BioTechnology

An Indian Journal

Biodiesel production from *Nannochloropsis oculata* oil using immobilized lipase with methyl acetate as an acyl acceptor

D.Surendhiran, M.Vijay*

Bioelectrochemical Laboratory, Department of Chemical Engineering,
Annamalai University, Annamalainagar, Tamilnadu-608002 (INDIA)
E-mail : drmvijay2009@gmail.com

ABSTRACT

The present investigation dealt with conversion of marine microalgal oil from *Nannochloropsis oculata*, to biodiesel, using immobilized lipase along with methyl acetate as acyl acceptor. Lipase, a versatile biocatalyst in biotechnological process, was isolated from *Burkholderia cepacia* MTCC4684. The crude extract of enzyme was entrapped in alginate beads. Using immobilized lipase the biodiesel conversion (%) was evaluated by optimizing the process parameters. The conditions yielding maximum conversion were 3g immobilized lipase, 1:12 oil to methyl acetate ratio, 35^0C, 6% water, 60 h reaction time and agitation rate of 400rpm. The immobilized beads retained their stability even after repeated uses of 20 cycles. The optimal conditions gave 95.36% of biodiesel conversion. The fatty acids predominantly constituting FAME (Fatty acid methyl esters), analysed using GC-MS, were lauric (C12:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and arachidic (C20:0) acids. Due to high content of oleic acid, biodiesel could be resistant to oxidation and stored for a longer period. This study is, thus an ecofriendly "green process", involving reusable and potential immobilized biocatalyst for biodiesel production.   © 2014 Trade Science Inc. - INDIA

KEYWORDS

Biodiesel;
Methyl acetate;
FAME;
Nannochloropsis oculata.

INTRODUCTION

The world is entering into a period of declining fossil fuel resources and their uses associated with accumulation of greenhouse gases in the atmosphere leads global warming[1]. Biodiesel (monoalkyl esters of long chain fatty acids) is a potential biofuel which is renewable biodegradable, non-toxic, having no net carbon dioxide and free from sulphur[2-4]. Microalgal biomass has become one of the emerging source of biodiesel-convertible lipids[5], because recent research has proved that oil production from microalgae is clearly superior to that of terrestrial plants such as palm, rapeseed, soybean or jatropha. Generally, the doubling time of microalgal biomass is within 24 h, achieving large biomass yields[5,6].

Currently biodiesel is being produced by acid and alkali transesterification by converting triglycerides to fatty acid methyl esters within shorter period[7,8]. Demerits of such methods include high energy input, elimi-

FULL PAPER

nation of salt, difficulty in recycling glycerol, soap formation and need waste water treatment[9-15].

To overcome this problem, recently enzymatic production of biodiesel has become an alternative for biodiesel production due to easy recovery of byproduct glycerol, salt and catalyst can be avoided, waste water treatment is not required, high production yield under milder conditions and ecofriendly process[16-18]. One such enzyme used in biodiesel production is lipases. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are produced by microorganisms, plants and animals, out of which microorganisms are highly suitable for the large scale production[19].

However, the enzymatic production of biodiesel is not yet commercialized due to high cost of enzyme. The problem can be overcome by immobilization of lipase by repeated use[20-22]. Use of lipase for producing biodiesel in a solvent free system is nowadays focused worldwide, since systems are advantageous over solvent aided transesterification by avoiding separation, toxicity, flammability and high cost of organic solvents.

In the present investigation, the acyl acceptor used is methyl acetate since it did not produce glycerol rather produces triacetylglycerol which do not inactivate lipase. An extensive literature survey revealed that there was no study yet reported on use of methyl acetate as acyl acceptor to interesterify marine microalgal oil for biodiesel synthesis using lipase enzyme.

MATERIALS AND METHODS

Culture condition

Nannochloropsis oculata was obtained from CMFRI, Tuticorin, Tamilnadu, India and cultivated in 25 L photobioreactor (PBR) using sterile Walne medium. The filtered sterilized sea water was enriched with required quantity of Walne's medium containing (gL^{-1}): $NaH_2PO_4 \cdot 2H_2O$, 20.0; Na_2EDTA, 4.0; H_3BO_3, 33.6; $MnCl_2 \cdot 4H_2O$, 0.36; $FeCl_3 \cdot 6H_2O$, 13.0; vitamin B_{12}, 0.001; vitamin B_1, 0.02; and $NaSiO_3$, 6.6. The trace metal solution contained (g L^{-1}): $ZnSO_4 \cdot 7H_2O$, 4.4; $CoCl_2 \cdot 6H_2O$, 2.0; $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 0.9; and $CuSO_4 \cdot 5H_2O$, 2.0. The medium was adjusted to pH 8 and autoclaved at 121^0 C for 20 min. The filter sterilized vitamins were added after cooling. Mixing was provided by sparging air from the bottom of the PBR

and lighting was supplied by cool-white fluorescent light with an intensity of 5000 lux under 12/12 light/dark cycle for 15 days.

