Pretreatment of waste oil and biodiesel preparation catalyzed by immobilized lipase

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As a new kind of environment-friendly renewable energy, the high cost of raw materials limits the application of biodiesel. Producing biodiesel from waste oil could effectively reduce its material cost and the environmental pollution caused by waste oil. The complex composition of waste oil could lead to catalyst poisoning. In order to meet the raw material requirements of biodiesel production, the oxidation of hydrogen peroxide combined with the adsorption of activated white soil and carbon was investigated in this paper. The treatment process was optimized as follows: the waste oil was bleached with 5% hydrogen peroxide based on the oil weight for 30 min at 60°C, then was treated for 20 min at 70°C with 5% mixed adsorbent (the ratio of active carbon and bentonite was 1:1) based on the oil weight. The biodiesel was then catalyzed by immobilized lipase at 0.2 g/mL packing density in cyclic reactors at 40°C for 12 h with 0.6 mL/min flow rate. The study showed that two-step processing could improve the quality of waste oil significantly and meet the needs of biodiesel producing. A 90% conversion rate could be reached through the transesterification reaction catalyzed by immobilized lipase. Further refining was needed for the product because the acid value of biodiesel was slightly high.

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Introduction

Biodiesel is a new kind of environment-friendly renewable energy, which can be used as alternative fuel for petrochemical diesel and reduce the demand for fossil fuels [1, 2]. Compared with the traditional petroleum diesel, there is no SO_2 in the automobile exhaust using biodiesel, or less hydrocarbons and carbon monoxide, which should greatly reduce the pollution to the environment [3]. At present, the price of biodiesel is 1.5 times of petrochemical diesel, mainly due to the cost of raw materials [4].

Biodiesel is mainly made from soybean oil, rapeseed oil, and animal oil which account for 75-80% of its production cost. Also, the struggles for food and land have led to short supply of raw materials. Therefore, cheap and readily available raw materials are the key to reduced cost of biodiesel [5].

Illegal cooking oil is a kind of inedible oil produced from animal and plant oils after repeated frying including the waste oil in catering industry and the oil separated from wastewater containing grease. Illegal cooking oil is rich in glycerides and free fatty acids, which are cheap and widely available, and could be used as an ideal raw material for biodiesel production [6, 7].

Edible oil consumption has reached more than 20 million tons in recent years in China. If the waste oil produced in use was collected to be

used to produce biodiesel, nearly 1.5 million tons of biodiesel could be obtained. With the increasing of global demand for energy, the production of biodiesel from waste oil (gutter oil) was regarded as a charming way to develop new energy sources by more and more people [8-10].

The production of biodiesel from gutter oil has certain advantages in cost but requires advanced technology. After repeated use at high temperature and over frying, fatty acids and glycerides in oils could form colored substances or potentially colored substances [11]. Proteins, phospholipids, and other substances begin to decompose, degenerate, or react with other substances to form a large number of dark substances.

A series of reactions including bi-degree hydrolysis, oxidation, and polymerization gradually occur during the storage of gutter oil. The productions including polycyclic aromatic hydrocarbons, peroxides, decomposition products of low-molecular, free fatty acids, and dimers of fatty acids should result in dark brown, opaque, and high freezing point of waste oil. In addition, the presence of aldehydes, ketones, and other substances should generate special sour and foul odor. The complex composition of gutter oil could lead to catalyst poisoning, which greatly hinders the catalytic reaction [12, 13].

In order to remove impurities in gutter oil and improve its color and luster, the method of removing intermediate products was adopted to reduce its influence on esterification reaction [14]. The traditional treatment process of waste oil was to adsorb pigment and impurities dispersed in oil using active white soil, which was simple to operate, but the complex composition of waste oil made it difficult to achieve good treatment effect with only physical adsorbent. It has been found that the oxidation of hydrogen peroxide has a significant effect on the treatment of dark waste oil, which provides a feasible method for the pretreatment of gutter oil [15, 16].

In order to meet the raw material requirements of biodiesel production, the oxidation of hydrogen peroxide combined with the adsorption of activated white soil and carbon were investigated in this paper.

