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Lipase-catalyzed in situ reactive extraction of oilseeds with short-chained alkyl acetates for fatty acid esters production

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Abstract

Substituting short-chained alkyl acetates for short-chained alcohols as acyl acceptors for fatty acid esters production, the negative effects of glycerol and alcohol on lipase can be eliminated. Short-chained alkyl acetates, like other short-chained esters, are also suitable solvents for seed oil extraction. Thus, methyl acetate and ethyl acetate were adopted as extraction solvents and transesterification reagents at the same time for in situ reactive extraction of *Pistacia chinensis* Bunge seed and *Jatropha curcas* L seed in this work. Fatty acid methyl esters and ethyl esters were, respectively obtained with higher yields than those achieved by conventional two-step extraction/transesterification. The improvement ranged from 5.3% to 22%. The key parameters such as solvent/seed ratio and water content were further investigated to find their effects on the in situ reactive extraction. The highest *P. chinensis* Bunge and *J. curcas* L methyl/ethyl esters could attain 92.8%, 89.5%, 86.1% and 87.2%, respectively under the optimized conditions.

Keywords: In situ reactive extraction; Short-chained alkyl acetates; Fatty acid esters; *Pistacia chinensis* Bunge seed; *Jatropha curcas* L seed

1. Introduction

Reserves shortage and price increase are causing a growing substitution of fossil fuels with fuels derived from vegetable origin such as fatty acid esters (biodiesel). Different processes are currently available to achieve transesterification of oils for the production of fatty acid esters, which include chemical or enzyme catalysis or supercritical alcohol treatment [1–4]. Although fatty acid esters can be successfully produced by chemical approach, there are several associated problems, such as recovery of catalyst and glycerol, as well as high energy requirements [5,6]. Use of biocatalysts (lipases) in transesterification of oils for fatty acid esters production overcomes these problems and offers an environmentally more attractive option to the conventional processes [7–9]. However, there are two bottlenecks in enzymatic approach for fatty acid esters production. One is the high cost of lipase and its short operational life caused by the negative effects of excessive short-chained alcohol and by-product glycerol [10–14]. It has been demonstrated that more than 0.5 M equivalent methanol are insoluble in vegetable oils and the immobilized lipases are easily inactivated by contacting with insoluble methanol existing as drops in the oils. By-product glycerol is hydrophilic and insoluble in the oil, so it is easily adsorbed onto the surface of the immobilized lipase also leading to negative effect on lipase activity and operational stability [9,15,16]. The other bottleneck for lipase-catalyzed fatty acid esters production is the high cost of the feedstock. Perhaps the largest impediment to wider adoption of fatty acid esters is its cost. When produced from refined oils, feedstock cost contributes more than 70% to the cost of the product.

Recently the use of methyl acetate and ethyl acetate as acyl acceptor for the transesterification of vegetable oils has been reported [17–19]. This transesterification method eliminates the risk of deactivation of enzyme by short-chained alcohol and glycerol, as short-chained alcohol is substituted with short-chained alkyl acetate and no glycerol is produced in the reaction (Scheme 1). Furthermore, short-chained alkyl acetates such as methyl acetate and ethyl acetate have also been shown to be suitable extraction solvents for oilseeds extraction [20]. Consideration of the current route from oilseeds to fatty acid esters

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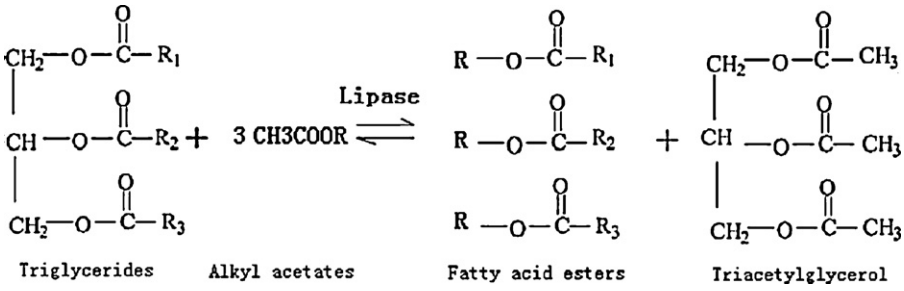

Scheme 1. Interesterification of triglycerides and alkyl acetates for fatty acid esters production. $R_{1,2,3}$ represent long-chained fatty acids, R represents CH_3 or CH_2CH_3.

caused us to inquire whether isolation of the oil from the seed, and its refining, were necessary. In contrast, transesterification reagents such as methyl acetate and ethyl acetate might be able to access acylglycerides resident in oilseeds and achieve their transesterification directly, in situ. Such a route to fatty acid esters could eliminate the expense associated with solvent extraction and oil cleanup, and simplify the steps in fatty acid esters production. This could result in a decrease in the cost of the product. Therefore, Haas et al. had tried "in situ transesterification" using a chemical method [21,22]. However, we investigated the use of methyl acetate and ethyl acetate as extraction solvents and the in situ reactive extraction of oilseeds yielding directly fatty acid esters using a biological method in this work.

