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# EVALUATION OF BIOTECHNOLOGICAL POTENTIALS OF SOME INDUSTRIAL FUNGI IN ECONOMICAL LIPID ACCUMULATION AND BIOFUEL PRODUCTION AS A FIELD OF USE

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□ *Considering the vast number of scientific reports on various potential uses of fungi, there was an attempt to select the best lipid producer of some fungi at optimized conditions (*Aspergillus versicolor, Rhizopus oryzae, Rhizopus arrhizus, Tramates versicolor*). The aim was to offer new fields of use to the industries already culturing and using such materials.* Aspergillus versicolor *mycelia were found to be accumulating the highest amount of lipids. Experiments to improve lipid accumulation and transesterification properties were performed in molasses medium; the first steps were testing the effects of different pH values and different nitrogen sources on lipid accumulation. Various concentrations of* $KNO_3$ *(0.5, 1.0, 1.5* $g\,L^{-1}$*) and molasses (6%, 8%, 10%) were tried in order to find the optimum carbon and nitrogen requirements. Maximum lipid content was 22.8% in the samples containing 6% molasses solution and 1.0* $g\,L^{-1}$ $KNO_3$ *at pH 4 after 10 days of incubation. The highest fatty acid ethyl ester yield of these samples was 77% (5.0 ethanol:oil, 0.4 sulfuric acid:oil at 30° C for 6 hr). Since the crude lipids were rich in C16 and C18 fatty acids, this was considered as suitable feedstock for biodiesel production.*

**Keywords** biodiesel, fungi, microbial lipid, molasses, transesterification

## INTRODUCTION

In today's world, petroleum-based fuels are decreasing day by day because of their rapid consumption. Besides their limited amount, their usage causes some problems to the environment.[1–3] In this context, researchers have focused on finding alternative energy sources. The biofuels derived from renewable biological sources are promising solutions to solve the increasing energy problems.[4–6] Especially in the biofuel sector, industrial biotechnology processes have some advantages, such as using molecular biology and synthetic biology methods compared to the chemical processes.[7]

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As is well known, biodiesel (mono-alkyl esters of long-chain fatty acids) is a promising alternative biofuel source because of its similarity to diesel fuel in properties.[8] Transesterification is the most popular way of converting oils to biodiesel.[9,10] The transesterification reaction is affected by reaction conditions such as molar ratio and type of alcohol, type and amount of catalysts used, reaction time, and temperature.[11]

The cost of biomass takes up a major part of the biodiesel production process. This is the main reason making commercialization of biodiesel production difficult,[12] which highlights the importance of selecting an appropriate feedstock for biodiesel production.[13]

It is known that the biodiesel industry competes with the food industry for raw materials such as rapeseed or sunflower seed for oil crops.[14] In this context, microbial oils are promising feedstocks for producing biodiesel, as relatively cheaper material with higher lipid content.[15] There is a vast literature on microbial oils produced by yeast, fungi, and algae.[16–22]

The usage of fungal oils as a raw material for biodiesel production did not receive its deserved place in the literature. Although these organisms grow rapidly even in the wastewaters, there are limited studies in the literature about usage of fungal lipids for biodiesel production.

This picture leads us to study the optimization of conditions to stimulate the selected fungi to synthesize and accumulate lipids that could be used in biodiesel production as efficiently as possible. The aim is to offer new fields of use to the industries already culturing and using such materials also as feedstock for biodiesel production. This application was selected as an example of promising fields of use of fungal lipids for the future at least, and experiments were designed to improve lipid accumulation and transesterification properties.

This work aims to suggest an economically competitive raw material for biodiesel production by using mycelia. After selection of the highest performing species in lipid production, further experiments were performed to study various key parameters of reaction conditions affecting the biodiesel production capacity of this species, as described in the following. Molasses was used as a carbon and energy source in the experiments to reduce the cost of lipid production. To our knowledge this is the unique report about investigation of key parameters and the lipid-accumulating properties of *Aspergillus versicolor* mycelia in the media containing molasses.

