase production of 111 U mL^{-1} was obtained. Similar pH optima of 8.0 for maximum lipase production have been reported by Immanuel *et al*.[18] and Singh *et al*.[20] for *Serratia rubidaea* and *Pseudomonas aeruginosa*, respectively.

Changes in pH of fermentation media by Aeromonas sp. S1

The pH of fermentation media may change during fermentation due to production or utilization of acidic or alkaline compounds[12] by the microbes. The samples from fermented media with varying initial pH values were aseptically withdrawn after 24 h of growth and pH determined. As shown in Fig. 3(a), the pH of both low (acidic) and high value (alkaline) were changed to neutral to slightly alkaline range (6.6–8.6) after 24 h of growth at 30 °C. Increase in pH from an initial value of 5.0 to a final value of 8.0 might be due to the presence of proteolytic activity since proteases production was observed during fermentation (data not shown). Increase in pH due to protease activity is also reported by Gombert *et al*.[21] during lipase production by *Penicillium restrictum* in solid state fermentation using Babassu cake. In submerged fermentation a similar change in pH was reported by Freire *et al*.[22] and Carzo and Rivah.[23] Because, in that study, the pH for maximum enzyme production was also found to be 8.0–9.0, this supports current findings. This property of changing pH is very interesting, and has useful applications in the treatment of wastewater. This will reduce the cost of treatment, by minimizing the steps required for usual treatment, especially in the case of dairy wastewater treatment, where the pH is first neutralized with some chemicals and then additional treatments/steps are done.

Effect of incubation temperature on lipase production

Temperature is one of the most important physical factors for growth and enzyme production. To check the optimum temperature in the present case, incubation was carried out at different temperatures in the range 15 to 40 °C. It is clear from Fig. 3(b) that lipase production increased with increasing temperature with maximum production (195 U mL^{-1}) at 30 °C. Further increase in temperature resulted in decreased enzyme production showing 74.1 and 55 U mL^{-1} of enzyme activities at 35 and 40 °C, respectively. At 25 °C, 171.6 U mL^{-1} of lipase activity was detected. Singh *et al*.[20] and Kanwar *et al*.[24] also reported optimized temperatures of 30 °C and 34 °C for lipase production by a newly isolated strain of *Pseudomonas aeruginosa* and *Pseudomonas* sp. respectively.

Effect of additional carbon and nitrogen sources

Lipases are generally inducible in nature and carbon and nitrogen sources act as important factors for lipase production.[2] Among nitrogen sources, generally organic nitrogen sources like peptone and yeast extract are preferred for lipase production, while triacylglycerols, fatty acids, sugars, sugar alcohol and polysaccharides are preferred as carbon sources.[2] The effect of added carbon and nitrogen sources on lipase production was tested for *Aeromonas* sp. S1 also. Since the fermentation in this study was carried out in nutrient rich media with sufficient carbon and nitrogen sources, and additional sources also available from SDOCE, this might be the reason that supplementation of extra carbon and nitrogen sources did not enhance the lipase yield by *Aeromonas* sp. S1. Among the carbon sources explored, Mustard oil (1% v/v) did not affect the lipase production; however 43% of lipase activity was observed in the presence of coconut oil (1% v/v) compared with the control (Fig. 4(a)). Other carbon sources such as maltose (1% w/w) and glucose (1% w/w) had an inhibitory effect, resulting in 17 and 5.5% lipase activities, respectively, compared with the control. The inhibitory nature of glucose and maltose on lipase production by *Bacillus* sp. strain 42 was also reported by Eltaweel *et al*.[25] Li *et al*.[26] showed lower production of lipase from *Acinetobacter radioresistens* using olive oil and suggested that its oleic acid suppressed lipase synthesis. Inhibitory and inducible properties of triglycerides on lipase production were also suggested by Immanuel *et al*.[18]

Because most of the organic nitrogen sources were available from the media, the effect of different inorganic nitrogen sources (1% w/w), i.e. sodium nitrate, ammonium chloride and ammonium sulphate was tested on lipase production by *Aeromonas* sp. S1. Lipase production was found to be inhibited with supplemented inorganic nitrogen sources as shown in Fig. 4(b). There was 161.85, 152.1 and 75.27 U mL^{-1} of lipase activity in the presence of sodium nitrate, ammonium sulphate and ammonium chloride

