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Energy Procedia 54 (2014) 518 – 529

Energy Procedia

4th International Conference on Advances in Energy Research 2013, ICAER 2013

Isolation, Purification and characterization of lipase from *Microbacterium* sp. and its application in biodiesel production

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Abstract

Presently the world is facing the problem of scarcity of fossil fuel as it is a non-renewable source of energy. Biodiesel can be an alternative energy source having the advantage of being renewable as well as environment friendly. Enzymes are better catalysts for the production biodiesel as enzymes are more stable and their production is more convenient and safer. Microalgae *Scenedesmus* sp. was used as a feedstock for biodiesel production. Lipase producing bacteria was isolated from the pulp and paper mill and identified as *Microbacterium* sp. using 16S rDNA sequencing method. Lipase enzyme was purified by sequential methods of ammonium sulphate precipitation and Sephadex G-100 gel column chromatography. The molecular weight of purified enzyme was 40 kDa on SDS-PAGE. This purification procedure resulted in 2.1fold purification of lipase with a 20.8 % final yield. The purified lipase exhibited maximal hydrolytic activity at a temperature of 50 ^{0}C and a pH of 8.5. The Km of lipase was 3.2 mM and the Vmax 50 µmol/min/mg. Lipase activity was stimulated by Triton X-100 and SDS and inhibited by Tween 20 and Tween 80. Biodiesel was prepared through sodium hydroxide, potassium hydroxide and Lipase (Celite and charcoal bound as substrate) catalyzed transesterification process, which enabled a yield of 72.5%, 90% (95.1%, and 15.5%) respectively determined by gas chromatography/mass spectrometry (GC/MS) analysis.

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Keywords: Lipase ; *Microbacterium* ; Biodiesel

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1. Introduction

Energy is the basic requirement for economic development. The Growing consumption of energy has resulted the world is becoming increasingly dependent on fossil fuels. Carbon dioxide concentration in the atmosphere has reached to 450 ppm and is very harmful to the environment [1]. Alternative fuels like second generation biofuels have the ability to reduce pollutant emissions of greenhouse gases by their capacity to trap and use the carbon dioxide (CO_2). Microalgae have been emerging as a potential source for biodiesel production and *Scendesmus* sp.

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doi:10.1016/j.egypro.2014.07.293

can be used as a source. Fats and oil are made up of triglycerides and contains glycerol esters with saturated and unsaturated fatty acids. Fats contain triglycerides, di and monoglycerides, free fatty acids (FFAs), and hydrocarbons [2-5]. On the industrial scale, production of biodiesel is being carried out by using oils and alcohol (methanol or ethanol) and inorganic catalyst at different conditions and the process is known as transesterification. Alkali-catalyzed process has its drawback due to its sensitivity to both water and free fatty acid contents in the feedstock. Saponification occurs when free fatty acids and water react with the alkali catalyst [6-7]. Saponification consumes the alkali catalyst and formation of soap occurs which can produce emulsions that create contamination in the form of glycerol [8-9]. Acid-catalyzed transesterification is not referred due to its slower reaction rate [5]. Enzymes are better catalysts for the production of biodiesel as enzymes are more stable and their production is more convenient and safer. Microbial lipase has its use in industrial applications in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture and production of cosmetics and pharmaceuticals. *Microbacterium* sp. is capable of producing lipase was isolated from the pulp and paper industry. Immobilization of lipase prevents the contamination of the product (biodiesel) and increase the active life of the enzyme [5].Immobilization hastens the rate of reaction as it provides the rigid external backbone to lipase molecule [10]. Physical adsorption is the most useful method for immobilization because it is easy, inexpensive, intoxicant, able to retain the activity and feasible for regeneration [11]. Celite, obtained from the fossilized silica remains of diatoms is an inexpensive solid support for the adsorption of catalysts, and is found in a variety of particle size, shape and porosity [11]. Charcoal can also be used as an adsorbent due to its high surface area. Lipase should be used again and again without losing its stability and catalytic activity to reduce the cost in biodiesel production. Therefore, in this study, algae *Scendesmus* sp. ISTGA1 was used as feedstock for biodiesel production. Lipase producing bacteria was isolated from pulp and paper mill effluent and purified using as catalyst in transesterification process.

2. Material and methods

2.1. Cultivation and harvesting of Microalgae

The microalgae *Scendesmus* sp. isolated from marble mining rock was cultured in BG-11 media. The biomass of cultured cells was harvested by centrifugation for oil extraction.

