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ORIGINAL PAPER

Enzymatic Biodiesel Production from *Manilkara Zapota* (L.) Seed Oil

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Abstract Oil obtained from the seeds of *Manilkara zapota* (L.) was transesterified with methanol using lipases as biocatalysts. Four commercially available lipases, such as, porcine pancreas, *Candida rugosa, Pseudomonas cepacia* and *Candida antarctica*-B were used for biodiesel preparation. Novozyme-435 (*C. antarctica* lipase-B immobilized on acrylic resin) and CLEA (cross-linked enzyme aggregate) of *C. antarctica* lipase-B were compared for their biodiesel production potential. Under optimized reaction conditions, Novozyme-435 gave 96% biodiesel yield in 12 h; whereas, CLEA of *C. antarctica*-B gave 84% yield of biodiesel. Novozyme-435 was reused for six cycles and 72% biodiesel was formed at the end of 6th cycle. The deactivated Novozyme-435 was regenerated by incubating it in soybean oil, 2-butanol and *tert*-butanol.

Keywords *Manilkara zapota* seed oil · Lipase · Transesterification · Biodiesel · Sustainability

Introduction

The world at large relies upon petroleum based fuels for daily energy needs. Most countries are dependent on handful of nations for the supply of fossil fuel—a finite resource. Such over dependency on fossil fuels is risky, since continuous fuel supply is dependent on international relations between countries. Therefore, all-time supply of fossil fuels from this region cannot be guaranteed. In addition, consumption of fossil fuel also causes increase in air pollution. Hence, developing and developed countries are investing heavily on the development of various alternative fuels. Along this line, biodiesel (fatty acid methyl esters) is a potential liquid fuel that is synthesized from a variety of oils, fats, and greases [1]. Biodiesel is commercially available in many countries. In general, biodiesel is considered beneficial for farmers because it has created a defined market for oils, fats and oil rich feedstocks; farmers have the opportunity to grow biofuel plants and in turn the generated biofuels can then be used in agriculture-related processing industries and sufficient biodiesel production will decrease dependency on Gulf countries. Biodiesel is mainly produced from edible oils (feedstocks) such as soybean, canola, and corn oil [2]. Typically, feedstock accounts for 70–80% of the total biofuel cost [3]. To make biodiesel cost effective, new oil-rich low-cost feedstocks viz. non-edible oils, discarded fats, food waste, sludge, and waste cooking oils are being investigated [4–10].

There is an ongoing necessity to identify oil rich plant species that are growing well on marginal lands. In the above context, oil rich seeds obtained from native marginalised plants and agricultural sources can be used as potential sources for biodiesel preparation. In this regard, *M. Zapota L.*, a small tree native to Mexico and tropical America is now spread throughout regions with tropical climate [11, 12]. In India, it is called "Chiku or Sapota" by local people. Fruits of *M. Zapota* L. plant are sold widely in India. The fruits are sweet with high quantities of energy, vitamins, and antioxidants [13–17]. After eating the fruits, the resulting seeds are generally discarded due to lack of awareness and collection facilities. The seeds of *M. Zapota* L. are highly underutilized. Such underutilized seeds are potential candidates for biodiesel production. Local people

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in the rural areas collect the seeds and prepare oil from it. In some parts of India, this oil is used for lamp lighting. *Manilkara Zapota* L. plant oil was investigated for biodiesel production using chemical catalysts. Kumar et al. obtained 94.83% of biodiesel via KOH catalysed transesterification [18, 19]. Chemo-catalysts, in particular, KOH and NaOH are highly moisture sensitive. Presence of moisture can lead to saponification; which is known to affect the yield of biodiesel adversely.

Lipases are enzymes that hydrolyse triglycerides as natural substrates. In both conventional and non-conventional media, lipases are known to catalyse esterification, transesterification and inter-esterification reactions [20–25]. As an alternative to the chemical catalysts, lipases can be used for biodiesel production; since, they are moisture tolerant [7, 26]. Lipase catalysed biodiesel production is carried out under mild reaction conditions. Furthermore, they can be used for many reaction cycles. Therefore, lipases are extensively investigated for biodiesel production using different feedstocks. In this work, lipases are used for the preparation of biodiesel from *M. Zapota* L. seed oil (Fig. 1).