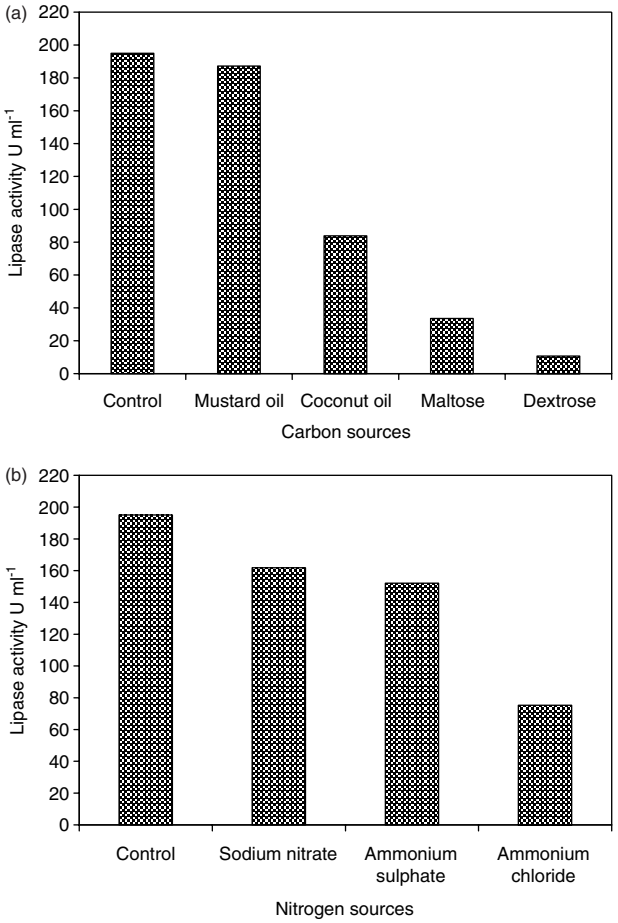

Figure 4. (a) Effect of different carbon sources on lipase production by *Aeromonas* sp. S1. Fermentation was carried out in nutrient media with 3% (v/v) SDOCE at pH 8.0, 30 °C for 24 h as described in the text. Lipase activity in media containing only 3% (v/v) of SDOCE at pH 8.0 was taken as control. (b) Effect of different inorganic nitrogen sources on lipase production by *Aeromonas* sp. S1. Fermentation was carried out in nutrient media with 3% (v/v) SDOCE at pH 8, 30 °C for 24 h. Lipase activity in media containing only 3% (v/v) of SDOCE at pH 8.0 was taken as control.

respectively. The inhibitory effect of sodium nitrate and other inorganic nitrogen sources was also reported by Eltaweel *et al*.[25] in the case of lipase production by *Bacillus* sp. Strain 42. However, no detectable lipase activity could be determined with *Bacillus megaterium* AKG-in the presence of ammonium chloride as nitrogen source.[27]

The optimized culture conditions for lipase production from *Aeromonas* sp. S1 were established as following: nutrient media containing 3% (v/v) SDOCE, pH 8.0 and incubation temperature 30 °C.

Under all optimized conditions, the lipase yield was enhanced 24-fold (195 U mL^{-1}) after 24 h growth compared with the initial 8.13 U mL^{-1} at 24 h. This level of lipase production (195 U mL^{-1}) from *Aeromonas* sp. S1 using SDOCE as additive nutrient source was comparable with the lipase yields of 72 U mL^{-1} and 92.27 U mL^{-1} using *Rhodotorula mucilaginosa* – MTCC 8737[28] and *Rhizopus delemar*,[29] respectively, in the presence of molasses. In contrast, Lotrakul and Dharmsthiti[16] reported high levels (450 U mL^{-1}) of lipase production by *Aeromonas sobria* in the presence of soybean meal and whey. In another report, combination of brewery co-product, yeast extract, malt extract, Tween 80 and olive oil with cheese whey provided an average increase of 15 U mL^{-1} in the lipase activity using *Candida rugosa*, through experimental design and response surface methodology.[30]

Enzyme characteristics

Since this lipase preparation was required for dairy wastewater treatment, it was worthwhile to investigate its enzymatic characteristics.