2. Materials and methods

2.1. Materials

Novozym435 (lipase B from *Candida antarctica*, a nonspecific lipase immobilized on a macroporous acrylic resin with a specific activity 10,000 propyl laurate unit (PLU) g^{-1} and water content 1–2% (w/w), PLU is based on a reaction between propyl alcohol and lauric acid) was bought from Novo Nordisk Bioindustrials Inc. The *Pistacia chinensis* Bunge seed and *Jatropha curcas* L seed used in this study were standard products of Chinese agriculture. The seeds were milled using a standard coffee grinder before use. Methyl and ethyl esters of palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, and heptadecanoic acid were obtained from Sigma and were chromatographically pure. Methyl acetate and ethyl acetate were obtained from commercial supplier (Shanghai Chemical Company, China). All other chemicals and reagents were obtained commercially and were of analytical grade. All the organic solvents were treated with molecular sieve 4 A for several days before use.

2.2. Solvent extraction of oilseeds

Solvent extraction of oilseeds was performed according to the method of Veronique et al. [23].

Ground seeds (10 g) was mixed with 100 ml solvent in screw-caped glass vials, the extraction was carried out at 50 °C and 180 rpm for 6 h in a shaker fitted with a thermostat. After filtration, the ground seed mixture was mixed with another 50 ml solvent and extracted at the same condition for another 2 h. The two filtrates were pooled and centrifuged at 17,400 × *g* for 10 min, the supernatant was collected into a round bottom flask and the solvent was evaporated using a rotary evaporator. The amount of lipid recovered was then measured gravimetrically. The theoretical oil content was determined according to AOCS Am 2-93 (FOSFA method) using a Soxtec extraction unit with *n*-hexane. Results of two identical experiments differed not more than 2%.

2.3. Water content of oilseeds

Water content of oilseeds was measured gravimetrically using the method AOCS Ai2-75 at 103 °C.

2.4. Procedure for two-step extraction/transesterification and in situ reactive extraction

Two-step extraction/transesterification: ground seeds (5 g) were extracted as in Section 2.2 with *n*-hexane and then the crude oil obtained was transesterified with methanol or ethanol at 50 °C and 180 rpm in the presence of Novozym435, the amount of lipase was 30% (w/w) based on theoretical oil content and the molar ratio of alcohol to oil was 3:1.

In situ reactive extraction: ground seeds (5 g) were mixed with methyl acetate or ethyl acetate in screw-caped glass vials, 30% (w/w) of Novozym435 based on theoretical oil content was added, the reactions were carried out at 50 °C and 180 rpm.

Samples (100 μl) taken from the reaction mixture at specified times were centrifuged at 17,400 × *g* for 10 min and the supernatants (30 μl) were mixed with 7.5 μl of 40 mM heptadecanoic acid methyl ester (served as internal standard) and 262.5 μl of *n*-heptane, and were analyzed by gas chromatography.

2.5. GC analysis of the samples

Samples prepared as described above were analyzed by injecting 1 μl of *n*-heptane solution and internal standard into an Agilent 6890 gas chromatography, equipped with a HP-5 capillary column (5% phenyl methyl siloxane capillary, 30.0 m × 320 μm × 0.25 μm nominal). The column temperature was kept at 180 °C for 1 min, heated to 300 °C at 10 °C/min, and then maintained for 2 min. The temperatures of the injector and detector were set at 260 and 280 °C,

respectively. All samples were measured in triplicate. The ester yields were calculated by following equations: yield (%) = 100 × (A + B + C + D + E)/(3 × F), where A, B, C, D, E, and F are the peak areas of methyl/ethyl esters of palmitic, stearic, oleic, linoleic, linolenic, and heptadecanoic acids, respectively.