2.2. Isolation and identification of lipase producing bacteria

2.2.1. Strain isolation, screening and culture conditions

Sludge and sediment samples were collected from effluent of M/s Century Pulp and Paper Mill, Lalkuan (79° 28′E longitude and 29°24′N latitude), Nainital, Uttarakhand, India and stored at 4°C. 10 g sludge sample was dissolved in 100ml of autoclaved distilled water and stirred. The supernatant was spread into Luria Broth (LB) agar plates. Bacterial cells were grown on LB-agar plates. Different bacteria were screened for lipase production using phenol red plate assay containing tributyrin [12].The bacterial strain which showed the maximum lipolytic activity was taken for the lipase production. The genomic DNA was isolated using DNA Kit (Qiagen Inc.,USA). The 16S rDNA gene was selectively amplified from genomic DNA by using PCR with oligonucleotide universal primers. The Strain was identified on the basis of 16S rDNA gene sequences by using appropriate software (nucleotide-nucleotide BLAST) in "National Centre for Biotechnology Information" resource. The lipase producing bacteria was grown in LB medium and transferred into Minimal Salt Media (per litre salt solution) $MgSO_4$,0.2g; $Fe(CH_3COO)_3NH_4$,0.05g; $NaNO_3$,0.085 and (per litre buffer solution) $Na_2HPO_4.2H_2O$,7.8g; KH_2PO_4,6.8 g [13] and 1% (v/v) tributyrin as a carbon source and the pH was maintained at 8.5. The culture was incubated at 37^0C for 72 hours. The culture medium was removed after 12, 24, 36, 48, 60 and 72 h for determining the growth pattern and Lipase activity. The growth patterns of bacterial strains were taken at O.D. at 595 nm with a spectrophotometer (Cary, 100 Bio, Varian Co, Australia).

2.3. Assay for lipolytic activity

Lipolytic activity was determined by a spectrophotometric assay using pNPP (Para nitro phenyl palmitate) as a substrate [14]. The reaction mixture consisted of 0.1 ml enzyme extract, 0.8 ml of 0.05 M Tris buffer (pH 8) and 0.1 ml of 0.01M of p-NPP dissolved in isopropanol. The reaction mixture was kept at 60°C for 20 min in a water bath and 0.25ml of 0.1M Na_2CO_3 was added to stop the reaction. The reaction mixture was centrifuged at 11,000 g for 15 min and the Optical density (O.D.) was determined at 410 nm. One unit of lipase activity was defined as the amount of enzyme which liberated 1 μmol of p-nitro phenol per min from p-nitro phenyl palmitate.

2.4. Lipase purification

Bacterial culture grown in MSM media and tributyrin was centrifuged at 8000g for 20 min at 4 °C in a refrigerated centrifuge. Cell free supernatant was saturated with (0-70%) ammonium sulphate with continuous stirring at 4°C followed by centrifugation at 14,000 rpm for 20 minutes [15]. Ammonium sulphate fraction was dialyzed against 50mM Tris-Chloride buffer (pH 8.0) for 6 hours at 4°C in a Dialysis bag. The concentrated enzyme after dialysis was loaded onto sephadex G-100 column. The enzyme was eluted from the column at a flow rate of 1 ml/min. Enzyme fractions (5ml each) were collected and the protein content was measured spectrophotomerically at 280 nm. Lipase assay was performed using fractions containing highest protein content.

2.5. Characterization of Lipase enzyme

2.5.1. Effect of pH and Temperature on Lipase activity

The optimal pH was determined in the range of 3 to 11 at 50 °C in buffer solutions of pH values ranging from 3 to 11 using p-NPP as a substrate and spectrophotometric assay method was performed as described above.
The Optimal temperature for activity was determined by the spectrophotometric assay using p-NPP as substrate at different temperatures (10-90℃) at pH 8.

2.5.2. *Effect of different concentrations of substrate*

Enzyme activity at different substrate concentrations (0.9-20 mM) was determined at 50^0C by measuring absorbance at 410 nm. Km and Vmax values were calculated at pH 8.5. Specific activity of enzyme was tested with increasing concentrations of substrate. The kinetic parameter Km and Vmax were estimated from Michaelis-Menten equation. The data were fit in curve of the single rectangular hyperbola (equation y= ax/b+x).From this Vmax and Km values have been derived. The rate of reaction was expressed as the number of micromole of substrate changed /min/mg of protein in the sample. One Unit specific activity was defined as one micromole substrate conversion per mg per minute.

2.5.3. Effect of detergent and organic solvents on Lipase activity

Effect of detergent and organic solvents was determined by using different detergents 1% V/V(Triton X-100, Tween 20, Tween 80 and SDS) and organic solvents (Acetone, Propanol, Butanol and Methanol), were added to the enzyme solution and incubated at 30 °C for 60 min. Activity was measured spectrophotometrically using pNPP as a substrate.

2.5.4. Effect of metal ions and enzyme inhibitors on Lipase activity

The effect of different metal ions and inhibitors on lipase activity was determined by using 1mM of $BaCl_2$, $CaCl_2$, $MgCl_2$, KCl, LiCl, NaCl $ZnSO_4$, EDTA, β-mercaptoethanol, and phenylmethyl-sulfonyl fluoride (PMSF) in 0.05M phosphate buffer (pH 8), at 60 ℃.

2.6. Immobilization of Lipase on celite and charcoal

Celite 545 was obtained from Loba chemicals. Activated charcoal was prepared from Sugarcane bagasse. The sugarcane bagasse was obtained from Modi sugar mills, Modi Nagar, U.P., India. Immobilization of Lipase enzyme was performed. 4 g of celite and charcoal were dissolved in 50 ml of 50mM sodium phosphate buffer (pH 7.0) and stirred at 30°C for one hour. The fine particles were decanted three times. Washed Celite and charcoal were stirred at 4°C overnight. Celite and charcoal bound enzyme was washed with 50 mM sodium phosphate buffer. The bound enzyme suspended in 20 ml of 50mM phosphate buffer and kept at 4°C. The protein concentration in the lipase solution was determined by Bradford protein assay using bovine serum albumin (BSA) as the standard.