Harvesting of cells and oil extraction

After the culture reached stationary phase, the biomass was harvested by centrifugation at 8500 rpm for 10 min to get thick algal paste. Then the microalgal paste was rinsed with distilled water to remove residual salts and then dried in hot air oven at 60^0 C for 8 hr. Dried biomass was subjected to oil extraction by Bligh and Dyer (1959) with slight modification[23]. In brief, the biomass suspension was mixed with chloroform: methanol (1:2) ratio, vortex it for few minutes and incubated on ice for 10 minutes. Then, chloroform was added followed by addition of 1M HCl and again vortexed it for few minutes. Finally the whole suspension was centrifuged at maximum speed for 2 minutes. Bottom layer containing lipid was transferred into fresh previously weighed beaker. Chloroform was added to reextract the lipid from the aqueous sample. The solvent system was evaporated using rotary evaporator at 30^0 C.

Fermentation of lipase production using Burkholderia cepacia MTCC4684

The lipase production was carried out in 250ml Erlenmeyer flask using 100 ml basal medium containing 1% olive oil, 0.2% $CaCl_2 \cdot 2H_2O$, 0.01% $MgSO_4 \cdot 7H_20$, 0.04% $FeCl_3 \cdot 6H_2O$ and 5% NaCl. The contents were incubated for 48 h at 37^0C at 200 rpm. The pH was maintained at 7. After incubation, the culture was centrifuge at 10,000 rpm for 10 min at 4^0C. The supernatant of crude lipase was quantified using lipase assay and used for immobilization.

Immobilisation of crude lipase

Crude lipase (6ml) was mixed with 14ml of sodium alginate solution (2%). The mixer was dripped into cold sterile 0.2 M $CaCl_2$ using sterile syringe from a constant distance and was cured at 4^0 C for 1 h. The beads were hardened by suspending it again in a fresh $CaCl_2$ solution for 24 h at 4°C with gentle agitation. After immobilization, the beads were separated by filtration and washed with 25 mM phosphate buffer (pH 6.0) to eliminate excess calcium chloride and enzyme. Then the beads were preserved using 0.9 % NaCl solution for future use[24,25].

Lipase assay and protein determination

Lipase activity was determined for free and immobilized enzymes according to Burkert et al (2004) and Padihla et al (2012)[26,27]. The olive oil emulsion was prepared by mixing 25ml of olive oil and 75ml of 7% Arabic gum solution in a homogenizer for 5 min at 500rpm. The reaction mixture containing 5ml of emulsion, 2ml of 10mM phosphate buffer (pH 7.0) and 1ml of the culture supernatant was incubated at 37⁰C for 30 min in orbital shaker. The reaction was stopped by addition of 15ml of acetone-ethanol (1:1v/v), and the titration of liberated fatty acids was done with 0.05 N NaOH. One unit of lipase activity was defined as the amount of enzyme, which liberated 1 μmol of fatty acid per minute. The protein content in the crude enzyme was determined by Lowry et al (1951) with BSA as a standard[28].

Optimization of enzyme interesterification process by solvent free system

The enzymatic interesterification reaction was carried out in 20 ml screw cap glass vial. No solvent was added in this reaction. The composition of reaction mixture was 5g of microalgal oil, 2g of immobilized enzyme and methyl acetate. The oil to acyl acceptor (methyl acetate) was optimized ranging from 1:2, 1:4, 1:6, 1:8, 1:10, 1:12 and 1:14. The effect of temperature was studied at various intervals of 20, 25, 30, 35 and 40⁰C. To study the effect of water, enzymatic interesterification was carried out by adding water at the concentration of 0, 2, 4, 6, 8, 10 and 12 weight % of the total reaction mixture. The reaction was allowed for 48h at constant speed of 200 rpm. The biodiesel yield was calculated according to Umdu et al (2009)[29]:

$$\text{Biodiesel yield (Weight \%)} = \frac{\text{Amount of biodiesel (g) in upper mixture}}{\text{Amount of microalgal oil (g)}} \times 100$$