The optimum pH determined for lipase activity was in the range 9.0 to 10.0, with maximum activity at pH 10.0. At a higher pH value of 11.0, the enzyme activity was 70%, while only 29% activity was obtained at pH 7.0 with respect to the maximum activity at pH 10.0 (data not shown). Lipase was found to be most active at 55 °C, although it showed considerable activity over the range 35–45 °C, while lipase activity was reduced to 32.3% and 22.7% at 65 °C and 75 °C, respectively (data not shown). Similar pH and temperature optima were also reported for *Bacillus licheniformis* strain H1.[31] *Bacillus alcalophilus* shows optimum pH of 10.6 and temperature of 60 °C.[32] The optimum temperature of lipase produced by *Aeromonas sorbia* LP004 was found to be 45 °C and was highly stable in alkaline conditions up to pH 9.5.[16]

Preliminary treatment of dairy wastewater

Dairy wastewater contains a high concentration of lipids in the form of O&G and proteins that have low biodegradability and can cause severe environmental pollution if not treated properly.[4] High concentrations of O&G often cause problems in the various wastewater treatment processes. According to Cammarota and Freire[4] and Alberton *et al*.[5], in the activated sludge process, high O&G levels lead to the formation of films around the biological flocs, hindering the transfer of oxygen and substrate to the floc microorganisms and leading to the proliferation of filamentous microorganisms. In the case of anaerobic digestion, excessive amounts of O&G inhibit the action of acetogenic bacteria and methanogenic archaea.[5] Such problems necessitate the pretreatment of wastewater for removal of O&G. Various physicochemical methods are employed for such pretreatment. However, in recent years the use of microbial cultures and enzymes to increase hydrolysis during or prior to the biological treatment process[4,33,34] is a topic of attention due to its sustainable and environment-friendly approach. Such pretreatment methods generally consist of the cultivation of lipase-producing microbial strains in the effluents or the direct addition of crude or pure lipase preparations (fermented solid or liquid media containing enzyme) to the sample. In the present study, treatment of dairy wastewater was carried out using both methods individually; by inoculation of lipase-producing *Aeromonas* sp. S1 as well as by addition of its crude lipase preparation. A control set was also set up in which no culture or enzyme preparation was added extraneously.

Wastewater characterization

The wastewater used in the present study was collected from a local dairy industry, New Delhi, India. Physico-chemical and biological analysis of the sample presented the following characteristics: pH = 10; temperature = 20 °C; COD = 1200 ± 200 mg L^{-1}; BOD = 850 ± 50 mg L^{-1}; TSS = 425 ± 62 mg L^{-1}; O&G = 219 ± 27 mg L^{-1}; total nitrogen = 20.2 mg L^{-1}; total phosphorus = 2.4 mg L^{-1} and total coliforms = 7500 MPN per 100 mL. As the sample was found to contain higher values of COD, TSS, and O&G than Indian standard limits for dairy effluent discharge,[35,36] its treatment before disposal is required. Similar characteristics of

dairy wastewater with COD, O&G (total fat content) and TSS values of 1792 mg L^{-1}, 360 mg L^{-1} and 320 mg L^{-1}, respectively, were reported by Davery *et al*.[36]

Microbial treatment of dairy wastewater

The potency of *Aeromonas* sp. S1 was evaluated for reduction of COD, O&G and TSS from dairy wastewater. Figure 5(a) clearly shows that there is a remarkable decrease in COD, O&G and TSS contents after treatment with *Aeromonas* sp. S1. The strain reduced COD and TSS in wastewater by 93% and 47%, respectively, after 96 h of treatment. The O&G content was also reduced by up to 75% of the initial value after 96 h. The reduction in BOD and O&G from dairy wastewater to dischargeable limits using different individual microbes and in combination as consortia was also reported by Prasad and Manjunath.[37] The formulated mixture of microbes (*P. aeruginosa* LP602, *Bacillus* sp. B304, *Acinetobacter calcoaceticus* LP009) reported by Prasad and Manjunath,[37] effectively reduced initial 3200 mg L^{-1} BOD and 25 000 mg L^{-1} lipid content to 40 and 80 mg L^{-1}, respectively, during 12 day aerobic incubation at 30 °C and 200 rpm. Mongkolthanaruk and Dharmsthiti[38] also reported the use of mixed culture composed of *P. aeruginosa* LP602, *Acinetobacter calcoaceticus* LP009 (both lipase-producing bacteria) and *Bacillus* sp. B304 (an amylase- and protease-producing bacterium) for the treatment of lipid rich wastewater. The rapid attainment of dischargeable values for COD in the case of *Aeromonas* sp. S1 also clearly shows the efficacy of the isolate (Table 1.).

