J. Int. Environmental Application & Science, Vol. 15(2): 135-140 (2020)

Research Paper

Improvement of Fungal Oil Production from Apple Processing Industry Wastewater

H. Duygu BİLGEN*, Süreyya DÖŞLÜ ÇETİNKAYA

Mersin University, Engineering Faculty, Department of Environmental Engineering, 33343, Mersin, TURKEY

Received April 04, 2019; Accepted June 12, 2020

Abstract: Turkey has an important place in the world ranking in terms of apple production area and apple processing products. During the process, apple processing industries produce high amounts of fruit washing water with high Chemical Oxygen Demand (COD) and acidic properties, which are highly suitable for use as a fungal substrate. It is also known that fungal oils produced by oleaginous fungi from wastewater is also suitable to produce low-cost biodiesel as an alternative fuel. In this study apple processing industry wastewater was used as an alternative substrate for *Mucor circinelloides* and the C/N/P ratio was changed to improve fungal lipid production. Maximum oil content of dry fungal biomass was 17.7 %. Composition of fatty acids from the fungal oil were also analyzed and gas chromatography analyses showed that the major fatty acid was oleic acid (C18:1, 31.62 %) which is very suitable for biodiesel production. Results of the study indicated that fungal oil produced from the low-cost substrate apple processing industry wastewater can be useful as an alternative source for different industries especially in the bio-energy for the future.

Keywords: Apple processing industry, wastewater, fatty acid, fungal oil, *Mucor circinelloides*, value-addition.

Introduction

Rapid population growth and rapid industrial development have led to an increase in new food products and agricultural practices, and consequently the rapid depletion of natural resources. Therefore, the increase of industrial and agricultural wastes and the decrease of fossil fuels constitute the most important environmental problems (Owusu & Asumadu-Sarkodie, 2016). To minimize the environmental damages of these wastes, on the other hand, to find alternative energy sources is the focus of recent researches. Biofuels, on the other hand, are an alternative fuel source for reducing dependence on fossil fuels. Biofuels have many advantages, such as energy independence, reduction of greenhouse gas emissions and economic sustainability. It is known that the oils of various plants are used in the production of biodiesel which is a type of biofuel. However, the use of primary foods and favorable agricultural land for biodiesel production has recently led to controversy.

It is known that microbial oils (single cell oils) can be produced by many microorganisms and these oils have a very high potential as a raw material for biodiesel production. In addition, microorganisms are not affected by changing seasonal and climatic conditions, having a high amount of oil content in the cells. Microorganisms can be produced in large quantities in a short time compared to plants, which makes biodiesel production superior to microbial oils (Xue, 2006). In recent years, oil accumulating fungi have been seen as a preferred raw material for the sustainable biodiesel industry (Sankaran *et. al*., 2010; Khot *et al*, 2012; Bhanja *et.al*., 2014; Amoozegar; 2019).

Among the oil-producing fungi, the genus *Mucor* is of great biotechnological importance since they can accumulate high levels of triacylglycerol in the mycelium. Concerning lipid-producing *Mucor spp*, several previous studies in the literature have reported that *Mucor circinelloides* offer polyunsaturated fatty acids-rich lipids that can be used as alternative raw materials to obtain biodiesel (Annie, 2018).

The production of new food products and fruit juices from fruits leads to the formation of by-products or waste water containing high amounts of organic matter. Ucar *et al*. (2016) reported that the demand for apple products increases gradually both in the Turkey and the world. Countries with highest production of apples include China (49.10%), United States (5.05%), Turkey (3.87%), Poland (3.82%), and Italy (2.74%). Because of suitable climate, apple production in Turkey increases year by year resulting in significant amount of wastewater production from their processing industries. It is not possible to discharge this wastewater into the receiving environment without treatment, representing a significant

* *Corresponding: E-Mail: hduygubilgen@gmail.com; Tel: +903243610001/7102 Fax: +903243610032*

financial burden on industries. However, due to the presence of polysaccharides, nitrogen, phosphorus, various minerals and organic acids, it is possible to convert this wastewater into a suitable medium for the growth of fungi. The utilization of wastewater compounds by fungi can result in wastewater sufficiently clean to meet the discharge and/or recycling criteria while simultaneously increasing biomass yield and oil composition, the extent of which depending on the environmental conditions and species selected (Puyol *et al*., 2017).