## MATERIALS AND METHODS

### Microorganism and Cultivation Conditions

To determine the lipid accumulation properties, four fungi strains (*Tramates versicolor, Aspergillus versicolor, Rhizopus oryzae, Rhizopus arrhizus*)

were used in the study. Among these strains, *A. versicolor* was selected for the further experiments due to its higher lipid content.

The strains were provided from the Ankara University Faculty of Science Laboratories culture collection.

The composition of the growth medium was molasses solution (approximately equivalent to $10\,g\,L^{-1}$ sucrose), $1.0\,g\,L^{-1}$ $NH_4SO_4$, and $0.5\,g\,L^{-1}$ $KH_2PO_4$.[23] All the chemicals used in the study were purchased from Merck.

Batch experiments were performed under aerobic conditions in an orbital shaker (New Brunswick Scientific Innova 4230, USA) at an agitation rate of 100 rpm and incubation temperature $30 \pm 1°C$.

## Lipid Extraction and FAME Analysis

Two different lipid extraction methods were used in the study. Fungal mycelia that were harvested at $4800 \times g$ for 15 min were used for lipid extraction. To calculate the lipid content of the fungus, mycelia were dried to constant weight in an oven at 80°C. Extraction of lipids from the biomass was performed to the procedure of Zhu et al.[24] Lipid was extracted with 2:1 chloroform–methanol solution. The total lipid was estimated by a gravimetric method.

For transesterification variables assays, the harvested biomass was washed with distilled water in order to remove molasses solution. Lipid extraction was performed with a Soxhlet extractor (Uniterm). Pure *n*-hexane (250 mL) was used to extract the lipid for 6 hr. After *n*-hexane was removed with a rotary evaporator (IKA RV10), extracted lipid was measured gravimetrically.[25]

## Lipid Accumulation Assays

To investigate the initial pH effect on the lipid accumulation of *A. versicolor*, the pH of the molasses medium was adjusted to 3, 4, 5, and 6 with dilute $H_2SO_4$ and NaOH solutions.

In order to examine the effect of different nitrogen sources on lipid accumulation, the cultures were grown in molasses media containing $1\,g\,L^{-1}$ $NH_4Cl$, $(NH_4)_2SO_4$, urea, $KNO_3$, and $NH_4NO_3$ at pH 4.

To determine the effect of increasing carbon and nitrogen amounts on lipid accumulation, the fungal mycelia were incubated in media containing 6, 8, and 10% molasses solution and increasing $KNO_3$ concentrations (0.5, 1.0, and $1.5\,g\,L^{-1}$) at pH 4.

## Assays on the Effects of Transesterification Variables

In order to examine the effect of catalyst type on the transesterification reaction, the microbial lipids obtained by Soxhlet extraction were reacted

with base (potassium hydroxide and sodium hydroxide) or acid (sulfuric acid) catalyst dissolved in methanol or ethanol.[27]

Alcohol:oil and catalyst:oil ratios ranging from 2.5 to 10.0 and from 0.2 to 0.8, respectively, were added to the reaction mixture in order to investigate the effects of these parameters on the transesterification reaction. To see the effect of reaction period on fatty acid methyl ester yield, 2, 6, and 24 hr of reaction times were tried.

Analysis of fatty acid profiles of transesterified microbial oils was performed by gas chromatography. After the transesterification step, a 1-μl sample was taken from the upper phase and the methylated fatty acids were analyzed by the GC-2010 gas chromatograph (GC; Shimadzu Japan). The condition of GC analysis was as follows: flame ionization detector (FID) 240°C; column TR-CN100, 60 m × 0.25 mm × 0.20 mm (Teknokroma); carrier gas $N_2$. Fatty acid peaks were identified against the chromatogram of a mixed fatty acid methyl ester standard (37 Comp. FAME Mi × 10 mg/ml in $CH_2Cl_2$; Supelco, USA).[26]

Each of these experiments and the measurements described in the following were performed in triplicate.