Enzymatic treatment of dairy wastewater

Many authors have reported the use of lipolytic enzymes in wastewater treatment.[34] High lipase activity is a critical prerequisite, if the enzymes are to be applied for industrial effluent treatment[4]. 5% (v/v) of enzyme extract containing 195 U mL^{-1} of initial lipase activity was used for the treatment of wastewater (9.75 U mL^{-1}). Figure 5(a) shows that there was significant reduction in COD, TSS and O&G compared with the control after treatment with this enzymatic preparation. Since, in the case of enzymatic treatment, there was 86% reduction of COD in 24 h (Fig. 5(a)), and thereafter it remained almost constant to 96 h, an experiment was set-up to check earlier COD removal, as enzymes are known to be fast in their reactions. Samples were withdrawn every 4 h during the treatment. Reductions of 44 and 69% in COD were observed in the first 4 and 8 h, respectively (Fig. 5(b)). After 12 h of treatment, COD reduction reached 86%, which remained constant at subsequent time intervals of 24, 48 and 96 h. This experiment confirmed that enzymatic treatment was very fast compared with microbial treatment, where only 71.5% COD reduction was observed after 24 h treatment. There

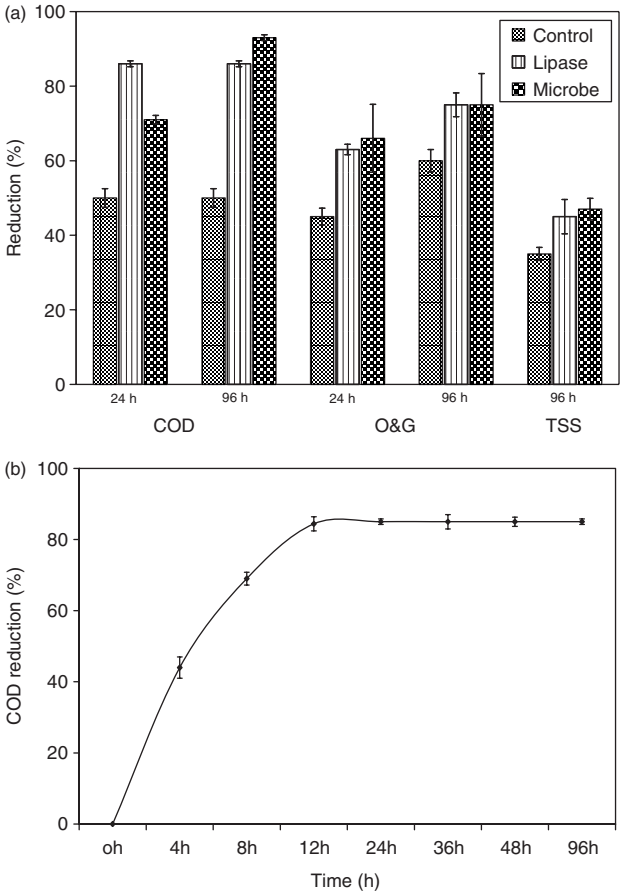

Figure 5. (a) Reduction of COD, O&G and TSS during lipolytic and microbial treatment of dairy wastewater. The percentage reduction values for COD and O&G are for 24 and 96 h of treatment period, whereas for TSS it is 96 h. In the control no microbial culture and lipase preparation were added extraneously. The initial value of COD, O&G and TSS were considered when calculating percentage reduction at the given times. The experiment was done in duplicate. Error bars show the percentage error. (b) Time course reduction of COD in dairy wastewater during lipase treatment. The initial value of COD was considered when calculating percentage reduction at the given times. The experiment was done in duplicate. Error bars show the percentage error.

was 75% and 45% reduction of O&G and TSS, respectively, after 96 h of Lipase treatment.