2.7. Oil Extraction and production of Biodiesel

Bligh and Dyer method [16] was used for algal Oil extraction. The transesterification process was performed using oil: methanol molar ratio (1:3), catalyst NaOH/ KOH (1%)/ celite immobilized lipase (50 mg)/charcoal immobilized lipase (50 mg) and shaken for 3 hours at 300 rpm. Samples were left overnight for settlement of different layers (fatty acid methyl ester and sediment layer).The biodiesel layer was separated from the sediment by centrifugation and FAMEs were extracted and analyzed through GC/MS. Determination of the free fatty acid content was done titrametrically. The titrant used was Sodium hydroxide with the phenolphthalein as an indicator and free fatty acid content was calculated [17].

2.8. Reusability of immobilized enzyme

The Lipase was removed from the transesterification reaction mixture by decantation and methanol was used for the washing. For further use, the enzyme was separated by decantation and the fresh reaction mixture was used for 10 different cycles. For using the enzyme after 24 hours it was also vacuum filtered and dried in dessicator. The immobilized enzyme was reused for the transesterification reaction and fatty acid methyl esters (FAMEs) content in the reaction medium were determined by gas chromatography (GC).

3. Results and discussion

3.1. Isolation, screening, identification and culture of lipase producing bacterial strain

Different types of bacterial strains appeared on the plates were isolated based on morphological differentiation of individual colonies. Eight different bacterial strains such as A, B, C, D, E, and L were screened for lipase positive strain on the phenol red plates containing 1% tributyrin. They produced yellow zone on the plates. In order to select the best possible lipase producer, strains with large clear yellow zone was collected and out of the 8 strains, one strain i.e. L strain produced the largest yellow zone and taken as lipase positive strain (Fig.1). The reason of the yellow zone formation is the decrease in pH due to release of fatty acids on lipolysis. The End point of the phenol red dye (pink) is at a pH (7.3) and it changes into yellow when there is a slight decrease in pH (6.9). Bacterial strain was isolated and identified by morphological characteristics like round, smooth, viscous, convex, opaque and yellow white. The Strain was identified by 16S rDNA sequencing. The Strain showed the maximum homology with the *Microbacterium* sp. genus. Phylogeny tree (Fig. 2) was drawn using MEGA 3.1 software [18]. Lipase production from *Microbacterium* sp. has been reported in the earlier studies [19]. There is occurrence of Lipolytic bacteria usually in nature and approximately 20% of them are of several lipase producers [20]. Result of the study indicated that the microbial growth and enzyme production was higher at 48 hours at the beginning of stationary phase (Fig. 3).The lipase activity was decreased in a late stationary phase may be due to the presence of proteases in the culture media. *Staphylococcus* warneri showed the highest lipase production at the beginning of the stationary phase after 24 hours [21]. According to Thomas et al., lipase production varies with species for the parameters like optimum temperature, optimum pH and enzyme specificity [22].