The fatty acids in the waste oil are higher than those in general oil materials, which make it more complex to esterification catalyzed by traditional catalytic including mineral acid and alkali. Compared to traditional chemical catalysts, enzyme catalysts can effectively catalyze both esterification and transesterification processes [17, 18]. Biodiesel production from waste oil catalyzed by enzyme could be industrialized if the stability and efficiency of enzyme in the reaction system are improved, which could alleviate the energy shortage at the present stage in China, and the waste cooking oil could be fully used [19].

At present, studies on the production of biodiesel by enzyme method are mostly limited to the small reaction stage in the laboratory, and most of them are under intermittent operation [20]. In addition, the high costs of industrial enzymes and raw materials make it difficult for this technology to be put into industrial application [21]. In this paper, the enzymatic synthesis of biodiesel was catalyzed with self-made immobilized lipase and waste oil was used as the raw material. Batching and continuous catalytic processes were studied to realize the high-efficient production of biodiesel.

Materials and Methods

Experimental design of pretreatment of waste oil

The waste oil (Shandong Jinjiang Bio-energy Technology Co., LTD, Yantai, Shandong, China) was pretreated following the process shown in Figure 1.

(1) Centrifugation: Raw waste oil was centrifuged at 10,000 rpm for 10 min to separate the top liquid oil from solid impurity.
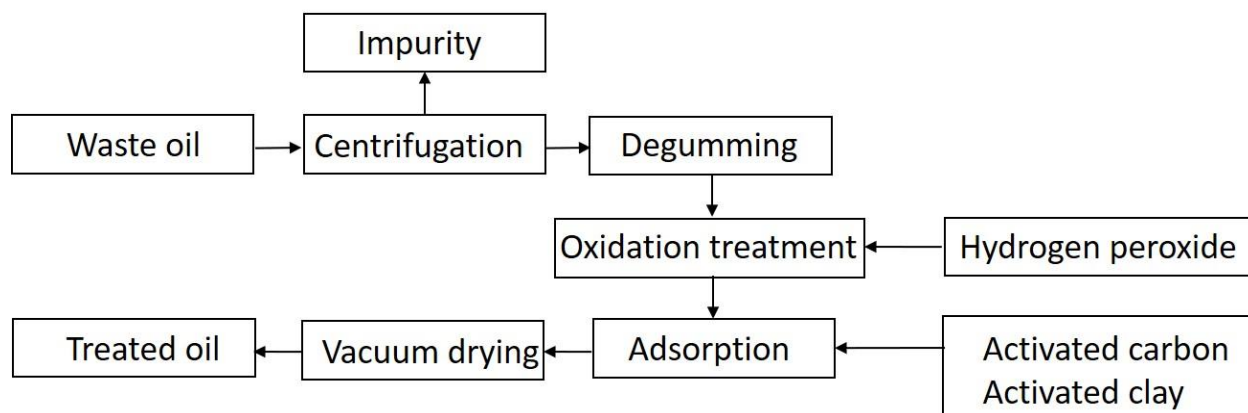
Figure 1. Pretreatment flow chart of waste oil.

(2) Degumming: Phosphoric acid was added into the upper oil by 2‰ weight of oil and stirred 10 min at 80℃, then hot water with 10% mass fraction was added. After stirred 10 min at 80℃, the mixture was centrifuged at 10,000 rpm for 10 min to obtain the upper liquid oil.

(3) Oxidation with hydrogen peroxide: After degumming, the waste oil was bleached with 5% hydrogen peroxide based on raw oil weight at 60℃ for 30 min. The upper oil was obtained after centrifuged at 10,000 rpm for 10 min. The treated effect was evaluated by using decolorization rate as indexing.

(4) Absorption with active bentonite: Activated clay (Jinan Hongqiao Chemical Co., LTD, Shandong, China) was activated at 150℃ for 2 h before use. Activated carbon (Jinan Hongqiao Chemical Co., LTD, Shandong, China) and active clay were added in the oil after oxidation at 5% of the oil weight with the mass ratio of 1:1 treated at 70℃ for 20 min. After centrifugation at 10,000 rpm for 10 min, the upper oil was obtained. The treated effect was evaluated by using decolorization rate as indicators.