In a similar kind of study, Dharmsthiti and Kuhasuntisuk[34] used 3.5 U mL^{-1} of crude lipase preparation from *Pseudomonas aeruginosa* LP602 for treatment of about 200 mg L^{-1} fat-containing restaurant wastewater. In their study, 70% reduction of lipid content was observed during the first 24 h of treatment, while

Table 1. Reduction in the initial COD, O&G and TSS values after treatment: the value of each parameter was obtained after 96 h treatment and compared with their standard permissible values. The parameters values obtained are of the average value of two independent experiments, with their standard deviations

		Final value (96 h)			
Parameter	Initial value	Control	Microbe	Lipase	Standard permissible value
COD (mg L^{-1})	1200 ± 200	600 ± 59	81.5 ± 9	168 ± 10	250
O&G (mg L^{-1})	219 ± 27	88 ± 25	55 ± 18	55 ± 7	10
TSS (mg L^{-1})	425 ± 62	276 ± 8	225 ± 12	234 ± 19	150

complete reduction of lipid occurred after 48 h. Enzymatic treatment of dairy wastewater using fermented solid containing the lipases of *Rhizopus microsporus* was also reported by Alberton *et al*.[5] They evaluated the efficacy of their treatment on the basis of BOD, COD and O&G analyses. During 72 h of treatment, the initial COD of 6908 mg L^{-1} was reduced to 2570 mg L^{-1} using solid fermented media containing lipase. The O&G content was also reduced to 250 mg L^{-1} during 72 h of treatment from an initial value of 1300 mg L^{-1}. They supplemented extra dairy fat to wastewater; initially it contained 400–600 mg L^{-1} of O&G.

In another report, Jung *et al*.[39] reported the effectiveness of enzymatic pre-hydrolysis on batch activated sludge systems dealing with oily wastewaters. They evaluated the biological treatment of dairy wastewater containing different O&G contents (400, 600 and 800 mg L^{-1}) with or without enzymatic pre-hydrolysis. 0.2% (w/v) of fermented Babassu cake containing 11 U g^{-1} of *Penicillium restrictum* lipase was used as enzymatic pool. COD removal efficiency was found to be better in the case of lipolytic pre-hydrolysed wastewater compared with the control. The COD reduction capacity was also observed to be decreased with increasing O&G content in control set, while it remained almost constant in the pre-hydrolysed case.[39]

As shown in Fig. 5(a), there was reduction in the control set (without addition of extraneous culture or enzyme) also. Since the control flask was kept under similar conditions to the test samples, i.e. with shaking of 200 rpm, this shaking might have provided enough oxygen and mixing conditions for growth of indigenous microbes in the wastewater, resulting in COD, TSS and O&G reduction. To confirm this, another control flask was also kept under similar experimental conditions but without shaking. The samples were withdrawn at 0 and 96 h, and surprisingly, there was no reduction of O&G and TSS, and negligible reduction of COD (less than 5%). This supported the previous assumption that, due to shaking, it was the growth of indigenous microorganisms that resulted in reduction of organic matter (COD).

The residual O&G and TSS values obtained in this study after 96 h of microbial and enzymatic treatments were close to the dischargeable value,[35] and for COD, the value was below the dischargeable limit,[36] as shown in Table.1. The above results show that both *Aeromonas* sp. S1 and its lipase could effectively be used for treatment of dairy wastewater.

CONCLUSIONS

The results obtained show that natural substrate in the form of SDOCE has good inducer properties for lipase synthesis. Thus it might be worthwhile to use this natural by-product as a cost-effective source for lipase production. The isolate *Aeromonas* sp. S1 and its lipase were found to be effective for treatment of wastewater originating from food processing industries. However, since the treatment was done as preliminary work, further studies are required regarding wastewater treatment in real situations with high O&G and COD contents.

ACKNOWLEDGEMENTS

The financial support provided by TRIFED (Tribal Co-Operative Marketing and Development Federation of India Limited), Ministry of Tribal Affairs, Government of India, is gratefully acknowledged. The first and second authors thank the Ministry of Higher Education, Iraq and University Grants Commission (UGC), Government of India, for research fellowships respectively. The authors also thank professor Subhash Chand and Dr S. K Khare, Indian Institute of Technology, Delhi for their valuable suggestions.