There are various studies on the use of fungal oils in biodiesel production. However, the use of industrial waste streams as a substrate for the production of fungal oil for subsequent biodiesel production has been limited.

Materials and methods

Wastewater supply and analysis

In this study, wastewater of apple processing industry (Anadolu Etap Agriculture and Food Products Industry) located in Mersin, Turkey was used as a substrate in fungal oil production.

Chemical Oxygen Demand (COD), pH, conductivity and Total Nitrogen (TN) analyzes were performed in order to characterize the wastewater supplied. TN analyzes were performed with Hach brand LCK138 nitrogen kits with a measuring range of 1-16 mg / L. COD analyzes were performed both with Hach brand LCK514 kits with a measuring range of 100-2000 mg / L and standard methods (5220 C) (Standard Methods, 2005). Total Phosphate (TPO_4^{3-}) analyzes were performed according to the phosphorolybdenum blue method with Hach brand LCK348 test kits. All analyzes performed for the characterization of wastewater were carried out in 3 replicates (Table1).

Renewal and inoculation of fungal cultures

0.1mL of *M. circinelloides* spore stocks in glycerin stored at -85 ^{o}C were used as the inoculum to inoculation of Potato Dextrose Agar (PDA) plates at aseptic conditions and incubated at 31 ^{0}C for 5 days (Mitra et al., 2012). At the end of the incubation period, agar fragments were cut to about 1 cm^2 from the solid culture of the fungi and inoculated into flasks containing sterilized Potato Dextrose broth. The flasks were incubated for 2 days in an orbital shaking incubator at 31 ^{o}C and 150 rpm.

Incubated *M. circinelloides* biomass in the flasks was separated from the liquid phase by filtration under sterile conditions and washed twice with sterile isotonic serum (0.09% NaCl solution). Therefore, the carbon source that is likely to come from the pre-culture medium is removed. Then the fungal biomass was rinsed with sterilized de-ionized water to remove any salt from the NaCl solution. The washed fungal biomass was placed into 250 mL Erlenmeyer flask containing 100 mL of sterile deionized water and homogenized under sterile conditions for 135 seconds at 13500 rpm to produce fungal inoculation solution for oil production experiments.

Determination of fungal biomass and oil yields

Homogenized *M. circinelloides* mycelium suspensions were then oven dried at 80 ^{o}C for 24 h. All of the experiments were performed in triplicate. Yields and coefficients were determined according to equations below (Carota et al., 2017; Chan et al., 2018):

The biomass yield was expressed as grams of dry biomass per liter of Apple Processing Industry Wastewater (g/L). Oil yield (ΔP) was calculated according to Eq. (1) (g/L):

$$\Delta P = \Delta X \times (C_L/100) \quad \ldots\ldots(1)$$

where ΔX is the biomass yield (g/L) and C_L is the intracellular oil content (%).

Optimization of growth and oil production conditions

Optimization of carbon /nitrogen /phosphorus (C/N/P) ratios of wastewater was investigated in order to increase fungal oil production. For this purpose, wastewater has been prepared in three different C/N/P ratios, 100/18/1, 100/10/1 and 100/5/1. Urea, D-Glucose, K_2HPO_4 and KH_2PO_4 were added to provide necessary ratios. The pH of wastewaters with different C/N/P content was then adjusted to 6.00, the optimum pH of *M. circinelloides* fungi, by addition of 1 M NaOH solution. Wastewaters with different C/N/P ratios were added to the 4L volume reactor and autoclaved with glass reactor at 121 ^{o}C for 15 minutes.

After sterilization, 100 mL of homogenized fungal biomass was inoculated into the reactor under aseptic conditions and incubated for 48 hours at 31 ºC, 200rpm agitation and the addition of oxygen from the bottom of the reactor with a diffuser. At the end of the incubation period, fungal biomass was harvested from wastewater and oven dried 60 ºC for 24 hours.

Intracellular fungal-oil extraction using high-power ultrasonication

The oven-dried fungal cells were subjected to ultrasonication for 30 min with a maximum power output of 400W and frequency of 40 kHz (Mercury) in the presence of toluene and methanol (1:1, v:v) to disrupt the cell wall of fungus and extraction of the intracellular oil (Christi et al, 2010; Mitra et al., 2012). After the ultrasonic bath, the biomass was filtered (whatman no. 1) into the flask and washed with methanol-chloroform (2:1, v:v) solutions. The flasks were then kept in an oven at 60 ºC until the organic solvents became volatile. After that step, the flasks were kept in desiccator for 30 minutes and the extracted oils were weighed (Mitra et al., 2012).