(5) Drying: The pretreated oil was vacuum dried to constant weight.

Preparation of biodiesel with cyclic reaction
Figure 2 shows the test device of fixed bed reactor with 80 mL filling volume in a glass column with jacket. Lipase fermented by *Aspergillus niger* with the specific activity of 5,000 U/g (Shenzhen Leveking Biological Engineering Co., LTD, Shenzhen, Guangdong, China) was immobilized according to the method described in patent ZL201210062682X [22] with 0.3-0.9 mm particle diameter and 500 kg/m^3 bulk density. The enzyme activity of immobilized lipase was 4,100 U/g. Lipase and glass beads (Taishan Steel Ball Factory, Taian, Shandong, China) were stacked alternately to increase the contact area between enzyme and raw material in the reactor.

Reaction liquid in the flat bottom flask was preheated and stirred evenly by magnetic stirrer, and then was pumped into the reactor through a peristaltic pump from the top of the reactor in a set volume flow to keep the volume of reaction liquid in the reactor to 20 mL. The product flowed from the bottom of the reactor into the flat bottom flask and cycled back to reaction with the peristaltic pump many times until the end of the reaction. The outer jacketed of glass reactor was filled with circulating water to keep the reactor temperature constant at 40℃.

The reaction liquid consisted of 30 g pretreated waste oil mixed with methanol, n-hexane, and deionized water in set proportion into a volume of about 50 mL, which were cycled in the reactor. Every 1 h, reaction liquid was sampled to analyze the esterification rate. The effects of the filling
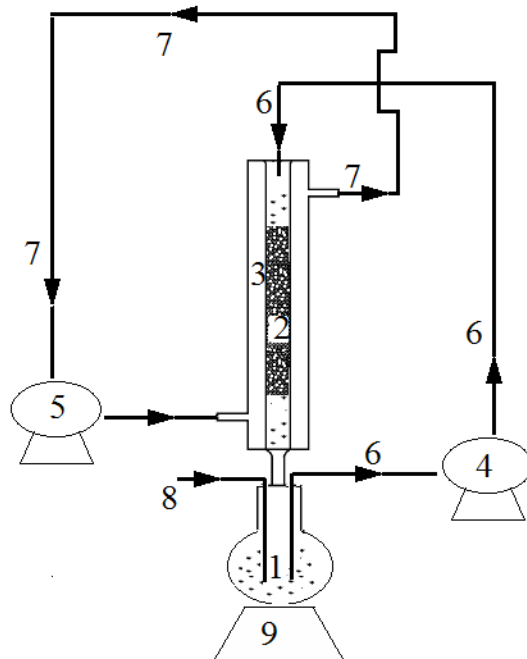
Figure 2. Process of biodiesel produced by fixed bed reactor. **1.** Round bottom flask. **2.** Mixture of enzyme and glass beads. **3.** Fixed bed reactor. **4.** Peristaltic pump. **5.** Circulating water. **6.** Reaction fluid. **7.** Circulating water. **8.** Methanol. **9.** Thermostatic magnetic stirrer.

density of lipase, the amount of reaction liquid, the molar ratio of methanol and oil in reaction liquid were investigated.

Analytical method

(1) Physicochemical index of oil
The acid value of oil was determined according to GB/T 5530-2005 [23], while the saponification value and the peroxide value were determined according to GB/T 5534-2008 [24] and GB/T 5538-2008 [25], respectively. The contents of water and phospholipid in oil were measured according to GB/T9696-2008 [26] and GB/T5537-2008 [27], respectively.

(2) Decolorization rate
The absorbance of oil at 520 nm was determined with petroleum ether following the method described by Nghiep *et al.* [28]. The decolorization rate was calculated as follows:

$$\text{Decolorization rate (\%)}=\frac{A_0\text{-}A_1}{A_0}\times 100\%$$

Where A_0 and A_1 are the absorbance of waste oil before and after treatment.

(3) Recovery rate
Recovery rate was calculated as follows:

$$\text{Recovery rate (\%)}=\frac{m_1}{m_0}\times 100\%$$

Where m_0 and m_1 are the weight of waste oil before and after treatment.