REFERENCES

1 Treichel H, Débora DO, Mazutti MA, Marco DL and Oliveira JV, A review on microbial lipases production. *Food Bioprocess Technol* **3**:182–196 (2010).
2 Gupta R, Gupta N and Rathi P, Bacterial lipases: an overview of production, purification and biochemical properties. *Appl Microbiol Biotechnol* **64**:763–781 (2004).
3 Babu J, Ramteke PW and Thomas G, Cold-active microbial Lipases: some hot issues and recent developments. *Biotechnol Adv* **26**:457–470 (2008).
4 Cammarota MC and Freire DMG, A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. *Bioresource Technol* **97**:2195–2210 (2006).
5 Alberton D, Mitchell AD, Cordova J, Peralta-Zamora P and Krieger N, Production of fermented solid containing lipases of *Rhizopus microsporus* and its application in the pre-hydrolysis of a high-fat dairy wastewater. *Food Technol Biotechnol* **48**:28–35 (2010).
6 Pandey A, Soccol CR and Mitchell D, New developments in solid state fermentation, I: bioprocesses and products. *Process Biochem* **35**:1153–1169 (2000).
7 Pandey A and Soccol CR, Economic utilization of crop residues for value addition – a futuristic approach. *J Sci Ind Res* **59**:12–22 (2000).
8 Ramachandran S, Singh KS, Larroche C, Soccol CR and Pandey A, Oil cakes and their biotechnological applications – a review. *Bioresource Technol* **98**:2000–2009 (2007).
9 Patnaik S and Mohaptra MD, Sal Seed -a losing proposition or an untapped resource? Community forestry, http://www.banajata.org/pdf/articles [Last accessed on 25 March 2011].
10 Chandra R, Vijay VK and Subbarao PMV, A study on biogas generation from non-edible oil seed cakes: potential and prospects in India, in *2nd Joint International Conference on Sustainable Energy and Environment*, 21–23 November, Bangkok, Thailand, E-007 (P) (2006).
11 Gandhi VM, Cherian KM and Mulky MJ, Detoxification of Castor seed meal by interaction with Sal seed meal. *JAOCS* **71**:827–831 (1994).
12 Mahanta N, Gupta A and Khare SK, Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* Pse A in solid-state fermentation using *Jatropha curcus* seed cake as substrate. *Bioresource Technol* **99**:1729–1735 (2008).
13 Kirschman HD and Pomeroy R, Determination of oil in oil-field wastewaters. *Anal Chem* **21**:793–797 (1949).
14 APHA, *Standard Methods for the Examination of Water and Wastewater*, 19th edn. American Public Health Association, American Water Works Association & Water Environment Federation, Washington, DC (1998).
15 Leal MCMR, Freire DMG, Cammarota MC and Jr Sant'Anna GL, Effect of enzymatic hydrolysis on anaerobic treatment of dairy wastewater. *Process Biochem* **41**:1173–1178 (2006).
16 Lotrakul P and Dharmsthiti S, Lipase production by *Aeromonas sobria* LP004 in a medium containing whey and soybean meal. *World J Microbiol Biotechnol* **13**:163–166 (1997).
17 Rajagopalan G and Krishnan C, α-Amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. *Bioresource Technol* **99**:3044–3050 (2008).
18 Immanuel G, Esakkiraj P, Jebadhas A, Iyapparaj P and Palavesam A, Investigation of lipase production by milk isolate *Serratia rubidae*. *Food Technol Biotechnol* **20**:60–65 (2008).
19 Gunasekaran V, Kotay SM and Das D, Alkaline lipase production by *Citrobacter freundii* IIT-BT L139. *Indian J Expt Biol* **24**:485–489 (2006).
20 Singh S, Kaur G, Chakraborti AK, Jain RK and Banerjee UC, Study of the experimental conditions for the lipase production by a newly isolated strain of *Pseudomonas aeruginosa* for the enantioselective hydrolysis of (±)-methyl *trans* -3(4-methoxyphenyl) glycidate. *Bioprocess Biosyst Eng* **28**:. 341–348 (2006).
21 Gombert AK, Pinto AL, Castilho LR and Freire DMG, Lipase production by *Penicillium restrictum* in solid-state fermentation using Babassu oil cake as substrate. *Process Biochem* **35**:85–90 (1999).
22 Freire DMG, Teles EMF, Bon EPS and Jr Sant'Anna GL, Lipase production by *Penicillium restrictum* in a laboratory-scale fermentor: media composition, agitation and aeration. *Appl Biochem Biotechnol* **63**:409–421 (1997).

