Fungal Oil Production from Oleaginous Fungi *Mucor circinelloides* and *Aspergillus oryzae* Cultivated on Sugar Beet Pulp

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Abstract

Fungal oil production from *Mucor circinelloides* and *Aspergillus oryzae* grown on sugar beet pulp was investigated. The average annual production of sugar in Turkey is ca. 3.1 million metric tons. Sugar beet pulp is one of the byproducts of sugar production and it is possible to use this byproduct as a substrate for cultivating beneficial fungi. Oleaginous fungi can accumulate high level of lipids, which is a promising alternative oil for producing biodiesel. *M. circinelloides and A. oryzae* were inoculated into sugar beet pulp and incubated during 13 days. At the end of the incubation, fungal biomass was harvested from the pulp and fungal oil extracted. The oil content was about 25 and 4% of dried biomass in *M. circinelloides* and *A. oryzae*, respectively. High biomass yield showed that sugar beet pulp was a good substrate to cultivate *M. circinelloides.* Although oil yield was low compared with other substrates in literature, sugar beet pulp can be a good alternative raw material to produce fungal oil because of ready availability and low cost.

Keywords: *Aspergillus oryzae*, Fungal oil, *Mucor circinelloides*, Oleaginous fungi, Sugar beet pulp

1. Introduction

High energy prices and concerns about petroleum supplies triggered searches to find renewable biofuels. One of the most promising renewable biofuels is biodiesel, which is produced from vegetable oils, animal fats or waste cooking oils [1, 2].

The global biodiesel production has increased to 18.2 billion liters per year from 2000 to 2010 [1, 3]. Currently, the major source of biodiesel is vegetable oils, with 95% of biodiesel produced from plant oils [4]. The global biodiesel market growth, inspired many researchers to investigate the resource cost of biodiesel. According to Van Gerpen (2004), when plant oil is used for biodiesel production, the raw material accounts for 70–85% of the whole production cost [5, 6]. The use of plant oils competes with the food industry. Therefore, to lower the cost of oil raw materials, much attention has been paid to the development of microbial oils [1].

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Microbial oils (also called single-cell oils), especially triacylglycerols (TAGs), which are the main materials for biodiesel production, can be produced from oleaginous microorganisms. Oleaginous microbes can accumulate more than 20% of their dry mass as oil inside their cells when grown in certain environmental conditions [7, 8]. It has been found that many microorganisms, such as bacteria, microalgae, and fungi, including yeasts. The advantages include abundant and cheap raw materials, no competition with food industry, rapid growth, independence from season and climate, and easier to scale up [9, 10, 11]. Recently, many researches have focused on the use of oleaginous microorganisms for biodiesel production. Among the oleaginous microorganisms, *Mucor circinelloides* accumulates some of the highest levels of lipids in its mycelia [6]. More significantly, its oil has been found to be rich in linolenic acid, which has a growing market demand due to its high value as a nutraceutical [12]. *Aspergillus oryzae* have also been found able to accumulate lipid up to 12–25% of biomass [4, 13, 14]

The most important difficulty for microbial oil production is the high cost of raw materials. It is estimated that 70% of the total production cost goes to raw materials. Thus, to be economically feasible utilization of cheap materials needs to be explored [4]. Various low-cost substrates have been studied for microbial lipid production such as corn-ethanol stillage, cassava starch hydrolysate and rice straw, sweet potato stillage [15], rice hull, sorghum biodiesel derived glycerol [4] and wastewater [16].

Sugar beet pulp (SBP), a major byproduct of the sugar refining industry, is a potential raw material for microbial oil production. Turkey has a total production capacity of sugar of 3.1 million metric tons per year[17]. SBP contains 20–25% cellulose, 25–36% hemicellulose, 20–25% pectin, 10–15% protein, and 1–2% lignin on a dry weight basis [18].

The work described here focused on fungal oil production by *Mucor circinelloides* and *Aspergillus oryzae* cultivated on SBP. The aim of this study was to convert this abundant low-value coproduct to lipid using oleaginous fungi.

2. Materials and Methods

2.1. Sugar Beet Pulp

Sugar beet pulp (SBP) was obtained from a sugar refining plant in Turkey. SBP was dried at 80 ^{0}C for 2 hours, granulated (Fig 1) and stored in sterile 5 L carboys at 4 ^{0}C prior to use.