Materials and Methods

Candida antarctica lipase-B (Novozym 435, immobilized on polyacrylic resin), *C. antarctica* lipase-B CLEA (cross-linked enzyme aggregate), *Candida rugosa* (Type VII), porcine pancreas (Type II) and *P. cepacia* were obtained from Sigma–Aldrich. All other chemicals such as methanol, *n*-heptane, ethyl acetate, soybean oil, tert-butanol, 2-butanol and sodium sulphate were reagent grade. Crude *M. Zapota* (L.) oil was purchased from oil mills of Koraput, Odisha, India. Incubator (Orbitrek, India) was used for all experimental purposes. The methanol was distilled by simple laboratory distillation procedure using round bottom flask, condenser, cooling water, receiving flask and stirrer cum heat plate. The obtained methanol was stored in molecular sieves before its use in transesterification. ^{1}HNMR spectra were recorded using a Bruker (400 MHz) spectrometer (Bruker, Massachusetts, USA). Tetramethylsilane (TMS) was used as an internal standard and $CDCl_3$ was used as a solvent. Molecular weight of *M. zapota* oil 873.95 g/mol was used for all calculation purposes [18, 19].

Screening of Lipases

Generally, transesterification reactions were carried out in 50 mL screw capped conical flasks containing *M. Zapota* (L.) oil (1.31 g, 1.5 mmol), methanol (0.351 mL, 4.5 mmol) and different types of lipases (10 wt% with respect to oil). All reactions were performed at 40 °C and 200 rpm for 4 h. Enzymes were filtered off after the reaction and the resulting samples were analysed by ^{1}H NMR spectrometry.

Transesterification of *M. Zapota* (L.) Oil at Different Lipase Loadings

Transesterification reactions were carried out exactly as described in the "screening of lipases" protocol using various amounts of *C. antarctica* lipase-B (5, 10, 15, 20 and 25 wt%). All the reactions were performed at 40 °C and 200 rpm for 4 h. After completion of the reactions, the lipase was carefully filtered using whatman qualitative filter paper and conical flask. The obtained samples were analysed by ^{1}H NMR.

Transesterification of *M. Zapota* (L.) Oil via "Sequential Addition of Methanol"

Transesterification reaction was initiated by addition of methanol (0.351 mL, 4.5 mmol) to a mixture of *M. Zapota* (L.) oil (1.31 g, 1.5 mmol) and Novozyme-435 (10 wt%). The reaction was carried out in a shaker (200 rpm) at 40 °C. Additional methanol (0.351 mL, 4.5 mmol × 2) was added in a stepwise manner to the reaction after 4 and 8 h. Then, the reaction was let to proceed until 24 h. In total, 13.5 mmol of methanol was reacted with 1.5 mmol of oil. Analysis and workup of the reaction were done as described in the "screening of lipases" method.

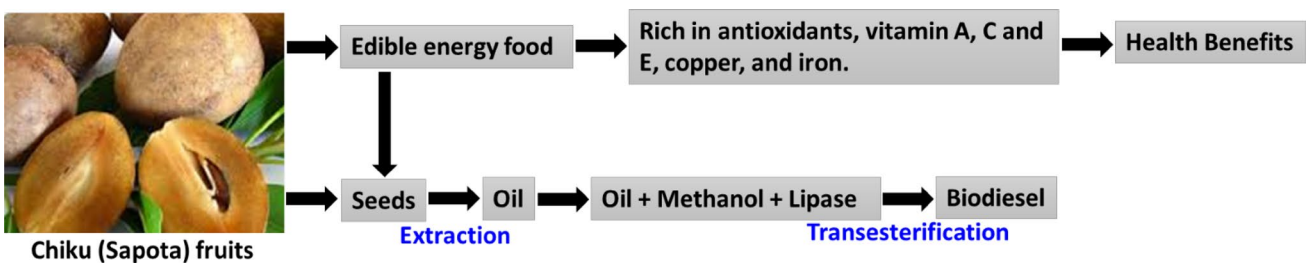

Fig. 1 Enzymatic preparation of biodiesel from the seed oil of *M. Zapota* L.