GC analysis of fatty acid methyl esters

Fatty acid methyl ester composition of biodiesel produced from *Nannochloropsis oculata* oil was analysed by Gas Chromatography-Mass Spectrometry (GC-MS-QP 2010, Shimadzu) fitted with VF-5 MS capillary column (30mm length, 0.25mm diameter and 0.25μm film thickness). The column temperature of each run was started at 70⁰ C for 3 min, then raised to 300⁰ C and maintained at 300⁰ C for 9 min. GC conditions were: column oven temperature-70⁰ C, injector temperature-240⁰C, injection mode split, split ratio-10, flow control mode-linear velocity, column flow-1.51ml/min, carrier gas- helium (99.9995% purity) and injection volume-1μl. MS conditions were: ion source temperature-200⁰ C, interface temperature-240⁰ C, scan range-40-1000m/z, solvent cut time-5 min, MS start time-5 min, end time-35 min and ionization-EI (-70eV) and scan speed-2000.

RESULTS AND DISCUSSION

Effect of enzyme loading

Effect of enzyme loading was learnt to enhance transesterification in the range of 1-4g. Figure 1 showed that the increasing enzyme loading resulted in biodiesel yield increment when the immobilized beads reached 3g. The methyl ester yield was decreased at higher enzyme concentration. This is in agreement with Jegannathan et al (2010) found that higher dosage of immobilized lipase results lower yield of biodiesel[8]. This is due to that the surplus amount of enzyme may aggregate which might have led to reduction in lipase activity[2].
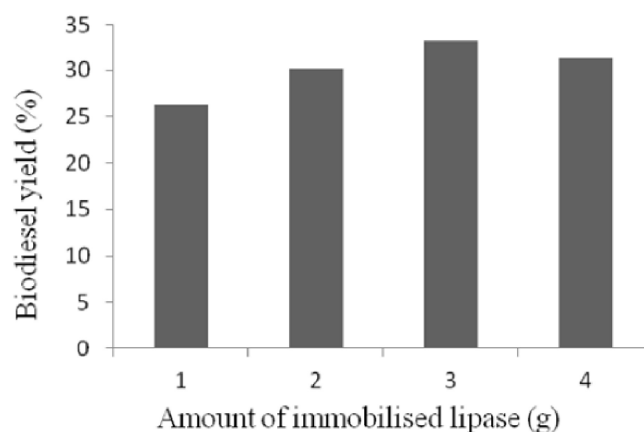

Figure 1 : Effect of immobilized lipase loading on biodiesel yield (%). Reaction conditions: 1:4 oil/methyl acetate molar ratio, 30⁰C, 200 rpm and 48h

Effect of oil and methyl acetate molar ratio

Effect of oil and methyl acetate ratio was studied and found that 1:12 molar ratio of oil to methyl acetate

FULL PAPER

gave maximum fatty acid methyl esters yield of 69.24% at 48 h in the absence of any solvents, similar to with previous study done by Ognjanovic et al 2009[11]. But the biodiesel yield was declined when the molar ratio was raised to 1:14 (Figure 2), which is due to the excessive amount of methyl acetate that diluted the oil resulting in poor yield of fatty acid methyl esters[30]. The conventional short chain alcohols such as ethanol and methanol inactivate lipase when exceeding 1:3 molar ratio. In support of this, Shimada et al (1999) reported that inactivation of immobilized lipase Novozym 435 from *C. antarctica* occurred at the molar ratio of 1:5 of plant oil and methanol[31]. In addition, during methanolic transesterification the main by product is glycerol that is hydrophilic and insoluble in oil resulted in decrease in the reactivity of immobilized lipase due to mass transfer resistance[30,32,33]. Methyl acetate produces triacetylglycerol instead of glycerol which do not inactivate lipase[32].
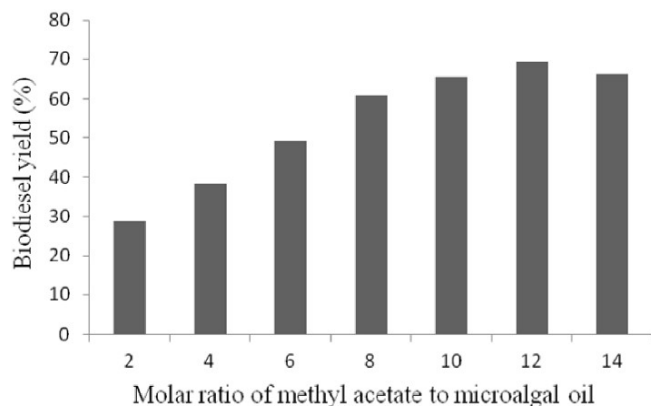