(4) Fatty acid composition
 1-2 mL waste oil was dissolved in 3 mL n-hexane. 1 mL of 0.4 mol/L potassium hydroxide methanol solution was then added to methyl esterification at 40°C for 10 min. 2-3 mL distilled water was injected to transfer the fatty acid methyl ester into the water phase. The supernatant was taken for chromatographic analysis.

Gas chromatography (GC) analysis of fatty acids was performed with an Agilent 6820 gas chromatograph (Agilent, Palo Alto, CA, USA)

equipped with a cross-linked PEG-20M silica capillary column (30 m × 0.32 mm × 0.25 μm), a Flame Ionization Detector (FID), and a split: splitless injector. Analysis was carried out using nitrogen (99.99%) as the carrier gas, and the flow rate was 15 cm/s. The column temperature was programmed from 180℃ to 250℃ at 5℃/min. The sample size was 1 μL, and the splitting ratio was 1:30. The injection port temperature was 250℃. The fatty acids indices were determined by co-injection of the sample with a solution containing a homologous series of n–hydrocarbons (C9 to C22) in a temperature-programmed run identical to that described above. The quantitative calculation was based upon the relative areas of the corresponding GC signals.

(5) Conversion rate of biodiesel

After the preparation reaction of biodiesel, 20 mL of warm water was added into the reaction system, and shocked at 40℃ for 30 min. The by-product glycerin was shifted into the water. After static stratification, the lower aqueous phase was collected and diluted with water to 25 mL. The content of glycerin was determined by high iodized acid REDOX method [29] and the conversion rate was calculated according to the following formula:

$$\text{Conversion rate } (\%) = \frac{m_1}{m_2} \times 100\%$$

Where m_1 is the actual yield of glycerin in mg, and m_2 is the theoretical yield of glycerin in mg.

Results and Discussion

Treatment technology of waste oil
(1) Decolorization with hydrogen peroxide

The waste oil was bleached by H_2O_2 at different conditions. The influences were investigated including the dosage of H_2O_2, bleaching temperature and time. The results were showed in Table 1.

Table 1. Decolorization efficiency of waste oil treated by hydrogen peroxide at different conditions.

Influence Factor	Level of factor	Decolorization rate (%)
Dosage of H_2O_2	1%	10.35±0.57
	3%	32.57±1.25
	5%	35.78±1.06
	7%	26.85±1.34
	9%	20.12±0.96
Temperature	50℃	24.39±1.11
	60℃	35.36±1.02
	70℃	29.65±0.96
	80℃	20.36±1.26
	90℃	18.26±1.55
Time	10 min	23.68±1.05
	20 min	30.27±1.23
	30 min	35.67±0.96
	40 min	35.71±1.01
	50 min	35.76±0.65

The decolorization rate of the gutter oil increased with the increasing of the amount of H_2O_2 when it was lower than 5%, then the decolorization rate began to decrease with the increasing dosage of H_2O_2. This is because the number of free hydroxyl groups in the solution increased as the amount of hydrogen peroxide increased, which is conducive to the degradation of pigment polymers. However, excessive hydrogen peroxide itself can also react with hydroxyl groups rapidly to form colored substances [30]. The results showed that the decoloring effect reached the best when the amount of hydrogen peroxide was about 5%.

When the temperature was below 60℃, the effect of hydrogen peroxide improved obviously with the increase of temperature, which is due to the faster movement of molecular at higher temperature enhancing the contact between oil and hydrogen peroxide in the reaction system, and then improving the decoloring rate of waste oil. When the temperature continued to increase, the decolorization rate decreased because of the decomposition of hydrogen peroxide at too high temperature, as well as the

side reaction of complex components in the gutter oil [31].

The decolorization rate of waste oil increased rapidly with time and reached about 34% with reaction time of 30 min, then gradually tended to be stable with the prolonging of decolorizing time because of the decreased concentration of peroxide and basically oxidized reaction substance.