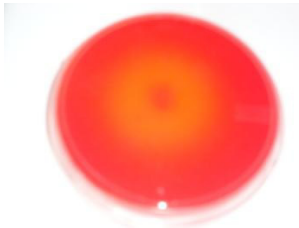

Fig.1. Phenol red plate showing lipase production

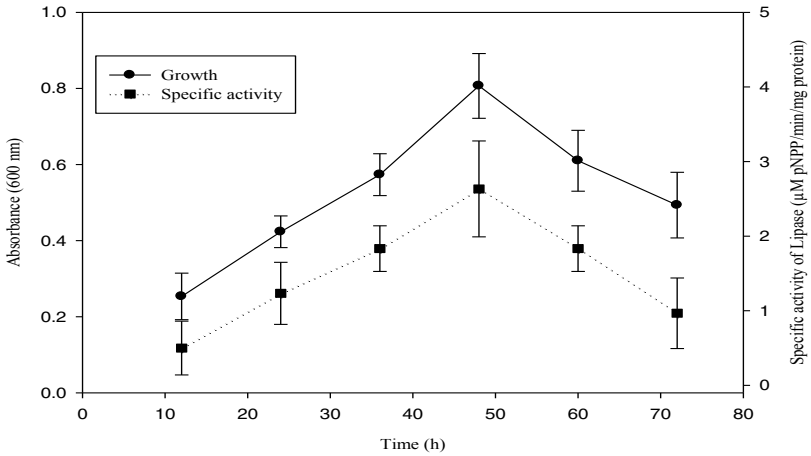

Fig.2. Growth curve and lipolytic activity of bacteria

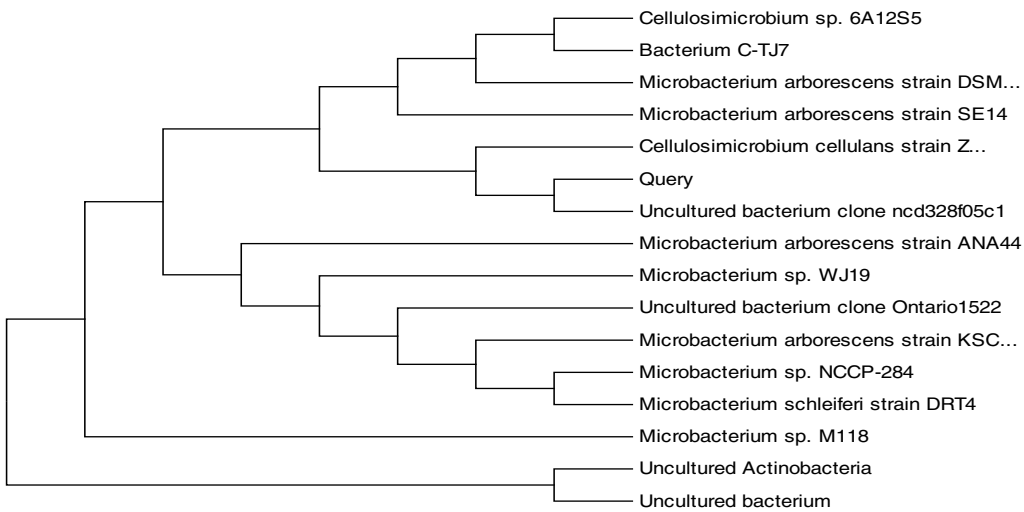

Fig. 3. Phylogenetic tree of lipase producing bacterium, *Microbacterium* sp. based on 16 S rDNA gene sequences. The bootstrap consensus tree was drawn by multiple sequence alignment with neighbour-joining method using software MEGA 3.1

3.2. Lipase purification

Lipase purification was done to get a protein of interest and to remove unnecessary one. Purification process of lipase occurs in sequential manner. The enzyme produced over 48 hours of culture was purified by ammonium sulpate precipitation for salting out the proteins. For increased enzymatic activity desalting was performed for removing the traces of salt. According to Pabai *et al.* [23] increased lipase activity depends on the concentration of ammonium sulphate. An extracellular lipase from *Microbacterium* sp. was purified by ammonium sulphate precipitation and Sephadex G-100 column chromatography with a total yield of 20.8 % and 2.1 fold purification (Table 1). It was also found that the total yield and purification fold were 35.6 % and 3 respectively in case of *Microbacterium* luteolum [19]. A low yield of the enzyme may be due to difficulty in removal of the high content of lipopolysaccharide present in *Microbacterium* sp. and coupled with lipid hydrolysis [24].

Table 1. Summary of the purification of lipase from *Microbacterium* sp.

Purification stage	Total activity in U	Total Protein in mg	Specific activity in U/mg	Purification Fold	Yield (%)
Crude	355	150	2.3	1	100%
Ammonium Sulphate precipitation	243	68	3.5	1.5	68%
Sephadex G-100	74	15	4.9	2.1	20.8%

Unit is expressed as $\mu mol^{-1}min^{-1}mg^{-1}$

Enzyme activity was determined which was concentrated and subjected to SDS-PAGE. Result of the study indicated molecular mass of the purified enzyme from *Microbacterium* sp. was 40 kDa on SDS-polyacrylamide gel electrophoresis (Fig.4).

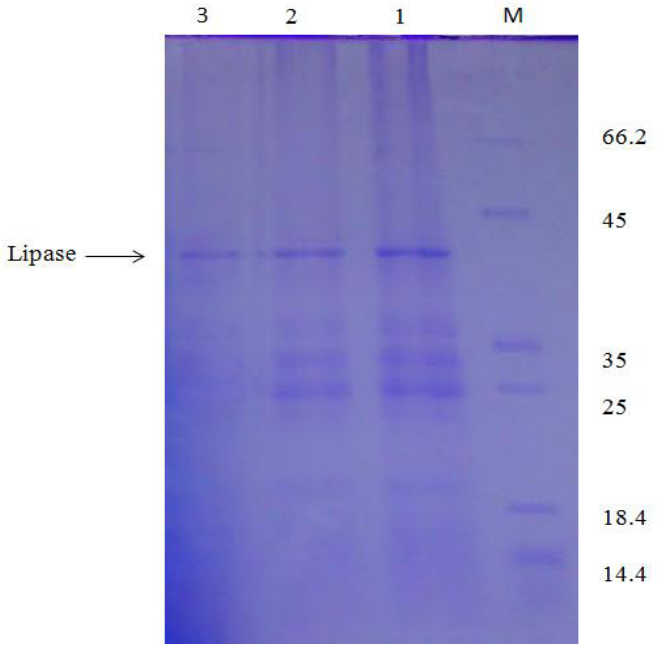

Fig .4. SDS-PAGE of *Microbacterium* sp. lipase at various stages of purification. Lane M molecular weight markers; Lane 1: crude extract; Lane 2: Ammonium sulphate precipitation; Lane 3: Sephadex G-100.

3.3.2. Lipase characterization

3.3.2.1. Effect of pH and temperature on lipase

Lipase showed the activity in a range of pH 3 to 11 and showed maximum activity at pH 8.5 (Fig.5a). Lipases from *Bacillus* thermoleovorans ID1 showed the optimal activity at pH 9 [25]. Lipases producing bacteria like thermophilic *Bacillus* which lie in the range of pH 7.2–8.5 [26–30, 31, 32] have been reported in earlier species.
Lipase from *Microbacterium* sp. showed activity in a range of 10–90°C. The optimum temperature for *Microbacterium* sp. lipase was found to be 50°C (Fig 5b). Similar results were found for lipase activity in *Bacillus* sp. J33 (60°C) [28], *Bacillus* sp. A30-1 (60℃) [32], and *Bacillus thermoleovorans* ID-1 (60–65°C) [34].