Reusability of *Candida antarctica* Lipase-B (Novozym-435)

The reaction was performed as stated for the "sequential addition of methanol" protocol. After each reaction, the enzyme was filtered off, washed using heptane (1 mL × 3) and then reused for the next cycle. Analysis and workup of all the reactions were carried out as described before. The lipase was reused for six consecutive cycles.

Regeneration of Novozym-435

Oil Washing

The lipase was washed using soybean oil (5 mL) three times and stored in soybean oil (5 mL) overnight in an incubator at 30 °C.

2-Butanol Washing

The enzyme was washed using 2-butanol (10 mL) three times and then it was washed using soybean oil. The lipase was separated by filtration and stored in an incubator overnight at 30 °C in a glass vial.

Tert-butanol Washing

The enzyme was washed using tert-butanol (10 mL) for three times. Later, it was washed using soybean oil (5 mL). The obtained lipase was stored in an incubator overnight at 30 °C in a glass vial.

Results and Discussion

Search for new feedstocks such as plant oils, food waste, sewage sludge, municipal organic waste for biofuel production is a part of ongoing research on renewable energy [27–30]. In this context, preparation of biodiesel from marginalized plant seed oil is of significant importance. Four commercially available lipases viz. *C. antarctica* lipase-B (Novozym 435, immobilized on polyacrylic resin), *Candida rugose* (Type VII), porcine pancreas (Type II) and *P. cepacia* were used for biodiesel production from crude *M. Zapota* oil. Conversion of oil to biodiesel was determined by ^{1}H NMR (Fig. 2). Among the lipases screened, *C. antarctica* lipase-B gave highest conversion (28%) at 1:3 molar ratio of oil to methanol and at 40 °C (Fig. 3). Therefore, *C. antarctica* lipase-B was chosen for the optimization of reaction parameters.

Effect of the lipase amount (*C. antarctica* lipase-B) on the yield of fatty acid methyl esters was studied. It was