23 Corzo G and Revah S, Production and characteristics of the lipase from *Yarrowia lipolytica* 681. *Bioresource Technol* **70**:173–180 (1999).
24 Kanwar L and Goswami P, Isolation of a *Pseudomonas* lipase produced in pure hydrocarbon substrate and its applications in the synthesis of isoamyl acetate using membrane-immobilized lipase. *Enzyme Microbial Technol* **31**:727–735 (2002).
25 Eltaweel MA, Abd Rahman RNZR, Salleh AB and Basri M, An organic solvent–stable lipase from *Bacillus* sp. strain 42. *Ann Microbiol* **55**:187–192 (2005).
26 Li CY, Cheng CY and Chen TL, Production of *Acinetobacter radioresistent* lipase using Tween 80 as the carbon source. *Enzyme Microbial Technol* **29**:258–263 (2001).
27 Sekhon A, Dahiya N, Tiwari RP and Hoondal GS, Production of extracellular lipase by *Bacillus megaterium* AKG-1 in submerged fermentation. *Indian J Biotechnol* **5**:179–183 (2006).
28 Potumarthi R, Subhakar C, Vanajakshi J and Jetty A, Effect of aeration and agitation regimes on lipase production by newly isolated *Rhodotorula mucilaginosa*– MTCC 8737 in stirred tank reactor using Molasses as sole production medium. *Appl Biochem Biotechnol* **151**:700–710 (2008).
29 Acikel U, Ersan M and Acikel YS, The effects of the composition of growth medium and fermentation conditions on the production of lipase by *R. delemar*. *Turk J Biol* **35**:35–44 (2011).
30 Tommaso G, Souza de Moraes B, Macedo GC, Silva GS and Kamimura ES, Production of lipase from *Candida rugosa* using cheese whey through experimental design and surface response methodology. *Food Bioprocess Technol* **4**:1473–1481 (2011).
31 Khyami-Horani H, Thermotolerant strain of *Bacillus licheniformis* producing lipase. *World J Microbiol Biotechnol* **12**:399–401 (1996).
32 Ghanem EH, Al-Sayeed HA and Saleh KM, An alkalophilic thermostable lipase produced by a new isolate of *Bacillus alcalophilus*. *World J Microbiol Biotechnol* **16**:459–464 (2000).
33 Lanciotti R, Gianotti A, Baldi D, Angrisani R, Suzzi G, Mastrocola D, *et al*, Use of *Yarrowia lipolytica* strains for the treatment of olive mill wastewater. *Bioresource Technol* **96**:317–322 (2005).
34 Dharmsthiti S and Kuhasntisuk B, Lipase from *Pseudomonas aeruginosa* LP602: biochemical properties and application for wastewater treatment. *J Ind Microbiol Biotechnol* **21**:75–80 (1998).
35 http://www.cpcb.nic.in/Industry-Specific-Standards/Effluent/DiaryIndustry.pdf. [Last accessed on 25 March 2011].
36 Daverey A and Pakshirajan K, Pretreatment of synthetic dairy wastewater using the sophorolipid-producing yeast *Candida bombicola*. *Appl Biochem Biotechnol* **163**:720–728 (2010).
37 Prasad MP and Manjunath K, Comparative study on biodegradation of lipid rich wastewater using lipase producing bacterial species. *Indian J Biotechnol* **10**:121–124 (2011).
38 Mongkolthanaruk W and Dharmsthiti S, Biodegradation of lipid rich wastewater by a mixed bacterial consortium. *Int Biodeter Biodeg* **50**:101–105 (2002).
39 Jung F, Cammarota MC and Freire DMG, Impact of enzymatic pre-hydrolysis on batch activated sludge systems dealing with oily wastewaters. *Biotechnol Lett* **24**:1797–1802 (2002).