Analytical methods for fatty acid analysis of fungal oil

Fatty acid profiles of microbial oils were performed by a Agilent 7890A gas chromatograph equipped with HP Innowax column (30 m length, 0.25 mm internal diameter, 0,25µm film thickness) and a flame ionization detector. The operating conditions were as follows: detector temperature at 330 ºC and initial oven temperature at 140 ºC held for 5 min then raised to 250 ºC at a rate of 10 ºC /min, then held at 250 ºC for 15 min. Carrier gas was helium (20 cm/sec), injection mode 1 µl, 280 ºC, split 100:1. Chromatographic peaks and retention times were identified by the comparison to a fatty acid methyl ester (FAME) standard mixture and individual peaks were quantified by means of external standards and their corresponding calibration curves.

Conclusion

Determination of optimum C/N/ P ratio of wastewater

According to the results of the determination of dry fungal biomass, 1 mL homogenized mycelial suspension contained 9.32 g/L fungal dry mass. In the studies to determine the optimum C/N/P content of the wastewater, it was observed that the development of mycelium was favored when using a C/N/P ratio of 100/10/1.

In regards to the biomass yield and composition, the amount of wet and dry fungal biomass harvested from wastewater with different C/N/P ratio and the mass of the oil extracted from the dry fungal biomass are given in Table 2. Picture depicting the various biomass oil yields are shown in Figure1.

Figure 1. Oils from fungal biomass developed in wastewater with different C/N/P ratios (left:100/5/1; middle:100/10/1; right:100/18/1).

As described in Table 2, the highest oil production yield (17.7%) was achieved with the use of wastewater with a 100/10/1 ratio. Previous studies indicated that the fungal oil yield of the oleaginous fungi growth in different substrates were obtained between 12% and 24% by different researchers. Vicente et al. (2009), investigated the effects of temperature, pH and solvents used for extraction on fungal oil yield and reported yield values ranging from 15.3% to 19.9%. Chan et al. (2018) reported that *M. circinelloides* oil yield reached 24% of dry biomass and 2.20 g/L whey permeate, at 168 h incubation period.

Table 1. Characterization of apple processing industry wastewater

Chemical Oxygen Demand (COD)	**2400 mg/L**
Total Nitrogen (TN)	428 mg/L
Total Phosphate (TPO_4^{3-})	2.47 mg/L
pH	4.70
Temperature	23°C
Suspended solid	740 mg/L

Table 2. Wet and dry fungal biomass harvested from wastewater with different C/N/P ratio and amount of obtained fungal oil.

Wastewater with different C/N/P ratio	**Wet Fungal Biomass (g/L)**	**Biomass Yield (ΔX)**	**Oil yield (ΔP)**	**C_L (%)**
100/5/1	184.180	7.898	0.408	5.17
100/10/1	330.406	17.072	3.015	**17.67**
100/18/1	165.386	10.609	0.698	6.58

The oil yield observed in our study is in agreement with the values reports in the literature using other substrates, although is below the maximum value reported. However, apple processing wastewater is considered as a good alternative for producing single cell oil due to its availability and low cost. The process herein developed can be further scaled up to pilot-and industrial-scale.

Fatty acid profile of fungal oil

The chromatogram of the fungal oil obtained from *M. circinelloides* growth in apple processing industry wastewater shown in Figure 2. Fatty acid types and contents (%) of the obtained oil are also given in Table 3.