(2) Adsorption with activated clay and activated carbon

The waste oil was adsorbed with activated clay and activated carbon. The effects of different conditions were investigated as showed in Table 2.

Table 2 showed that it was difficult to obtain a good decoloring effect for waste oil by using activated clay alone, and the decoloring rate was only 20%. Although the decoloring rate could reach 70% when activated carbon was used alone, much oil was also absorbed with pigment absorbing, which reduced the oil recovery rate to 30%. The treatment effect was obviously improved when activated clay and activated carbon were mixed in a certain proportion, and the proportion of 1:1 was relatively superior with around 60% decoloring rate and 75% recovery rate.

The decoloring rate of waste oil increased slowly with the increasing of the dosage of adsorbent when it was in the range of 1% to 15%, while the recovery rate decreased obviously. Considering the decolorization rate and recovery rate, the dosage of the mixed adsorbent was determined to be 5% of the oil weight.

The decoloring rate of gutter oil increased with the increasing of temperature. The high viscosity of waste oil made the decoloring effect poorly. With the increasing of temperature, the adsorption effect increased because the decreased viscosity enhanced the contact probability between adsorbent and pigment molecules [32]. When the temperature exceeded 70℃, the decoloring rate reduced with the rise of temperature. It may be that the higher temperature speeds up the desorption process of pigment, making the adsorption decoloring effect decrease obviously. In addition, the high temperature could lead to the rapid evaporation of free water in the adsorbent, resulting in the loss of surface activity of some adsorbents.

The decolorization rate increased with the extension of time within 20 min because the waste oil and adsorbent could fully contact and increase the decolorization rate significantly. Then the decolorization rate showed a decreasing trend with the extension of reaction time over 20 min because of the adsorption saturation and accelerated oxidation speed.

Treatment effect of waste oil
(1) Indicators of waste oil before and after treatment

Table 3 showed the physicochemical indexes of waste oil before and after treatment. After the treatment, the acid value increased due to the oxidation of some glycerol substances into acids by hydrogen peroxide, or the hydrolyzed triglycerides into free fatty acids [33].

Peroxide value is an indicator of oxidation degree of oil. The peroxide in gutter oil such as aldehydes, ketones, and other substances decreased obviously after several steps of treatment although the oxidation process of oil was irreversible. The saponification value indicated the length of fatty acid chain in oil (i.e. the size of fatty acid molecular weight), which decreased after treatment. The contents of water and phospholipids in oil were relatively small and had not much influence on subsequent reactions after treatment.

In addition, the raw waste oil was dark brown with a pungent smell but changed significantly after treatment into dark yellow with irritating smell disappearing, and so met the standards of industrial application.

(2) Fatty acid composition of pretreated oil

Table 2. Adsorption efficiency of waste oil with activated clay and activated carbon.

Influence Factor	Level of factor	Decolorization rate (%)	Recovery rates (%)
Ratio of activated clay to carbon (W:W)	1:0	20.01±0.54	85.32±1.55
	2:1	43.25±1.57	80.24±1.36
	1:1	60.85±3.06	73.25±1.47
	1:2	65.32±1.67	51.36±1.23
	0:1	70.23±1.33	30.05±1.28
Adsorbent contents (%)	1%	37.23±1.26	86.35±1.26
	5%	45.32±2.10	80.24±1.35
	10%	53.26±2.57	60.23±1.44
	15%	61.36±1.68	44.62±1.54
	20%	62.03±1.87	25.68±1.03
Temperature	60℃	38.62±1.25	63.52±1.89
	70℃	57.68±1.38	70.58±1.57
	80℃	55.38±1.22	61.25±1.24
	90℃	37.65±1.57	60.92±1.65
	100℃	16.85±1.54	62.38±1.87
Time	10 min	37.65±1.34	78.65±1.42
	15 min	42.36±1.51	71.55±1.53
	20 min	60.78±1.42	66.78±1.51
	25 min	43.12±1.56	59.36±1.36
	30 min	15.87±1.23	57.23±1.22

Table 3. Basic indexes of waste oil.