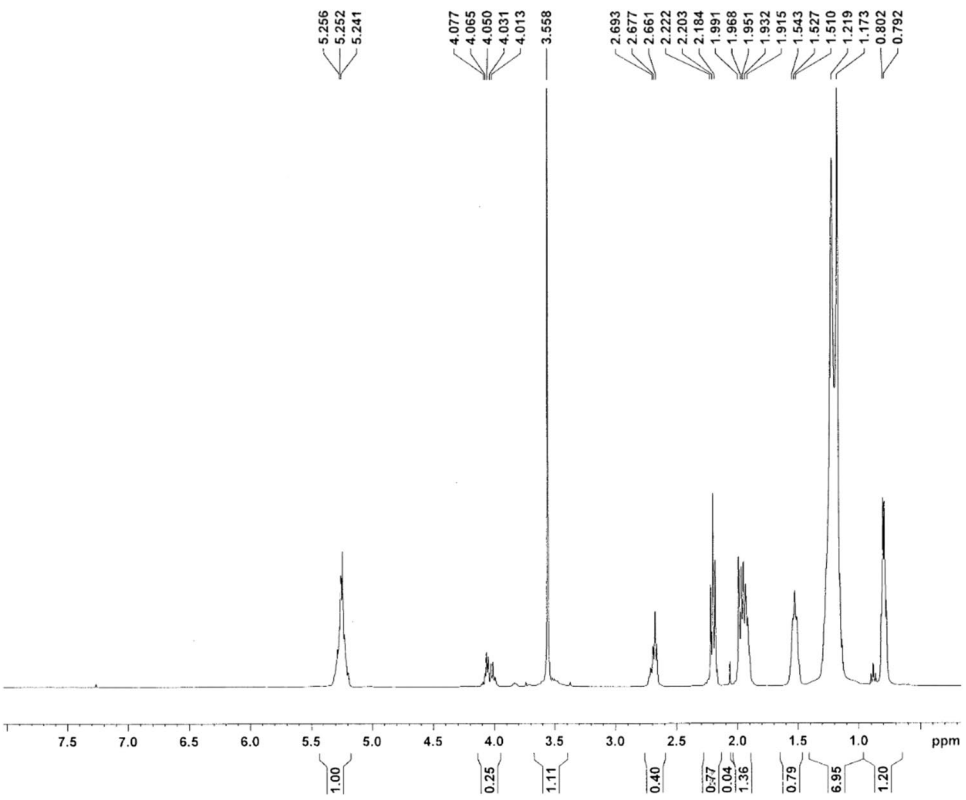

Fig. 2 ^{1}H NMR spectrum of the reaction mixture containing biodiesel and unreacted *M. Zapota* L. seed oil

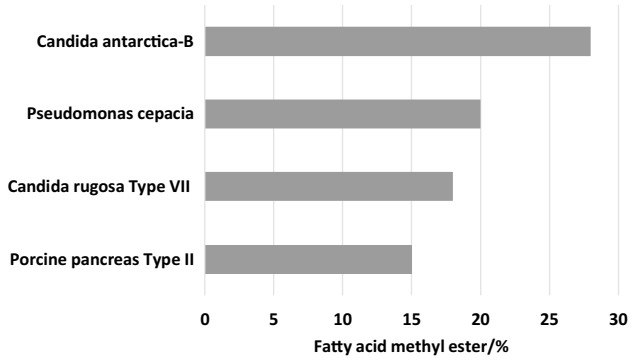

Fig. 3 Screening of different lipases for the preparation of biodiesel from *M. Zapota* (L.) seed oil

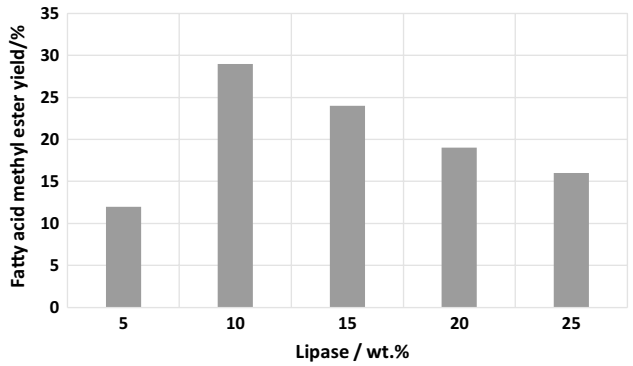

Fig. 4 Effect of lipase loading on biodiesel yield

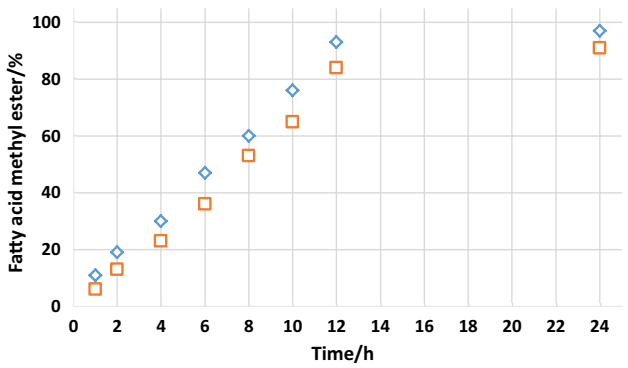

Fig. 5 Use of *Candida antartica* lipase-B (immobilized on acrylic resin, Novozyme-435, *open diamond*) and CLEA of *Candida antartica* lipase-B (*open square*) for biodiesel production