Figure 2 : Effect of molar ratio of methyl acetate to microalgal oil on biodiesel yield (%). Reaction conditions: 3g immobilized lipase, 30⁰C, 200 rpm and 48h

Effect of temperature

To study the effect of temperature on enzymatic biodiesel process the range studied was between 20 to 40⁰C with an interval of 5⁰C. The temperature was not exceeded more than 40⁰C, because sodium alginate dissolves at higher temperature. Tran et al (2012) reported that the FAME production decreased when the temperature increased to 50⁰C for fresh water microalgae *C.vulgaris* ESP-31 by enzymatic transesterification[33]. However, most of the enzymatic reaction does not require higher temperature[8]. In the current findings, 35⁰C gave the highest yield of 75.82
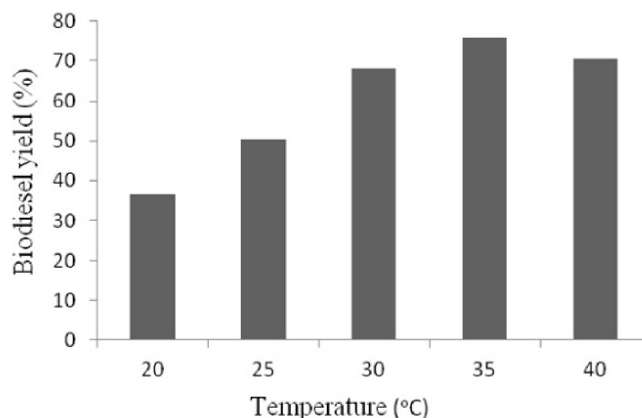

Figure 3 : Effect of temperature on biodiesel yield (%). Reaction conditions: 3g immobilized lipase, 1:12 oil/methyl acetate molar ratio, 200 rpm and 48h

% (Figure 3) thereby reducing the energy consumption since higher temperature had not been implemented.

Effect of water

For the biocatalyst mediated transesterification water act as a key factor for enhancing the lipase activity and unmasking the active sites of lipase by increasing interfacial area of oil water droplets[2,22,33,34]. Usually lipase activity relies on the availability for interfacial area[35]. Li and Yan (2010) reported that exceeding water content over 7 % of total volume of reaction mixture leads to decrease in the formation of FAME[2]. But in our study, there is no decrease of methyl esters until 8% water content was achieved, which was due to the formation of triacylglycerol that did not disturb the lipase activity. The highest yield of biodiesel was obtained as 87.34%. When the water content reached beyond 8%
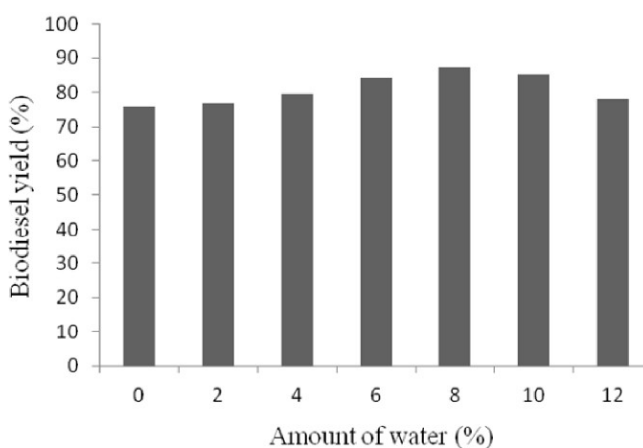

Figure 4 : Effect of water on biodiesel yield (%). Reaction conditions: 3g immobilized lipase, 1:12 oil/methyl acetate molar ratio, 35⁰C, 200 rpm and 48h

the yield was reduced (Figure 4), which is due to the excess of water content that reduce the transesterification reaction rate[35,36], as a result of excess water floods on pores of immobilised supporting material which inhibit the enzyme move to the reaction medium[34].