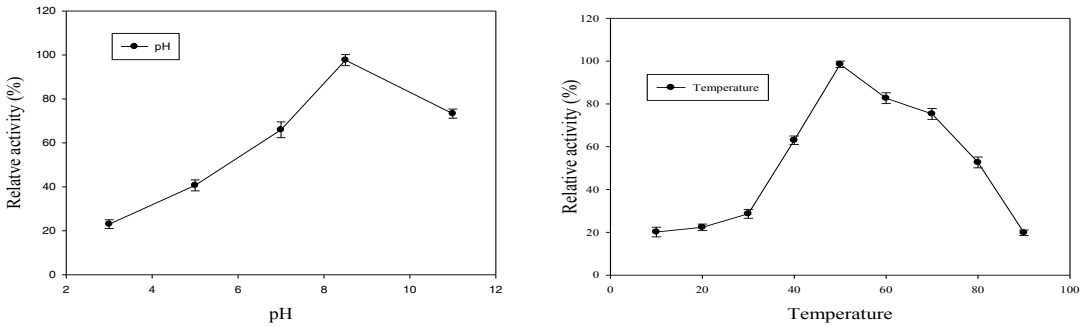

Fig.5. (a) Effect of pH on the activity of lipase obtained from *Microbacterium* sp.(b) Effect of Temperature on the activity of lipase

3.3.2.2. Effect of different concentration of substrate on lipase activity

Enzyme kinetics measured Km and Vmax from Michaelis- Menten plots of lipase activity at optimal pH and temperature using various concentrations of p-NPP substrate. The Km and Vmax values of lipase were 3.2 mM and 50 µM/min/mg, respectively (Fig. 6). It w as reported that Km and Vmax for a lipase from *Bacillus* sp. J33 were 2.5 mM and 0.4 M/mL/min., respectively, with pNPL as substrate. Km and Vmax values for lipase from *Bacillus Stearothermophilus* were 0.33 mM and 188 µM/min/mg, respectively, when using pNPP as substrate [35].

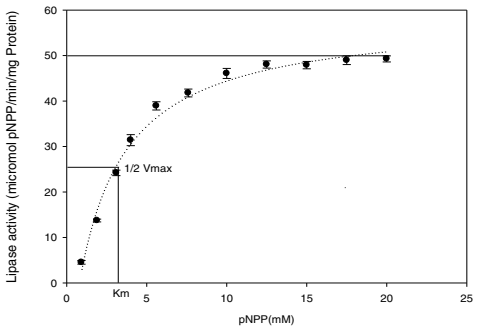

Fig. 6. Effect of different concentration of substrate on the activity of lipase obtained from *Microbacterium* sp.

3.3.2.3. Effect of detergent and organic solvents on lipase activity

The effects of detergent and organic solvents were observed in lipase activity in (table 2 and 3) respectively. The addition of 1% SDS and Triton x-100 increased the enzyme activity while on the addition of Tween-20 and Tween-80, it was inhibited. The same result was observed for the cynobacterium *Spirulina* platensis when treated with SDS [36]. As similar to the results, Schmidt- Dannert et al. [26] reported reduction of lipolytic activity in the presence of Tween 20 and Tween 80.

Table 2. Effect of detergents on *Microbacterium* sp. lipase activity

Detergent	Concentration	Relative activity % (1 h)
Control	-	100 ± 2.3
Triton X-100	1%	115 ± 1.3
Tween 20	1%	91 ± 0.5
Tween 80	1%	93 ± 2.9
SDS	1%	101 ± 1.9

Table 3. Effect of Organic solvent on *Microbacterium* sp. lipase activity

Organic solvent	Concentration	Relative activity % (1 h)
Control	-	100 ± 2.3
Acetone	70%	72.3 ± 3.1
Methanol	70%	91 ± 2.7
Ethanol	70%	84.4 ± 0.7
2-Propanol	70%	55 ± 1.7
Butanol	70%	93 ± 2.9

Enzymatic activity of lipase was determined in organic solvents. The addition of 70% methanol showed the maximum lipolytic activity while propanol showed the minimum activity. Similar methanol activity was reported in a lipase from *Photobacterium* lipolyticum [37]. The reason behind the high activity of lipase in the presence of solvents may be due to the conversion of the closed form of the enzyme to open form by the solvent used and stimulates the enzyme in this open confirmation [38].*Microbacterium* sp. has better methanol tolerance and lipase from it is applicable for the methylation of algal oil in transesterification reaction in biodiesel production.

3.3.2.4. Effect of detergent and organic solvents on lipase activity

Enzymatic activity of lipase was determined in presence of different metal ions and inhibitors (Table 4). Na^+ and Ba^+ salts did not affect the lipase activity, while Zn^{2+}, Mg^{2+}, K^+, and Li^+ ions decreased their activity. Ca^{2+} increased activity by 50%.It has been reported for other thermophilic lipases Ca^{2+} ions increased activity [29].Lipase activity was inhibited by 79% and 49.3% respectively in the presence of 1mM phenyl methyl sulfonyl fluoride (PMSF) and Ethylene diamine tetraccetic acid (EDTA),respectively.