## RESULTS AND DISCUSSION

The lipid accumulation properties of the four fungal strains were investigated by incubating them in the molasses medium with pH 5 for 10 days. The lipid concentrations were observed as 7.8% (±0.4) for *Tramates versicolor;* 9.1% (±0.5) for *Aspergillus versicolor*; 8.1% (±0.3) for *Rhizopus oryzae*; and 7.3% (±0.5) for *Rhizopus arrhizus*. Among these fungal strains, *A. versicolor* mycelia were found to be capable of accumulating higher amounts of microbial lipids in their mycelia. That is why *A. versicolor* was used for further lipid accumulation and transesterification reaction assays in the present study.

### Effect of Initial pH on Fungal Lipid Accumulation

To see the effect of the growth medium's pH value on lipid accumulation, the experiments were performed at pH 3, 4, 5, and 6 in molasses medium by incubating them for 8 days. *Aspergillus versicolor* could not grow when the medial pH value was 3, but succeeded in accumulating 7.9% (±0.4) and 4.3% (±0.2) lipid content at pH 5 and pH 6, respectively. At pH 4 the fungus showed its maximum lipid accumulation as 9.7% (±0.5). Lipid accumulation by *A. versicolor* mycelia has been found to be significantly affected by the changes in pH values, as expected.

In the previous papers, it was also shown that the lipid production capacity was affected by the pH, where the optimum pH values were found to be between 4.0 and 6.0.[12,28,29]

## Effect of Different Nitrogen Sources on Fungal Lipid Accumulation

Five different nitrogen sources ($NH_4Cl$, $(NH_4)_2SO_4$, urea, $KNO_3$, and $NH_4NO_3$) were tested in the molasses medium to investigate the effect of nitrogen source on the lipid production yield of *A. versicolor*. The initial nitrogen concentration was $1.0\,g\,L^{-1}$ for all of the nitrogen sources tried.

It is seen in Figure 1 that the lipid production yield of *A. versicolor* was similar for each experiment when the molasses medium was supplemented with 9.7% $(NH_4)_2SO_4$ and 10.2% $NH_4NO_3$. The lipid production yield of the fungal mycelia showed an increase up to 12.8% when $NH_4Cl$ was present in the medium. The highest lipid production yield was 17.0% in the medium containing $KNO_3$ at the end of 8 days of incubation at pH 4. The lowest lipid accumulation was seen as 6.9% in the urea-containing samples.

There are many papers in the literature on the effects of $(NH_4)_2SO_4$ and yeast extract regarding lipid accumulation in different fungal strains. In a study about oil production by *Mortierella isabellina* and *Cunninghamella echinulata* single cells, $0.5\,g\,L^{-1}$ yeast extract was used as a nitrogen source.[30] In another study, $0.5\,g\,L^{-1}$ initial concentrations of $(NH_4)_2SO_4$ and yeast extract were used for the lipid accumulation of *A. niger*.[28] As the highest lipid accumulation was found in the presence of $KNO_3$ here, it was used to determine the most favorable concentration of nitrogen source in the medium.

## Effect of Initial Molasses and $KNO_3$ Concentrations on Lipid Accumulation

To reduce the cost of microbial oil production, molasses was used as a carbon and energy source, and increasing concentrations of $KNO_3$ were added to the media containing 6%, 8%, and 10% molasses solutions in

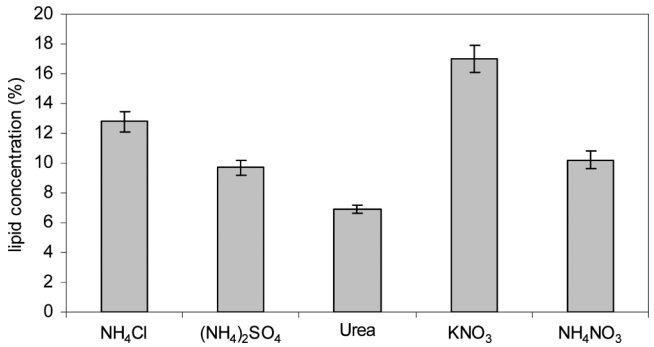


**FIGURE 1** The effect of different nitrogen sources on the lipid concentration of (%) *A. versicolor* mycelia (T: $30 \pm 1°C$; stirring rate: 100 rpm; $1\,g\,L^{-1}$).

order to find the optimum molasses and nitrogen quantities for lipid accumulation.