Effect of reaction time on biodiesel yield

Effect of reaction time was investigated in the range of 12-72 h. The optimized reaction time for conversion of microalgal oil to FAME by immobilized biocatalyst was observed as 60 h yielding 92.72% efficiency (Figure 5). Beyond the maximal reaction at 60 h a decrease in FAME was obtained. This is due to the increase in the water concentration during transesterification, which might trigger the hydrolysis of biodiesel[2].
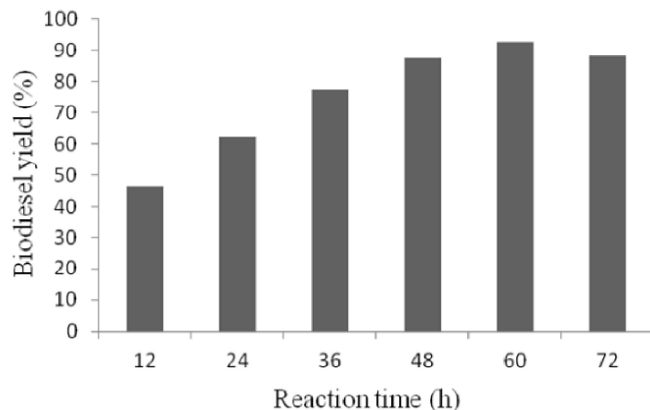

Figure 5 : Effect of reaction time on biodiesel yield (%). Reaction conditions: 3g immobilized lipase, 1:12 oil/methyl acetate molar ratio, 8% water (w/w) 35⁰C, 200 rpm and 48h

Effect of mixing on biodiesel yield

Study of agitation is one of the significant parameters in immobilized enzymatic transesterification. In the immobilization reaction system the reactants have to get transferred from the bulk liquid to the external surface of particle and then into the interior pores of catalyst[37]. Effect of mixing on biodiesel production was conducted between 100 to 400 rpm with the interval of 100rpm. Figure 6 shows the methyl ester production rate to their respective speed of agitation. The maximum yield of biodiesel was found 95.36% at 400 rpm thus agitation enhances the rate of reaction. Agitation reduces the mass transfer resistance between oil and acyl acceptor and immobilized lipase at the catalyzing interface, thus enhancing the reaction rate. In the other hand, when the speed reached beyond 200 the biodiesel yield was decreased. This is due to the damage of immobilized beads
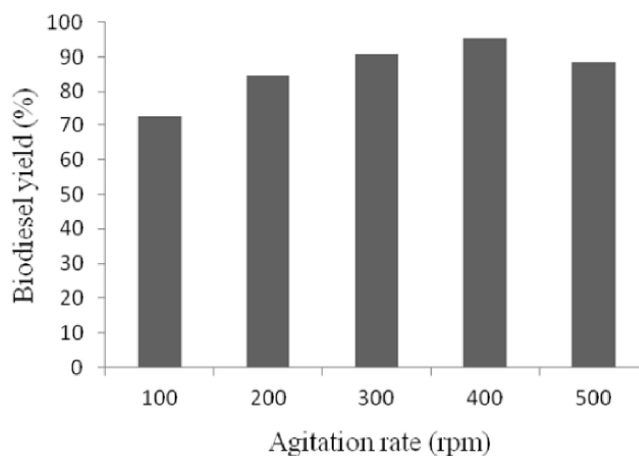

Figure 6 : Effect of agitation on biodiesel yield (%). Reaction conditions: 3g immobilized lipase, 1:12 oil/methyl acetate molar ratio, 8% water (w/w) 35⁰C and 60h

leads to inactivation of lipase by mechanical agitation[2,11,33].

Reusability of immobilized enzyme

One of the most meritorious features of immobilized enzyme is its reusability nature. Reusability of enzyme is the important parameter to decide possibilities of industrial scale enzymatic biodiesel production[18]. Stability and reusability of immobilized lipase from marine *B.subtilis* was investigated in this section. No major loss of lipase activity was observed even after immobilized enzyme beads were used for 20 cycles (Figure 7). As previously reported by Du et al (2004), there was no enzyme loss even after 100 cycles of repeated usage in the presence of methyl acetate[38]. When short chain alcohols (methanol and ethanol) were used as acyl acceptors, removal of glycerol from immobilized lipase
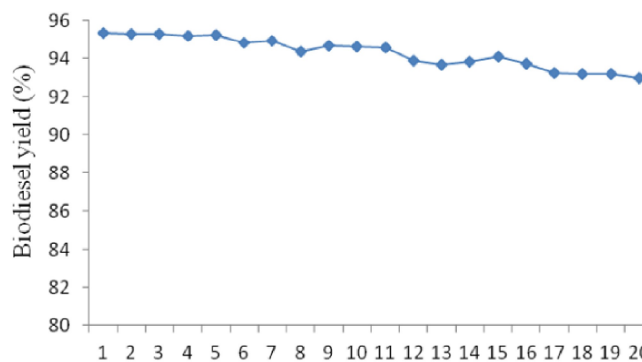