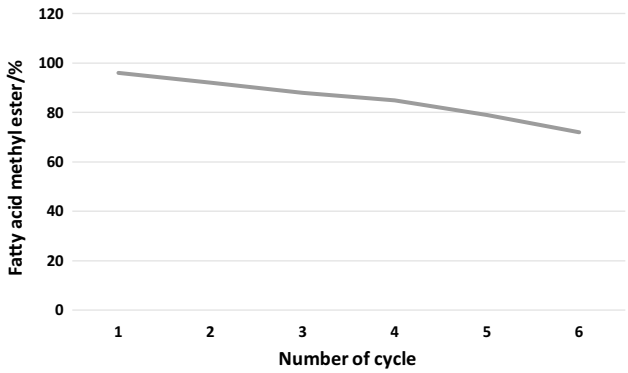

Fig. 6 Repeated use of Novozyme-435 for the biodiesel production

found that 10 wt% of lipase with respect to weight of oil gave highest biodiesel yield (29%) after 4 h of reaction (Fig. 4).

Addition of high quantities of methanol to oil causes deactivation of lipases during biodiesel preparation. Water-miscible polar organic solvents are known to deactivate lipases more than water-immiscible solvents [31]. Thus, to avoid deactivation of lipases, methanol was added in a step-wise manner to oil. Initially, methanol (4.5 mmol) was added to a mixture of *M. Zapota* (L.) oil (1.5 mmol) and Novozyme-435 (10 wt%). The reaction was performed at 40 °C and 200 rpm. Afterwards, additional methanol (4.5 mmol × 2) was added in a step-wise approach to the reaction mixture at 4 and 8 h (Fig. 5). Then the reaction was left to run for 24 h. Using *Candida antartica* lipase-B (Novozyme-435) 93% biodiesel was obtained after 12 h of reaction (Fig. 5). Under similar reaction conditions CLEA of *Candida antartica* lipase-B gave 84% of biodiesel (Fig. 5). Earlier report show that Novozyme-435 was efficiently used for biodiesel production from food waste lipid and 90% of FAME yield was achieved after 24 h of reaction [26]. Han and Kim reported the application of CLEA of Photobacterium lipolyticum lipase M37 for biodiesel preparation from olive oil in 64% yield [32]. Along this line, CLEAs of *C. antarctica* lipase B which are covalently bound to magnetic nanoparticles was employed for conversion of non-edible vegetable and waste frying oils to biodiesel [33].

Furthermore, reusability test of Novozyme-435 was conducted. After 6th cycle, 72% of biodiesel was obtained (Fig. 6). The deactivated lipase was regenerated by different washing methods [34]. For instance, *t*-butanol washing gave 91% of biodiesel; whereas, 2-butanol and oil washing gave 84 and 77% of biodiesel respectively (Fig. 7).

Conclusions

Oil from marginalized seed of *M. Zapota* (L.) was used as a feedstock for biodiesel production. Two immobilized forms of *Candida antartica* lipase-B, namely, Novozyme-435 and CLEA were found to be potential biocatalysts for the transesterification of *M. Zapota* (L.) seed oil. Novozyme-435 was reused up to 6th cycle with 21% loss of activity. The deactivated lipase was regenerated after tert-butanol washing. The protocol presented here, offers a facile, low-cost

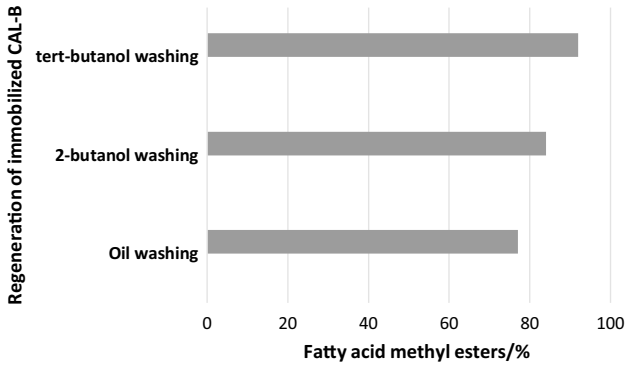

Fig. 7 Regeneration of deactivated Novozyme-435

and sustainable approach for synthesis of biodiesel from *M. Zapota* (L.) seed oil. This method can be extended to conversion of oils from other marginalized plant seeds and waste oils to biodiesel. Additionally, immobilized lipases can be exploited for utilization of low-cost biomass into liquid fuel and other value added products.

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