The results in Table 1 depict that the lowest fungal lipid accumulation (14.8%) was seen in the medium with 10% molasses solution and 0.5 g $L^{-1}$ $KNO_3$. However, the lipid accumulation was found to be higher (16.4%) when the $KNO_3$ concentration was 1.0 g $L^{-1}$. On the other hand, the lipid yields were close to each other in the media with 8% molasses and increasing $KNO_3$ concentrations. The table shows that higher lipid yields were measured in the media containing 6% molasses solution. The fungal lipid concentration was 18.2% in the medium with 0.5 g $L^{-1}$ $KNO_3$ and 6% molasses solution. The highest lipid accumulation yield was observed as 20.6% in the medium containing 1.0 g $L^{-1}$ $KNO_3$ and 6% molasses solution. It was seen that the lipid accumulation capacity was decreased when the initial nitrogen concentration was increased from 1.0 g $L^{-1}$ to 1.5 g $L^{-1}$ $KNO_3$. The same trend was also observed for the other molasses concentrations tried.

To investigate the effect of incubation time on lipid accumulation, *A. versicolor* was incubated for 4, 6, 8, and 10 days. The lowest lipid accumulation yield was observed as 18.6% at day 4 of incubation time. The lipid concentrations of the fungal mycelia were 21.0%, 21.6%, and 22.8% at 6, 8, and 10 days of incubation, respectively.

In the literature, researchers described some studies carried out on microbial lipid accumulation by cultivating the fungal mycelia on different carbon sources. In a study on *Mucor circinelloides*, researchers used 10 g $L^{-1}$ glucose in the growth medium.[12] In another study it was found that *Cunninghamella echinulata* could increase its lipid content from 7.1% to 25.6% when the carbon/nitrogen ratio was increased from 78 to 285.[30] The lipid accumulation yields were found as 19.9%, 19.0%, and 15.3% when chloroform:methanol, chloroform:methanol:water, and *n*-hexane lipid extraction solutions were used, respectively.[12] In our present study, the

**TABLE 1** The Effect of Initial $KNO_3$ and Molasses Concentration on the Lipid Concentration of (%) *A. versicolor* (T: 30 ± 1°C; Stirring Rate: 100 rpm; pH: 4)

| Molasses Solution (mL) | $KNO_3$ (g $L^{-1}$) | Lipid Concentration (%) |
|---|---|---|
| 6% | 0.5 | 18.2 ± 0.4 |
| | 1.0 | 20.6 ± 0.8 |
| | 1.5 | 15.4 ± 0.3 |
| 8% | 0.5 | 17.2 ± 0.5 |
| | 1.0 | 17.6 ± 0.4 |
| | 1.5 | 16.8 ± 0.7 |
| 10% | 0.5 | 14.8 ± 0.5 |
| | 1.0 | 16.4 ± 0.3 |
| | 1.5 | 15.4 ± 0.2 |

highest lipid amount of *A. versicolor* was found as 22.8% when the chloroform:methanol lipid extraction system was used.

## Effects of Variables on Transesterification Reaction

In the present study different kinds of key variables (catalyst and alcohol type, the amount of catalyst and alcohol, and the reaction time) were tried to improve the yield of transesterification reaction. To examine such effects of catalyst and alcohol types and their quantities on the reaction, acidic (sulfuric acid), and basic (potassium or sodium hydroxide) catalysts were used in the presence of methanol or ethanol.

Table 2 shows the effect of different catalyst and alcohol types and their quantities on the conversion yields of transesterification of *A. versicolor* lipids. The data depict that the highest fatty acid ethyl ester yield was obtained as 77% at the presence of following parameters: 5.0 ethanol:oil, 0.4 sulfuric acid:oil, incubated at 30°C for 6 hr. It is seen in the table that increasing sulfuric acid and ethanol did not increase the ethyl ester yield. The fatty acid methyl ester yields were close to each other when basic catalysts were used in the presence of methanol or ethanol.