Figure 7 : Reusability and stability of immobilized lipase on biodiesel yield (%). Reaction conditions: 3g immobilized lipase, 1:12 oil/methyl acetate molar ratio, 8% water (w/w) 35⁰C, 400 rpm and 60h

FULL PAPER

must be carried out using large amount of hydrophilic solvents which is a cost effective process and inhibit lipase activity. Thus, the current study indicates that immobilized lipase can be used for many repeated cycles for biodiesel production from microalgal oil with methyl acetate as acyl acceptor which will minimize the cost factor in the overall process.

Fatty acid methyl ester composition of microalgal biodiesel

TABLE 1 shows the six fatty acids are found in the *Nannochloropsis oculata* biodiesel. The results showed that lauric acid (C12:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and Arachidic acid (C20:0) are major fatty acids in the *Nannochloropsis oculata* oil. Moreover, palmitic acid and oleic acid are predominant in the FAME content synthesised by enzymatic interesterification. Feng et al (2011) reported that high content of oleic acid is relatively suitable for biodiesel[39]. Many researchers reported that the biodiesel cannot be stored for a longer period because of its oxidative sensitivity, but the high levels of oleic acid content make FAME highly stable to oxidation[40]. Since *Nannochloropsis oculata* contain more amount of oleic acid than the other fatty acids (TABLE 1), hence it is promising source for biodiesel production.

TABLE 1 : Fatty acid profile of marine microalgae Nannochloropsis oculata FAMEs

Lipid number	Common Name	Chemical name	Molecular Structure	%
C12:0	Lauric acid	Dodecanoic acid	$C_{12}H_{24}O_2$	9.86
C16:0	Palmitic acid	Hexadecanoic acid	$C_{16}H_{32}O_2$	19.39
C18:0	Stearic acid	Octadecanoic acid	$C_{18}H_{36}O_2$	10.76
C18:1	Oleic acid	9 - Octadecadienoic acid	$C_{18}H_{34}O_2$	35.22
C18:2	Linoleic acid	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	8.15
C20:0	Arachidic acid	Eicosanoic acid	$C_{20}H_{40}O_2$	16.62

CONCLUSION

Deterioration of fossil fuels had led the

biotechnologists to search for alternative fuels, which are renewable, harmless and biodegradable. Microalgal biodiesel was, hence a replacement of petroleum fuels. Immobilised lipase from *Burkholderia cepacia* was utilised to generate biodiesel by converting microalgal oil. The conditions for better interesterification were optimised and obtained a higher yield of 95.36%. Due to high content of oleic acid, the biodiesel produced is highly stable, resisting oxidation and thus its shelf life could also be prolonging. This study, thus involved an inexpensive and effective process of interesterification with reusable immobilised biocatalyst to generate environmental friendly biodiesel.

REFERENCES

[1] E.Z.Su, M.J.Zhang, J.G.Zhang, J.F.Gao, D.Z.Wei; Lipase-catalyzed irreversible transesterification of vegetable oils for fatty acid methyl esters production with dimethyl carbonate as the acyl acceptor. Biochem.Eng.J., **36**, 167–173 **(2007)**.

[2] Q.Li, Y Yan; Production of biodiesel catalysed by immobilized Pseudomonas cepacia lipase from Sapium sebiferum oil in micro-aqueous phase. Appl.Ener., **87**, 3148-3154 **(2010)**.

[3] D.G.Kim, H.J.La, C.Y.Ahn, Y.H.Park, H.M.Oh; Harvest of Scenedesmus sp. with bioflocculant and reuse of culture medium for subsequent high-density cultures. Bioresour Technol., **102**, 3163-3168 **(2011)**.

[4] A Ali, M.Kaur, U.Mehra; Use of Immobilized Pseudomonas sp. as Whole cell Catalyst for the Transesterification of used Cotton Seed Oil.J.Oleo.Sci., **60**, 7-10 **(2011)**.

[5] Surendhiran D.Vijay; Microalgal Biodiesel - A Comprehensive Review on the Potential and Alternative Biofuel.Res.J.Chem.Sci., **2**, 71-82 **(2012)**.