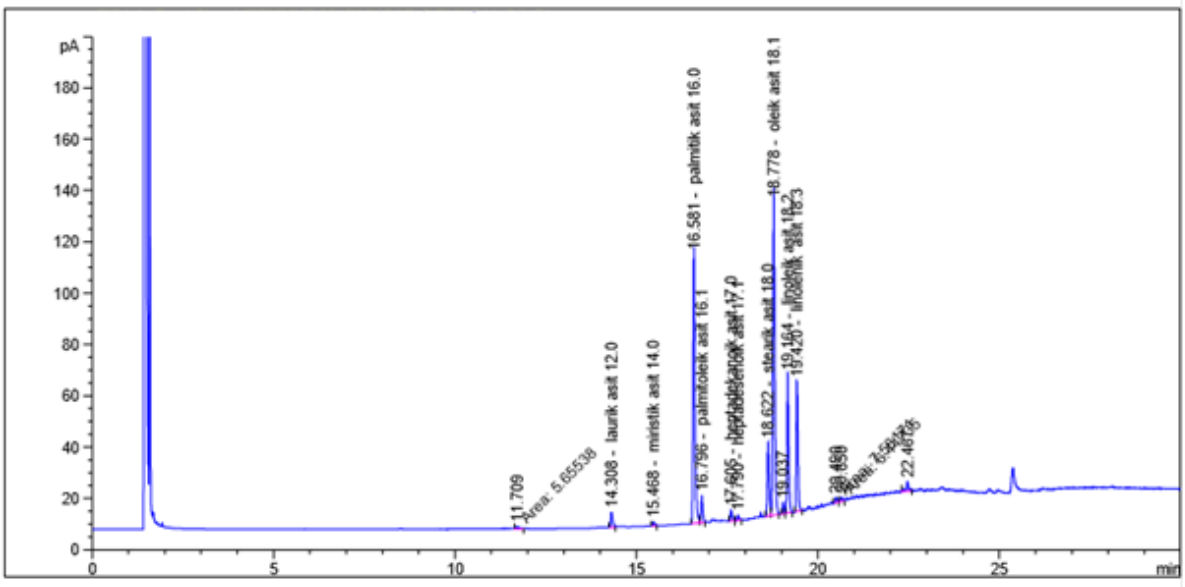

Figure 2. Fatty acid profile of fungal oil obtained from *M. circinelloides* growth in apple processing wastewater.

When the fungal lipids are examined, it is known that the most common linolenic acid (C18: 3) is the precursor of omega-6 fatty acids. Linoleic acid is converted to gamma linolenic acid (GLA), an important food supplement in the body. Therefore, the fungal biomass is known to be a rich source of gamma linolenic acid (Ratledge, 2004).

Table 3. Fatty acid composition and contents (%) of the fungal oil obtained from the cultivation of *M. circinelloides* in Apple Processing Wastewater.

Fatty acid types	Fatty Acid Standard Number	Content (%)
Oleic acid	C18:1	31.62
Palmitic acid	C16:0	27.74
Linoleic acid	C18:2	12.24
Linolenic acid	C18:3	11.64
Stearic acid	C18:0	7.34
Palmitoleic acid	C16:1	2.34
Lauric acid	C12:0	1.53
Heptadecanoic acid	C17:0	1.07

The high content of oleic acid which is one of the monounsaturated fatty acids of the fungal oil obtained in the study shows that this oil can be a preferred substrate for biodiesel production (Durett et. al., 2008; Sit-up et al., 2013; Sitepu et al., 2014). Moreover, linolenic acid (11.64 %), was also reported to have beneficial effects for the prevention and treatment of inflammatory disorders, diabetes, cardio-vascular disorders, cancers and some other diseases (Horobbin, 1992; Fan and Chapkin, 1998; Lu and Zhu, 2015; Kim et al., 2012).

Acknowledgement: *This study is part of a research project (2017-2-AP3-2387) funded by Mersin University, Department of Scientific Research Projects. The authors would like to thank Anadolu Etap Agriculture and Food Products Industry for providing wastewater.*

References

Amoozegar MA, Safarpour A, Noghabi KA, Bakhtiary T, Ventosa A, (2019) Halophiles and Their Vast Potential in Biofuel Production, *Front. Microbiol.* **10,**1895, 1-17.

Annie L, Laurence MC, Stéphanie MS, Emmanuel C. Jean-Luc J, Georges B, Erwan C, (2019) Comparative analysis of five Mucor species transcriptomes, *Genomics*. **111**, 1306-1314.

Bhanja A, Minde G, Magdum S, Kalyanraman V, (2014) Comparative Studies of Oleaginous Fungal Strains (*Mucor circinelloides* and *Trichoderma reesei*) for Effective Wastewater Treatment and Bio-Oil Production", *Biotech. Res. Int*., **2014**, 1-7.