3.4. Lipase immobilization

Lipase is an efficient biocatalyst for the synthesis of alkyl esters by alcoholysis of lipids. The Enzyme was immobilized on celite and charcoal through physical adsorption. The protein loading capacity and immobilization yield were (2.5 mg/g support and 0.5 mg/g support) and (25% and 10%) in celite and charcoal support respectively. Lipase enzyme, Lipozyme TL-100L immobilized on micro porous polymeric matrix synthesized from styreneedivinyl benzene (STY-DVB) showed the protein loading capacity and immobilization yield 4.79 mg/g and 23.70% respectively [39].There is the utilization of the lipase in industrial biodiesel production (transesterification process). In another study it was found that *Candida* rugosa lipase immobilized on cilite showed the protein content and tranesterification activity 1.2 (mg/g enzyme) and 2 (unit/mg protein) [40]. Lipase enzyme should be immobilized to enhance the stability, recovery and reusability of the enzyme. The Lipase was immobilized by the physical absorption method as it is simple and economical. According to Malcata et al. [41] there are various methods for the immobilization of lipase on different carriers and celite is a suitable carrier in one of them. The

immobilization by physical adsorption was more effective using celite whereas that obtained with charcoal immobilization exhibited a relatively low percentage of adsorbed enzymes. The adsorption of an enzyme onto a carrier is dependent on experimental variables such as pH, the nature of solvent, ionic strength, concentration of enzyme, adsorbent, and temperature. The mechanism of the immobilization of lipase is adsorption on celite as an immobilized carrier because of hydrophilic surface of celite. Thus, the catalytic activity of the lipase immobilized on celite is generally higher compared with the lipase immobilized on charcoal.

Table 4. Effect of metal ions and inhibitors on *Microbacterium* sp. lipase activity

Metal ions (1 mM)	Relative activity %
Control	100 ± 2.3
$BaCl_2$	99.1 ± 2.2
$CaCl_2$	150.0 ± 2.1
$MgCl_2$	86 ± 0.31
KCl	77 ± 0.25
LiCl	59 ± 1.39
NaCl	97.2 ± 5.09
$ZnSO_4$	65 ± 1.03
Inhibitors (1 mM)	
EDTA	50.7±1.2
PMSF	21.0± 2,7
β-Mercaptoethanol	90.3± 5.1

3.5. Determination of FAME and free fatty acid (FFA)

Transesterification reaction of algal oil was carried out with methanol and catalyst as sodium hydroxide, potassium hydroxide and lipase enzyme immobilized onto (celite and charcoal) support. The % FAME yields were 72.5 %, 90% (95.1 and 15%) respectively. The highest FAME yield was 95.1% with physical adsorption onto celite. On the other hand, FAME yield was very low (15%) in case of charcoal. Almost similar results were observed when the lipase enzyme (Lipozyme TL-100L) with physical adsorption onto STY-DVB provided the FAME yields of 92.41% [41]. This may be due to the inefficiency of the charcoal substrate to bind the lipase enzyme. FAMEs present in biodiesel using different type of catalysts are shown in table 5. 9-Octadecenoic acid methyl ester (Oleic acid methyl ester) was present in the major amount which is best suited for biodiesel production. The FFA content present in algal oil was calculated as 3.5%.There will be no effect of free fatty acid present in the feedstock using lipase as a catalyst because there is no soap formation and they have the ability to esterify the free fatty acid in one step without the need of washing step. In case of alkali (NaOH) catalysed transestreification, free fatty acid content may be the cause may be the cause of comparatively low yield of % FAME.

3.6. Reusability of Lipase enzyme

Immobilized enzyme can be reused for a long time. Reusability of the immobilized *Microbacterium* sp. lipase in hydrolysis of algal oil was determined. The residual activity of the immobilized enzyme is presented in Fig 7. Reuse of immobilized lipase retained 78.3% of its activity after 10 reuses (Fig.7). Almost similar results were observed by Hung and co-workers, when *C. rugos*a lipase on chitosan showed 74% residual activity after 10 reuses [42]. It was also earlier reported that after 10 times of reuse the activity of immobilized lipase of *C. rugosa* on alanine chitosan beads retained 77% of the initial activity [43]. The immobilized enzyme provides the reusability for many cycles, thus reduces the enzyme cost [44]. In another study, P. *cepacia* lipase immobilized on celite showed its activity up to 4 cycles in transesterification of Jatropha oil [45].

Table 5. Fatty acid composition (% as methyl esters) in *Scenedesmus* sp.

Fatty acid methyl ester (%)	NaOH	KOH	Lipase (Celite)	Lipase (Charcoal)
Hexadecanoic acid methyl ester	16	15	17	1
9-Hexadecenoic acid methyl ester	1.71	ND	2	2
Pentadecanoic acid, methyl ester	2.1	1.5	2	1.5
Heptadecanoic acid, methyl ester	1.3	2	2.3	ND
9, 12-Octadecadienoic acid, methyl ester	8.04	15	9	10
9-Octadecenoic acid , methyl ester	16	21	23	ND
10-Octadecenoic acid , methyl ester	4	ND	3	ND
11- Octadecenoic acid, methyl ester	3.1	4	4	ND
9,12,15-octadecatrienic acid , methyl ester	1.84	6	4	ND
8-Octadecenoic acid, methyl ester	3	6	5	ND
12-Octadecenoic acid, methyl ester	5	4.9	3.4	ND
8,11-Octadecadienoic acid, methyl ester	1.5	1.9	1.3	0.5
Octadecanoic acid , methyl ester	5	5	12	ND
Docosanoic acid, methyl ester	1	1.5	0.8	ND
Eicosanoic acid, methyl ester	2	2.3	3.1	ND
Nonadecanoic acid, methyl ester	1	3.9	3.2	ND
Total	72.5	90	95.1	15

(ND: Not detected)