[6] A.K.Bajhaiya, S.K.Mandotra, M.R.Suseela, K.Toppa, S.Ranade; Algal Biodiesel: the next generation biofuel for India: Review Article. Asian J.Exp.Biol.Sci.,, **1**, 728-739 **(2010)**.

[7] P.Shao, X.Meng, J.He, P.Sun; Analysis of immobilized Candida rugosa lipase catalyzed preparation of biodiesel from rapeseed soapstock. Food Bioprod Process, **86**, 283–289 **(2008)**.

[8] K.R.Jegannathan, L.J.Yee, E.S.Chan, P.Ravindra; Production of biodiesel from palm oil using liquid core lipase encapsulated in k-carrageenan. Fuel, **89**,

FULL PAPER

2272-2277 **(2010)**.

[9] K.Ban, S.Hama, K.Nishizuka, M.Kaieda, T.Matsumoto, A.Kondo, H.Noda, H.Fukuda; Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. J.Mol.Catal.B: Enzym, **17**, 157–165 **(2002)**.

[10] S.Al-Zuhair, F.W.Ling, L.S.Jun; Proposed kinetic mechanism of the production of biodiesel from palm oil using lipase. Process Biochem., **42**, 951–960 **(2007)**.

[11] N.Ognjanovic, D.Bezbradica, Z.K.Jugovic; Enzymatic conversion of sunûower oil to biodiesel in a solvent-free system: Process optimization and the immobilized system stability. Bioresource Technol., **100**, 5146–5154 **(2009)**.

[12] P.S.Bisen, B.S.Sanodiya, G.S.Thakur, R.K.Baghel, G.B.K.S.Prasad; Biodiesel production with special emphasis on lipase-catalyzed transesterification. Biotechnol.Lett., **32**, 1019–1030 **(2010)**.

[13] K.Kawakami, Y.Oda, R.Takahashi; Application of a Burkholderia cepacia lipase immobilized silica monolith to batch and continuous biodiesel production with a stoichiometric mixture of methanol and crude Jatropha oil. Biotechnol.Biofuels., **4**, 1-11 **(2011)**.

[14] R.C.Rodriques, M.A.Zachia Ayub; Effects of the combined use of Thermomyces lanuginosus and Rhizomucor miehei lipases for the transesterification and hydrolysis of soybean oil. Process Biochem., **46**, 682-688 **(2011)**.

[15] A.Yoshida, S.Hama, N.Tamadani, H.Noda, H.Fukuda, A.Kondo; Continuous production of biodiesel using whole-cell biocatalysts: Sequential conversion of an aqueous oil emulsion into anhydrous product. Biochem.Eng.J., **68**, 7– 11 **(2012)**.

[16] T.F.C.Salum, P.Villeneuve, B.Barea, C.I.Yamamoto, L.C.Cocco, D.A.Mitchell, N.Krieger; Synthesis of biodiesel in column ûxed-bed bioreactor using the fermented solid produced by Burkholderia cepacia LTEB11, Process Biochem., **45**, 1348–1354 **(2010)**.

[17] M.Gumbyte, V.Makareviciene, E.Sendzikiene; Esterification of By-products of Biodiesel Fuel Production with Methanol and Technical Glycerol Using Biocatalysts. Environ Res.Eng.Manage, **2**, 28-34 **(2011)**.

[18] N.Gharat, V.K.Rathod; Ultrasound assisted enzyme catalyzed transesterification of waste cooking oil with dimethyl carbonate. Ultrason Sonochem, **20**,

900–905 **(2013)**.

[19] M.S.Antczak, A.Kubiak, T.Antczak, S.Bielecki; Enzymatic biodiesel synthesis – Key factors affecting efûciency of the process: Review. Renew Energ, **34**, 1185–1194 **(2009)**.

[20] S.Tamalampudi, M.R.Talukder, S.Hama, T.Numata, A.Kondo, H.Fukuda; Enzymatic production of biodiesel from Jatropha oil: A comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. Biochem.Eng.J., **39**, 185–189 **(2008)**.

[21] R.Maceiras, M.Vega, C.Costa, P.Ramos, M.C.Marquez; Effect of methanol content on enzymatic production of biodiesel from waste frying oil. Fuel, **88**, 2130–2134 **(2009)**.

[22] C.H.Liu, C.C.Huang, Y.W.Wang, D.J.Lee, J.S.Chang; Biodiesel production by enzymatic transesterification catalyzed by Burkholderia lipase immobilized on hydrophobic magnetic particles. Appl.Ener., **100**, 41-46 **(2012)**.