	Acid value (mg/g)	Saponification value (mg/g)	Peroxide value (meq/kg)	Water content (%)	Phospholipid content （%）
Before treatment	29.57	200.45	58.61	0.85	1.07
After treatment	36.34	196.94	30.37	0.07	0.68

Table 4. Fatty acid compositions of pretreated oil.

Fatty acid	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
content/%	0.82	3.21	8.71	58.89	14.20	2.23	1.26	0.67

It can be seen from Table 4 that the content of fatty acid of pretreated oil was about 90%, among which, saturated fatty acid accounted for 63.61% and the unsaturated fatty acid was 26.40%, which satisfied the requirements for the preparation of biodiesel.

Biodiesel preparation in cyclic reactors
(1) Effect of packing density of immobilized lipase
When the reaction solution flowed through the continuous ester exchange reactor at 0.5 mL/min, the filling density of immobilized lipase had a certain effect on the conversion rate shown in Figure 3.
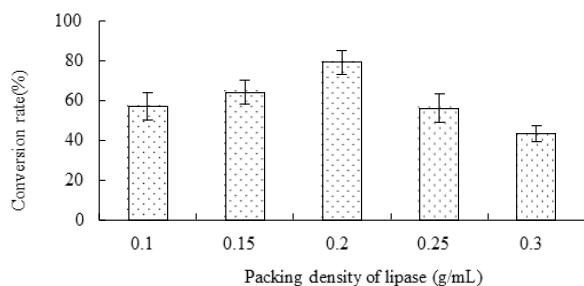

Figure 3. Effect of lipase packing density on biodiesel conversion.

It could be found in Figure 3 that the conversion rate of waste oil increased with the increasing of packing density when the packing density of lipase was small and reached the highest at the packing density of 0.2 g/mL. When the packing density of lipase increased further, the conversion rate of oil in the reactor decreased. That means oversize packing density of lipase could not increase the mass transfer effect of catalytic reaction and should lead to the decrease in the conversion rate because too much space was occupied by lipase, which led to the insufficient substrate [34]. As a result, the packing density of lipase in the reactor should be controlled at 0.2 g/mL.

(2) Effect of flow velocity of reaction liquor
Immobilized lipase was packed in the reactor with the density of 0.2 g/mL, and the volume flow of reaction fluid was adjusted by changing the speed of the peristaltic pump. The effect of flow velocity of reaction liquor on the esterification reaction is shown in Figure 4.

As shown in Figure 4, when the flow rate of the reaction fluid was 0.6 mL/min, the volume of oil

in the reactor remained at 20 mL. 1 hour was needed for a cycle of the reaction fluid, and the esterification rate of the waste oil reached the highest of about 80%. Lower conversion rate of biodiesel at lesser flow rate is perhaps because the fluid didn't reach fluidized state in the reactor, and the small space among immobilized lipase particles made it difficult for the substrate to fully contact with enzyme [35]. When the circulating velocity increased enough to suspend the enzyme in the reactor, lipase particles occupied the entire reactor volume and the space among lipase increased gradually, which led to more contact between the reactants and lipase, so the conversion rate increased gradually. If the reaction fluid flow continued to increase, the reactants should flow out of the reactor without sufficient reaction with the lipase, which in turn reduced the esterification rate [36].
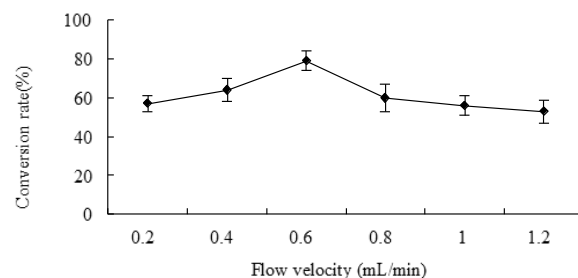

Figure 4. Effect of flow velocity on biodiesel conversion.

(3) Effect of reaction duration
Immobilized lipase was packed in the reactor with the density of 0.2 g/mL, and the volume flow of reaction fluid was adjusted around 0.6 mL/min. The effect of reaction time on the esterification reaction is shown in Figure 5.