3. Results and discussion

3.1. Extraction of oilseeds by short-chain alkyl acetates

Alkyl acetates, especially methyl acetate and ethyl acetate are important chemicals, which find extensive application such as a solvent and a starting material for organic synthesis. Substitution of short-chained alcohol with short-chained alkyl acetates as acyl acceptor in the production of fatty acid esters can overcome the negative effects of alcohol and glycerol. In order to investigate the feasibility of in situ reactive extraction of oilseeds with short-chain alkyl acetates for fatty acid esters production, we firstly investigated the effect of alkyl acetates as alternative solvents on the solvent extraction of the oilseeds. In many countries, like China, as edible oils are not in surplus supply, there is a need to search for alternative starting oils such as from non-edible oilseeds for biodiesel production. *J. curcas* L seed oil, due to the presence of toxic phorbol esters is considered as a non-edible oil. The seed kernel contains 40–60% (w/w) oil. *P. chinensis* Bunge seed oil is also not used as an edible oil because of its bitter taste. The seed kernel contains 42–55% (w/w) oil. Furthermore, they can be grown on barren land under harsh conditions and can be cultivated as a part of the strategy for reclaiming degraded lands. Thus, they offer obviously good potential for biodiesel production. Keeping all this in view, the China government has announced a 'National Mission on Biodiesel' for Jatropha and Pistacia plantations in wasteland regions in her "the tenth five-year-plan". Therefore, *P. chinensis* Bunge seed and *J. curcas* L seed were selected as the studying materials in this work.

P. chinensis Bunge seed and *J. curcas* L seed were extracted with methyl acetate and ethyl acetate and the results were compared to those obtained by extraction with *n*-hexane. Table 1 shows the oil content in g/100 g. Considering the experimental error margins (see Section 2.2) the results of the extraction of both oilseeds are very similar with oil contents not differing more than 2%. Insofar, methyl acetate and ethyl acetate are suitable solvents for seed oil extraction. In this stage, the content of minor constituents was not determined.

Table 1
Extraction of various oilseeds by *n*-hexane, methyl acetate and ethyl acetate

	Extraction with		
	n-Hexane	Methyl acetate	Ethyl acetate
Pistacia chinensis Bunge seed	36.69%	36.86%	36.19%
Jatropha curcas L seed	54.90%	55.92%	56.65%

Extraction conditions: 50 °C, 8 h, for details see Section 2.2.

3.2. Comparison of two-step extraction/transesterification and in situ reactive extraction

Since extraction and lipase-catalyzed transesterification with methyl acetate and ethyl acetate were carried out under the same conditions, they now can be easily combined to a two-step-one-pot in situ reactive extraction. The alkyl acetates now acted first as the extraction solvent and afterwards as the transesterification agent. Afterwards, the methyl/ethyl esters were obtained directly from the extraction process by simply removing the catalyst and defatted plant material (by filtration) and the solvent (by evaporation). Table 2 shows the results of the in situ reactive extraction *versus* the two-step extraction/transesterification method. In all cases the yields of in situ reactive extraction were better than those achieved by the conventional two-step technique; the improvement ranged from 5.3% (*J. curcas* L seed fatty acid ethyl esters) to 22% (*P. chinensis* Bunge seed fatty acid methyl esters). This could be explained by the fact, that in small-scale laboratory experiments, losses in multi-step operations were inevitable and the short-chained alcohol might impair the lipase activity to some extent. Furthermore, some minor components such as phospholipids may be extracted by *n*-hexane in two-step technique and have a negative effect on the activity of lipase [24]. In our further study we found that the content of phospholipids in the crude oil extracted with *n*-hexane was remarkably higher than those in the crude oil extracted with alkyl acetates (date not shown).

Table 2
In situ reactive extraction *vs*. two-step extraction/transesterification of various seed oils to methyl and ethyl esters

	Extraction with *n*-hexane and transesterification with		In situ reactive extraction with	
	Methanol[a]	Ethanol[a]	Methyl acetate	Ethyl acetate
P. chinensis Bunge seed Yield (%)	53.1%	62.5%	75.2%	76.4%
J. curcas L seed Yield (%)	45.9%	59.4%	63.9%	64.7%

Reaction condition: 5 g oilseeds, 50 ml solvent, 50 °C, 12 h, for details see Section 2.2.
[a] Two-step addition of methanol and ethanol at 0 and 6 h.