[23] E.G.Bligh, W.J.Dyer; A rapid method of total lipid extraction and purification. Can J Biochem Physiol, **37**, 911–917 **(1959)**.

[24] A.Vimalarasan, N.Pratheeba, B.Ashokkumar, N.Sivakumar, P.Varalakshmi; Production of biodiesel from cyanobacteria (Oscillatoria annae) by alkali and enzyme mediated transesterification. J.Sci.Ind.Res., **70**, 959-967 **(2011)**.

[25] S.S.S.Kavardi, I.Alemzadeh, A.Kazemi; Optimization of lipase immobilization. IJE Transactions C: Aspects, **25**, 1-9 **(2012)**.

[26] J.F.M.Burkert, F.Maugeri, M.I.Rodrigues; Optimization of extracellular lipase production by Geotrichum sp. using factorial design. Bioresource Technol., **91**, 77-84 **(2004)**.

[27] G.S.Padilha, J.S.Curvelo Santana, R.M.Alegre, E.B.Tambourgi; Extraction of Lipase from Burkholderia cepacia by PEG/Phosphate ATPS and its Biochemical Characterization. Braz Arch Biol.Technol., **55**, 7-19 **(2012)**.

[28] O.H.Lowry, N.J.Rosebrough, A.L.Farr, J.Randal; Protein measurement with the folin-phenol reagent. J.Biol.Chem., **193**, 265–275 **(1951)**.

[29] E.S.Umdu, M.Tuncer, E.Seker; Transesteriûcation of Nannochloropsis oculata microalga's lipid to biodiesel on Al_2O_3 supported CaO and MgO catalysts. Bioresource Technol., **100**, 2828–2831 **(2009)**.

[30] Y.Xu, W.Du, D.Liu, J.Zeng; A novel enzymatic route for biodiesel production from renewable oils in a solvent-free medium. Biotechnol.Lett., **25**, 1239-

FULL PAPER

1241 **(2003)**.

[31] Y.Shimada, Y.Wantanabe, T.Samukawa, A.Sugibara, I.L.Noda, I.I.Fukuda, Y.Tominaga; Conversion of vegetable oil to biodiesel using immobilized Candida antarctica lipase. J.Am.Oil Chem.Soc., **76**, 789-793 **(1999)**.

[32] N.I.Ruzich, A.S.Bassi; Proposed Kinetic mechanism of Biodiesel production through Lipase Catalysed Interesterification with a Methyl acetate acyl acceptor and Ionic liquid [BMIM] [PF$_6$] co-solvent. Can.J.Chem.Eng., **89**, 166-170 **(2011)**.

[33] D.T.Tran, K.L.Yeh, C.L.Chen, J.S.Chang; Enzymatic transesteriûcation of microalgal oil from Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized Burkholderia lipase as the biocatalyst. Bioresource Technol., **108**, 119–127 **(2012)**.

[34] A.E.Ghaly, D.Dave, M.S.Brooks, S.Budge; Production of Biodiesel by Enzymatic Transesterification: Review. Am.J.Biochem.Biotechnol., **6**, 54-76 **(2010)**.

[35] N.Dizge, B.Keskinler; Enzymatic production of biodiesel from canola oil using immobilized lipase. Biomass Bioenerg, **32**, 1274–1278 **(2008)**.

[36] S.Hama, S.Tamalampudi, T.Fukumizu, K.Miura, H.Yamaji, H.Kondo Afukuda; Lipase localization in Rhizopus oryzae cells immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production. J.Biosci.Bioeng., **101**, 328-333 **(2006)**.

[37] A.Kumari, P.Mahapatra, V.Kumar Garlapati, R.Banerjee; Enzymatic transesterification of Jatropha oil. Biotechnol Biofuels, (doi:10.1186/1754-6834-2-1), **2**, 1 **(2009)**.

[38] W.Du, Y.Xu, D.Liu, J.Zeng; Comparative study on lipase-catalysed transesterification of soybean oil for biodiesel production with different acyl acceptors. J.Mol.Catal.B: Enzyme, **3**, 125-129 **(2004)**.

[39] D.Feng, Z.Chen, S.Xue, W.Zhang; Increased lipid production of the marine oleaginous microalgae Isochrysis zhangjiangensis (Chrysophyta) by nitrogen supplement. Bioresource Technol., **102**, 6710–6716 **(2011)**.

[40] M.Ballat, H.Ballat; Progress in biodiesel processing. Appl.Ener., **87**, 1815-1835 **(2010)**.