Carota E, Crognale S, D'Annibale A, Gallo AM, Stazi SR, Petruccioli M, (2017) A sustainable use of ricotta cheese whey for microbial biodiesel production, *Sci Total Environ*., **584**,554–560.

Chan GL, Cohen LJ, Ozturk G, Hannebelle M, Taha AY, de Moura Bell JMLN, (2018) Bioconversion of cheese whey permeate into fungal oil by *Mucor circinelloides*, *J. Biol. Engin.,* **12**:25, 1-14.

Christi WW, Han X, (2010) Lipid Analysis- Isolation, Separation, Identification and Lipidomic Analysis, The Oily Press Lipid Library, **24**, eBook ISBN**:** 9780857097866.

Durrett TP, Benning C, Ohlroogee J, (2008) Plant triacylglycerols as feedstocks for the production of biofuels, *Plant J.*, **54**, 593-607.

Fan YY, Chapkin RS, (1998) Importance of dietary gamma-linolenic acid in human health and nutrition. *J. Nutr.*. **128**, 1411–1414.

Horrobin DF, (1992) Nutritional and medical importance of gamma-linolenic acid, *Prog Lipid Res*. **31**, 163–194.

Khot M, Kamat S, Zinjarde S, Pant A. Chopade B, RaviKumar A, (2012) Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel, *Microbial Cell Factories*, **11**, 71-84.

Kim DH, Yoo TH, Lee SH, Kang HY, Nam BY, Kwak SJ, Kim JK, Park JT, Han SH, Kang SW, (2012) Gamma linolenic acid exerts anti-inflammatory and anti-fibrotic effects in diabetic nephropathy, *Yonsei Med J*. **53**, 1165–1175.

Lu H, Zhu Y, (2015) Screening and molecular identification of overproducing gamma-linolenic acid fungi and cloning the delta 6-desaturase gene, *Biotech.l Appl Biochem*. **62**, 316–322.

Mitra D, Rasmussen ML, Chand P, Chintareddy VR, Yao L, Grewell D, Verkade JG, Wang T, van Leeuwen JH, (2012) Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus *Mucor circinelloides*, *Biore. Tech*. **107**, 368–375.

Owusu PA, Asumadu-Sarkodie S, (2016) A review of renewable energy sources, sustainability issues and climate change mitigation, *Cogent Engin.*, **3**, 1-14.

Puyol D, Batstone DJ, Hülsen T, Astals S, Peces M, Krömer JO, (2017) Resource Recovery from Wastewater by Biological Technologies: Opportunities, Challenges, and Prospects, *Front. Microbiol*. **7**, 1-23.

Ratledge C, (2004) Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production, *Biochimie*. **86**, 807–815.

Sankaran S, Khanal SK, Jasti N, Jin B, Pometto AL, Van Leeuwen JH, (2010) Use of filamentous fungi for wastewater treatment and production of high value fungal byproducts: a review, *Cri. Rev. in Environ. Sci. & Tech.*, **40**, 400–449.

Standard Methods for the Examination of Water and Wastewater, (2005) American Public Health Association/ American Water Works Association/Water Environment Federation (APHA/AWWA/WEF), 21st Washington DC, USA.

Sitepu RI, Jin M, Fernandez JE, da Costa Sousa L, Balan V, Boundy-Mills KL, (2014) Identification of oleaginous yeast strains able to accumulate high intracellular lipids when cultivated in alkaline pretreated corn stover, *Appl. Microbio. Biotech*. **98**, 7645-7657.

Sitepu RI, Sestric R, Ignata L, Levin D, German JB, Gillies LA, Amanda LAG, Boundy-Miles KL, (2013) Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast, *Biore. Tech*. **144**, 360-369.

Uçar K, Engindeniz S, Markovic T, Kokot Z, (2016) Analysis of Changes in Apple Production in Turkey, In: 27th International Scientific-Expert Congress of Agriculture and Food Industry, pp:147-151, Bursa-Turkey.

Vicente G, Bautista LF, Rodríguez R, Gutiérrez FJ, Sádaba I, Ruiz-Vázquez RM, Torres-Martínez S, Garre V, (2009) Biodiesel production from biomass of an oleaginous fungus, *Biochem. Eng. J*. **48**, 22–27.

Xue F, Zhang X, Luo H, Tan T, (2006) A new method for preparing raw material for biodiesel production, *Process Biochem*. **41**, 1699-1702.