3.3. In situ reactive extraction

In situ reactive extraction differs in that the oilseeds contact with alkyl acetates directly instead of reacting oil and alkyl acetates. This will give more benefit, because extraction and transesterification proceed in one step and the solvent is reusable. Therefore, the key parameters such as solvent/seed ratio and water content were further investigated to find their effect on the in situ reactive extraction of various oilseeds.

3.3.1. Effect of solvent/seed ratio

In the in situ reactive extraction the alkyl acetates act not only as an extraction solvent but also as a transesterification reagent. Du et al., Xu et al. and Modi et al. reported that a large excess of alkyl acetates was required to shift the transesterification in forward direction [17–19]. Therefore, a suitable amount of solvent should be able to extract the oil and shift the reaction in forward direction effectively. Different solvent/seed ratios were investigated in this work. As shown in Fig. 1, the highest ester yields were all obtained at solvent to seed ratio of 7.5:1 regardless of the kinds of seeds and alkyl acetates used. Shifting the ratio above or below the optimum value decreased the methyl/ethyl ester yields with both seeds. This might be because the solvents were not enough to extract the oilseeds when low ratio was used and too many solvents led to an excessive dilution of oil when higher ratios were used. Based on the result, solvent/seed ratio of 7.50 ml/g was used for the future studies.

3.3.2. Effect of water content of oilseeds

Amount of water present in the reaction media is another critical parameter, which is known to influence biotransformations in nonaqueous media [25]. The general picture available is that less than a monolayer of water is required for an enzyme to show biological activity. As the water level increases, it increases

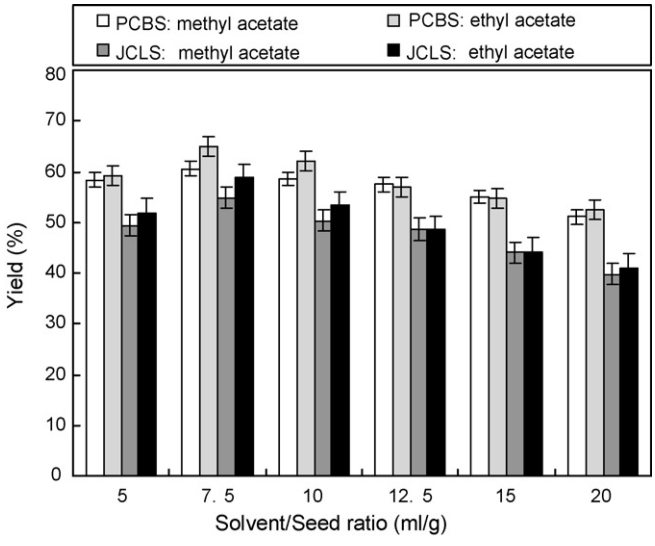

Fig. 1. Effect of solvent/seed ratio on the ester yields. PCBS: *Pistacia chinensis* Bunge seed; JCLS: *Jatropha curcas* L seed. Reaction conditions: 5 g oilseeds, 50 °C, 30% (w/w) of Novozym435 based on theoretical oil content, 10 h, 180 rpm.