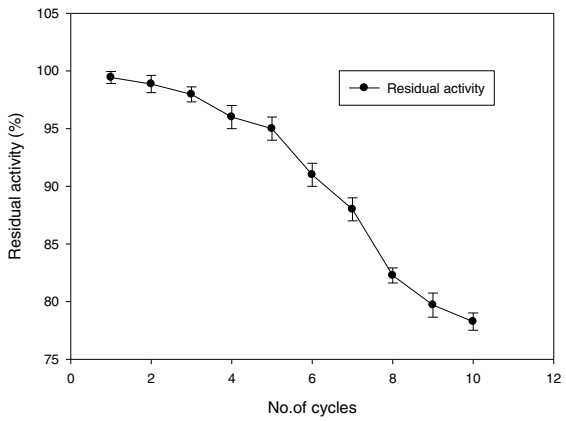

Fig. 7. Effect of repeated use of immobilized *Microbacterium* lipase on residual activity

4. Conclusion

The results of this study indicate that the *Microbacterium* sp. is valuable as a source of lipase isolated from pulp and paper industry. *Microbacterium* sp. showed maximum lipase production after 48 hours. An extracellular lipase was purified having molecular weight of 40 Kda. Lipase enzyme was immobilized onto celite-545 and it retained the enzymatic activity after reuse in transestrification process. Therefore, *Microbacterium* sp. can be a good source for the production of lipase enzyme to be used in transesterification reaction for biodiesel production.

Acknowledgements

This paper is supported by the research grants of Department of Biotechnology, Government of India, New Delhi, India. We thank Mr. Ajay Kumar of Advanced Instrumentation Research Facility (AIRF) Jawaharlal Nehru University, New Delhi for GC-MS analysis and Dr. Mihir Tanay Das for his technical support. The author (Tripathi R) thanks Ministry of New and Renewable Energy, Government of India, New Delhi, India, for providing Research Fellowship.

References

[1] Pachauri RK, Reisinger A. IPCC (Intergovernmental Panel on Climate Change), Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, Eds., IPCC, Geneva: 2007 p.102.

[2] Gunstone FD, Padley FB. Lipid Technologies and Applications, Marcel Dekker, New York, NY, USA;1997.
[3] Gunstone FD. Vegetable Oils in Food Technology: Composition, Properties and Uses, Blackwell Publishing, Oxford, UK; 2002.
[4] Ali MF, Ali B ME, Speight JG. Handbook of Industrial Chemistry—Organic Chemicals, McGraw-Hill, New York, NY, USA; 2005.
[5] Al-Zuhair S. Production of biodiesel : possibilities and challenges, Biofuels, Bioprod Biorefin 2007; 1: 57–66.
[6] Liu KS. Preparation of fatty acid methyl esters for gas chromatographic analysis of lipids in biological materials, J Am Chem Soc 1994; 71: 1179–1187.
[7] Basu HN, Norris ME. Process for production of esters for use as a diesel fuel substitute using a non-alkaline catalyst. US Patent; 1996 p. 525-126.
[8] Freedman B, Butterfield, R.O. and Pryde, E.H. Transesterification kinetics of soybean oil. J Am Chem Soc 1986 ; 63: 1375–1380.
[9] Jeromin L, Peukert E, Wollmann G. Process for the pre-esterification of free fatty acids in fats and oils. US Patent ; 1987.
[10] Knezevic ZD, Siler-Marinkovic SS, Mojovic LV.Kinetics of lipase catalyzed hydrolysis of Palm oil in lecithin/isooctane reversed micelles. Appl Microbiol Biot 1998;49: 267–271.
[11] Wisodn RA, Dunnill P, Lilly MD. Enzymic interesterification of fats: factors influencing the choice of support for immobilized lipase, Enzyme Microb Tech1984; 6:443-446.
[12] Singh R., Gupta N, Goswami VK, Gupta R. A simple activity staining protocol for lipases and esterases. Appl Microbiol Biot 2006 ;70: 679- 682.
[13] Thakur IS Structural and functional characterization of a stable, 4 chlorosalicylic acid degrading, bacterial community in a chemostat. World J Microb Biotech 1995; 11: 643-45.
[14] Bulow L, Mosbach K. The expression in Escherichia coli of a polymeric gene coding for a esterase mimic catalyzing the hydrolysis of p- nitrophenyl esters. FEBS Lett 1987;210: 147-152.
[15] Gupta A, Joseph B, Mani A, Thomas G. Biosynthesis and properties of an extracellular thermostable serine alkaline protease from Virgibacillus pantothenticus. World Journal of Microbiology and Biotechnology 2008; 24:237–243.
[16] Bligh EG, Dayer WJ. A rapid method for total lipid extraction and purification Can J Biochem Phys 1959; 37: 911-917.
[17] Wan PJ, Pakarinen DR, Wakelyn PJ. Concerns for the determination of free fatty acid in cottonseed, J Am Chem Soc 1998; 75:1321-1324.
[18] Kumar S, Tamura K, Nei M. MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment brief. Bioinformatics 2004; 5:150–163.
[19] Joseph B, Shrivastava N, Ramteke PW. Extracellular cold-active lipase of Microbacterium luteolum isolated from Gangotri glacier, western Himalaya: Isolation, partial purification and characterization, J Gen Eng Biotechnol 2012; 10:137–144.
[20] Jaeger KE, Eggert T. Lipases for biotechnology. Curr Opin Biotech 2002; 13: 390–397.
[21] Winayanuwattikun P, Kaewpiboon C, Piriyakananon K, Chulalaksananukul W, Yongvanich T, Svasti J. Immobilized lipase from potential lipolytic microbes for catalyzing biodiesel production using palm oil as feedstock. Af J Biotechnol 2011;10(9):1666-1673.
[22] Thomas SB, Thomas BF. Psychrotrophic bacteria in refrigerated bulk-collected raw milk. Part II Dairy Ind 1973; 38: 61–70.
[23] Pabai F, Kermasha S, Morin A. Lipase from Pseudomonas fragi CRDA 323: partial purification, characterization and interesterification of butter fat. Appl Microbiol Biotechnol 1995b; 43:42–51.
[24] Gerday C, Aittaleb M, Arpigny JL, Baise E, Chessa JP, Garssoux G, Petrescu I, Feller G. Psychrophilic enzymes: a thermodynamic challenge. Biochimica et Biophysica Acta 1997; 1342: 119–131.
[25] Lee DW, Kim HK, Lee KW, Kim BC, Choe AC, Lee HS. Purification and characterization of two distinct thermostable lipases from the gram-positive thermophilic bacterium Bacillus thermoleovorans ID-1, Enzyme Microb Tech 2001; 29:363–71.
[26] Schmidt DC, Sztajer H, Stocklein W, Menge U Schmid RD. Screening, purification and properties of a thermophilic lipase from Bacillus thermocatenulatus. Biochimica et Biophysica Acta 1994;1214: 43–53.
[27] Kim HK, Sung MH, Kim HM, Oh, TK. Ocurrence of thermostable lipase in thermophilic Bacillus sp. Strain 398, Biosci Biotech Bioch 1994; 58: 961–2.