## Lipid Composition of the Fungal Mycelia

Fatty acid profiles of *A. versicolor* lipids extracted with the Soxhlet system were analyzed by gas chromatography technique. Figure 2 shows the distribution of C16 and C18 fatty acid ethyl esters fungal lipids at the highest conversion yield of transesterification in the presence of $H_2SO_4$. The ethyl ester contents in the reaction mixture indicated that C16 and C18 fatty acids

**TABLE 2** Effect of Different Catalyst and Alcohol Amounts on C16–C18 FAME Yields of *A. versicolor* Lipids (30°C, 200 rpm Magnetic Stirring, 6hr)

| | Methanol | | | Ethanol | | |
|---|---|---|---|---|---|---|
| | Catalyst:oil | Alcohol:oil | %FAME | Catalyst:oil | Alcohol:oil | %FAME |
| KOH | 0.2 | 2.5 | $55 \pm 3.4$ | 0.2 | 2.5 | $52 \pm 2.8$ |
| | 0.4 | 5.0 | $32 \pm 2.1$ | 0.4 | 5.0 | $52 \pm 2.3$ |
| | 0.6 | 7.5 | $28 \pm 1.7$ | 0.6 | 7.5 | $50 \pm 1.7$ |
| | 0.8 | 10.0 | $21 \pm 1.3$ | 0.8 | 10.0 | $46 \pm 1.5$ |
| NaOH | 0.2 | 2.5 | $50 \pm 2.8$ | 0.2 | 2.5 | $48 \pm 2.0$ |
| | 0.4 | 5.0 | $52 \pm 3.1$ | 0.4 | 5.0 | $47 \pm 1.5$ |
| | 0.6 | 7.5 | $41 \pm 2.2$ | 0.6 | 7.5 | $47 \pm 1.7$ |
| | 0.8 | 10.0 | $55 \pm 3.7$ | 0.8 | 10.0 | $31 \pm 1.1$ |
| $H_2SO_4$ | 0.2 | 2.5 | $41 \pm 2.6$ | 0.2 | 2.5 | $53 \pm 2.7$ |
| | 0.4 | 5.0 | $32 \pm 2.7$ | 0.4 | 5.0 | $77 \pm 3.9$ |
| | 0.6 | 7.5 | $34 \pm 2.3$ | 0.6 | 7.5 | $56 \pm 2.2$ |
| | 0.8 | 10.0 | $46 \pm 2.9$ | 0.8 | 10.0 | $57 \pm 2.6$ |

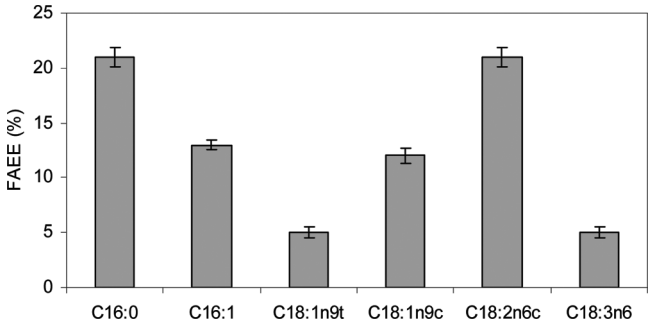


**FIGURE 2** Distribution of C16 and C18 fatty acid ethyl esters of *A. versicolor* mycelia (30°C, 200 rpm magnetic stirring, 6 hr).

dominated the crude lipid compounds. Palmitic acid (C16:0) and linoleic acid (C18:2n6c) were found at 21% in *A. versicolor* lipids. Our results showed there were in fungi lipids no methylated fatty acids with four or more double bonds, which are known to be limiting the biodiesel production.

## CONCLUSIONS

It is important to improve the variables in transesterification reaction for the development of biodiesel usage. However, there are limited numbers of studies focusing on the optimization of variables affecting chemical transesterification of microbial oils. In this sense the results obtained from the current work contribute to the literature.

The variables affecting transesterification reaction of fungal lipids were investigated in the present study. The highest C16 and C18 ethyl ester yields were found as 77% for *A. versicolor* in the presence of acid catalyst with 6 hr of reaction time. Furthermore, waste molasses could be used as a cheap and efficient feedstock for microbial lipid production.

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