As the extension of reaction time, more cycles of reaction fluid increased the contact between enzyme and substrate, and the catalytic efficiency of lipase was brought into full play to raise the conversion rate gradually. When the reaction time was extended from 3 h to 12 h, and the reaction liquid was circulated 12 times, the esterification rate increased to about 90%. Then

Table 5. Quality index of product compared with the standard of ethereal diesel oil in China and biodiesel in USA.

Quality index	ASTM6751-02	GB252-2000	Biodiesel produced by cyclic reaction
Acid value (mg KOH/g)	8	<0.9	2.75
Water content (10^{-6})	<500	Trace	432.6
Density (g/cm^3)	0.87-0.89	0.82-0.9	0.853
Content of free glycerine （%）	<0.02	-	0.014

Note: ASTM6751-02 is the biodiesel standard of USA, and GB252-2000 is the national standard of light diesel in China.

the esterification rate began to decrease after 12 h, perhaps too many products in the system led the reaction to the opposite direction.
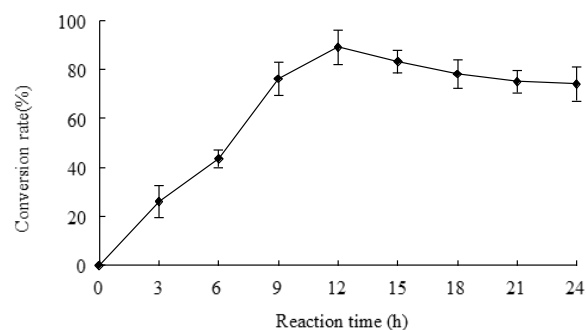
Figure 5. Effect of reaction time on biodiesel conversion.
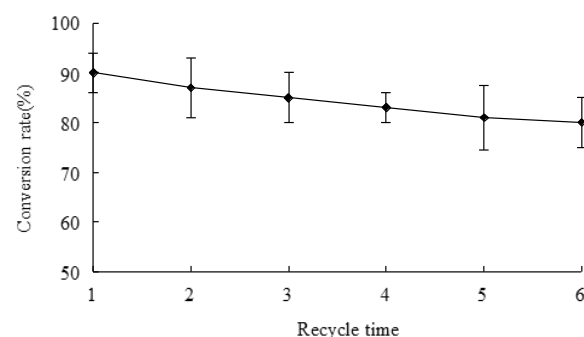
(4) Reuse of immobilized lipase
After the esterification reaction, acetone was added into the reactor to scour off residual oil and glycerin on the surface of immobilized lipase, and then a new batch of waste oil was flowed into reactor to continue reaction. The service life of the immobilized enzyme in circulation reactor was investigated using the esterification rate as an index. The results are shown in Figure 6.

With the increasing of catalytic times in circulating reactor, the conversion rate of biodiesel decreased slowly. After reused for 6 times, the conversion rate of waste oil could still reach more than 80%.

(5) Quality index of biodiesel
The density and water quality of biodiesel are related to the combustion and storage performance of oil products. It can be seen from

Table 5 that the water content and density of biodiesel produced by catalytic reaction all meet the standards of China's light diesel oil and USA's biodiesel, and the free glycerin content conformed to U.S. biodiesel standards [37]. However, the acid value of biodiesel is slightly higher than the standard, which is caused by the fact that biodiesel is only a crude product, which has not been refined and contains unreacted fatty acids [38]. During the using process of oil, too much free fatty acid could corrode oil nozzle, block the oil filter, and deposit around the nozzle. The oil refining could greatly reduce the acid value such as vacuum distillation and molecular distillation.

Figure 6. Effect of cycle times of immobilized lipase on biodiesel conversion.

Conclusions

Two-step processing of waste oil by hydrogen peroxide combined with mixed adsorbent could improve the quality of waste oil significantly and to meet the needs of biodiesel production. Transesterification reaction was catalyzed by immobilized lipase to produce biodiesel using

pretreated waste oil as materials in a self-designed circulation reactor, 90% conversion rate was received. The quality indexes of biodiesel conformed to the standard of light diesel oil in terms of the density, water and glycerol content of biodiesel, but the acid value of biodiesel was slightly higher that means further refining was needed for the product.

Acknowledgement

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