[28] Nawani N, Dosanjh NS, Kaur J. A novel thermostable lipase from a thermophilic Bacillus sp.: characterization and esterification studies, Biotechnol Lett 1998; 20 : 997–1000.
[29] Lee DW, Koh YS, Kim KJ, Kim BC, Choi, HJ, Kim DS. Isolation and characterization of a thermophilic lipase from Bacillus thermoleovorans ID-1. FEMS Microbiol Lett 1999; 179: 393–400.
[30] Dharmsthiti, S. and Luchai, S. Production. purification and characterization of thermophilic lipase from Bacillus sp. THL027. FEMS Microbiol Lett 1999; 179: 241–6.
[31] Imamura S, Kitaura S. Purification and characterization of a monoacylglycerol lipase from the moderately thermophilic Bacillus sp. H-257, J Biochem 2000;127: 419–25.
[32] Handelsman T. Shoham Y. Production and characterization of an extracellular thermostable lipase from a thermophilic Bacillus sp., J Gen Appl Microbiol 1994;40: 435–43.
[33] Wang Y, Srivastava KC, Shen GJ, Wang HY. Thermostable alkaline lipase from a newly isolated thermophilic Bacillus, strain A30-1 (ATCC 53841), J Ferment Bioeng 1995; 79: 433–8.
[34] Lee DW, Kim HK, Lee KW, Kim BC, Choe AC, Lee HS.Purification and characterization of two distinct thermostable lipases from the gram-positive thermophilic bacterium Bacillus thermoleovorans ID-1. Enzyme Microb Tech 2001; 29: 363–71.
[35] Kambourova M, Mandeva N, Derekova A. Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7.J Mol Catal B: Enzym, 2003; 22, 307-313.
[36] Demir BS, Tukel SS Purification and characterization of lipase from Spirulina platensis, J Mol Catal B-Enzymatic 2010; 64:123–128.
[37] Yang KS, Sohn JH, Kim HK. Catalytic properties of a lipase from Photobacterium lipolyticum for biodiesel production containing a high methanol concentration. J Biosci Bioeng 2009; 107(6): 599–604.
[38] Colton I J, Ahmed S N, Kazlauskas RJ. Isopropanol Treatment Increases Enantio selectivity of Candida rugosa Lipase toward Carboxylic Acid Esters. J Org Chem 1995;60: 212-217.
[39] Yucel Y, Demir C, Dizge N. Keskinler B. Lipase immobilization and production of fatty acid methyl esters from canola oil using immobilized lipase, Biomass Bioenerg 2011; 35:1496-1501.
[40] Pu W, Li-rong Y, Jian-ping W. Immobilization of lipase by salts and the transesterification activity in hexane, Biotechnol Lett, 2001; 23:1429–1433.
[41] Malcata FH, Reyes HR, Garcia HS, Hill CG, Amundson CH. Immobilized lipase reactors for modification of fats and oils—a review. J Am Chem Soc 1990; 67(12): 890-09.
[42] Hung TC, Giridhar R, Chiou SH, Wu WT. Binary immobilization of Candida rugosa lipase on chitosan. J Mol Catal B-Enzym 2003; 26: 69-78.
[43] Yi SS, Noh JM, Lee YS. Amino acid modified chitosan beads: Improved polymer support for immobilization of lipase from Candida rugosa. J Mol Catal B-Enzym 2009; 57:123-129.
[44] Fukuda H, Kondo A, Noda H. Biodiesel fuel production by transesterification of oils. J Biosci Bioeng 2001; 92(5): 405–416.
[45] Shah S, Gupta MN. Lipase catalyzed preparation of biodiesel from Jatropha oil in a solvent free system. Process Biochem 2007; 42(3): 409–414.