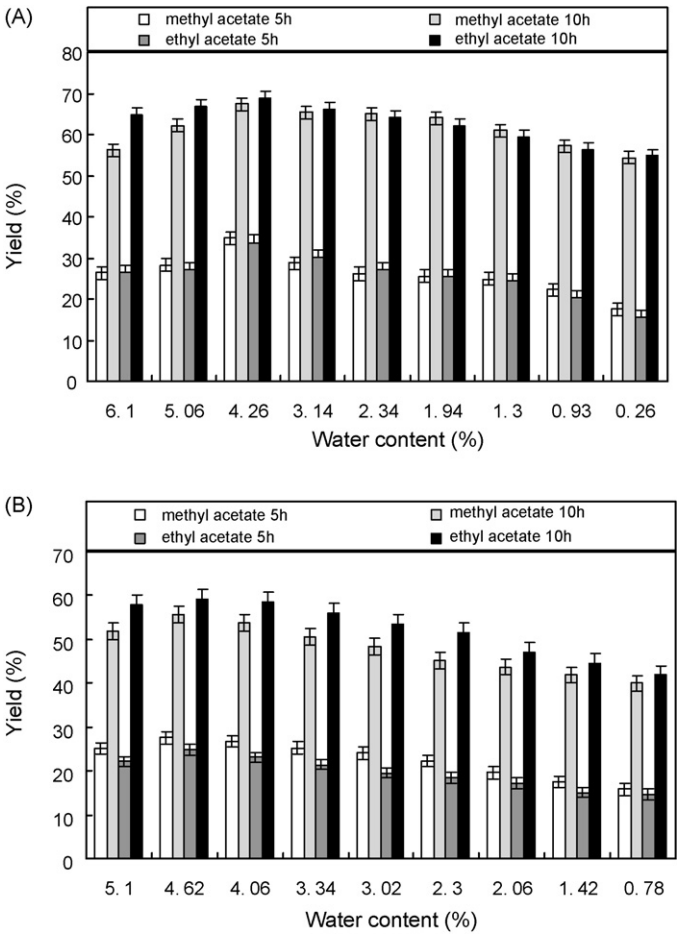

Fig. 2. Effect of water content of oilseeds on the ester yields: (A) *P. chinensis* Bunge seed; (B) *J. curcas* L seed. Reaction conditions: 5 g oilseeds, solvent/seed ratio of 7.5:1, 50 °C, 30% (w/w) of Novozym435 based on theoretical oil content, 180 rpm.

the enzyme flexibility and the expressed activity [26], after an optimum level of water, hydrolytic reactions become significant and transesterification yield is expected to go down. In the in situ reactive extraction the alkyl acetates contact with the oilseeds directly. Therefore, the water content of oilseeds will influence the ester yields inevitably. Fig. 2(A) shows the effect of water content of *P. chinensis* Bunge seed on the methyl/ethyl ester yields, the highest ester yields were attained at 4.26% of water content no matter methyl acetate or ethyl acetate used. The effect of water content of *J. curcas* L seed on the ester yields was showed in Fig. 2(B), the highest ester yields were achieved at 4.62% of water content regardless of methyl acetate or ethyl acetate used.

3.3.3. Time course

Using the above optimized reaction conditions, the *P. chinensis* Bunge and *J. curcas* L methyl/ethyl esters were prepared by varying the reaction period. The ester yields were increased rapidly before 16 h, and then very slowly until 36 h, practically were almost constant after 24 h of reaction with both seeds. The highest *P. chinensis* Bunge and *J. curcas* L methyl/ethyl esters could attain 92.8%, 89.5%, 86.1% and 87.2%, respectively (Fig. 3).

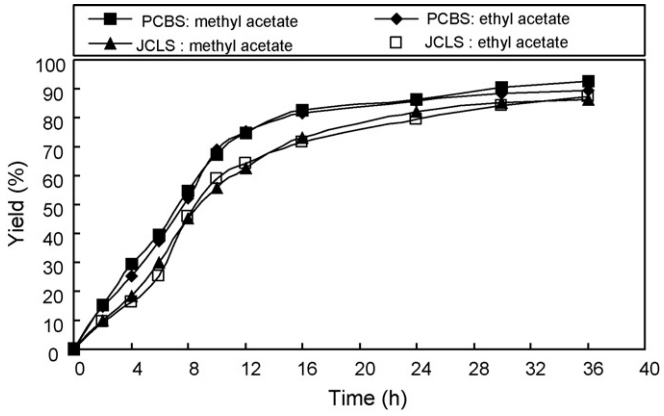

Fig. 3. Time course of ester formation during in situ reactive extraction. PCBS: *P. chinensis* Bunge seed, 4.26% of water content; JCLS: *J. curcas* L seed, 4.62% of water content. Five grams oilseeds, solvent/seed ratio of 7.5:1, 50 °C, 30% (w/w) of Novozym435 based on theoretical oil content, 180 rpm.

4. Conclusions

In conclusion, fatty acid esters can be produced satisfactorily by in situ reactive extraction of oilseeds. The highest *P. chinensis* Bunge and *J. curcas* L methyl/ethyl esters could attain 92.8%, 89.5%, 86.1% and 87.2%, respectively under the optimized conditions (solvent/seed ratio of 7.5:1, 4.26% of water content for *P. chinensis* Bunge seed, 4.62% of water content for *J. curcas* L seed, 50 °C, 30% (w/w) of Novozym435 based on theoretical oil content, 180 rpm). In comparison with the former works of other researchers [17–19], this is a very convenient method because the reaction steps are cut in half. Furthermore, such a route to fatty acid esters can eliminate the expense associated with solvent extraction and oil cleanup. This will result in a decrease in the cost of the product. Thus we think in situ reactive extraction will be a very promising method for fatty acid esters production.

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