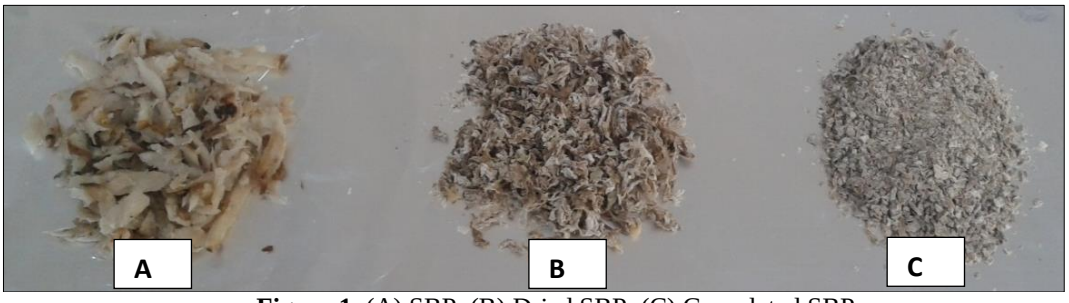

Figure 1. (A) SBP, (B) Dried SBP, (C) Granulated SBP

2.2. Fungal strains, media and inoculum preparation

A lyophilized culture of *M. circinelloides* f. lusitanicus CBS 277.49 was obtained from Iowa State University's fungal research group, Iowa State University, USA. *A. oryzae* were obtained from Environmental Biotechnology Laboratory, Environmental Engineering Department, Mersin University, Turkey. The cultures were conserved in glycerin at 4 ºC. Also, the strains were maintained in potato-dextrose agar (PDA; Merck) at 4 ºC and transferred every 20 d.

M. circinelloides was incubated in Petri dishes which contain potato dextrose agar (PDA) (Merck) at 30 ^{0}C until white sprulation was observed. *A. oryzae* was incubated in PDA at 37 ^{0}C [19] for 7 days. At the end of the incubation, *M. circinelloides* and *A. oryzae* were inoculated into flasks containing Yeast Malt (YM) extract broth (Sigma-Aldrich) shaken at 150 rpm, and incubated at 30 ºC and 37ºC, respectively, during 5-7 days until a dense growth of mycelia was observed in the broth. At the end of the incubation, each flask was filtered, washed twice with sterilized 0,009% NaCl (Sigma-Aldrich) solution and washed twice with sterilized distilled water. Washed biomass was homogenized at 13500 rpm for 30 seconds for use as inoculum in suspended solid experiments.

For further studies, carbon-deficient basal medium [20] was prepared in pH 5.0 phosphate buffer and contained, as micronutrients, 0.3 g/L $MgSO_4.7H_2O$; 0.3 g/L $CaCl_2.2H_2O$; 0.005 g/L $FeSO_4.7H_2O$; 0.0016 g/L $MnSO_4.2H_2O$; 0.0014 g/L $ZnSO_4.7H_2O$. This media is referred to as basal mineral medium (BMM) in the rest of study.

2.3.Fungal biomass calculation

Homogenized *M. circinelloides* and *A. oryzae* mycelium suspensions were then oven dried at 80 ^{0}C for 24 h. All of the experiments were performed in triplicate.

2.4.Fermentation experiment preparation

Suspended solid-state fermentation (SuSF) experiment was performed by adding, 10 g SBP and 80 mL carbon deficient BMM to 250 mL flasks, respectively. The bottles were sterilized at 121 ^{0}C for 20 min. and each bottle was inoculated with different amounts of homogenized mycelial suspension of *M. circinelloides* (5 mL and 10 mL) and *A. oryzae* (5 mL and 10 mL).

Control experiments were performed in 250 mL flasks. The first flask had 10 g SBP and 80 mL carbon deficient BMM added. Second and third flasks had 80 mL carbon deficient BMM only added. For the purpose of providing the same condition with SuSF experiments, all control flasks were sterilized at 121 ^{0}C for 20 min. After sterilization, the second of each three flasks thus prepared was inoculated with 5 mL *M. circinelloides* and 5 mL *A. oryzae* mycelium suspension.

All essays were performed in triplicate. The experimental flasks inoculated with *M. circinelloides* and *A. oryzae* were incubated at 30 ^{0}C and 37 ^{0}C during 13 days, respectively. The experiments are summarized in Table 1.

Table 1: Content of SuSF experiments

	Flask No	Inoculated *M. circinelloides* (10,3 ± 1,50 g dry mass/L) (mL)	Inoculated A. oryzae (6,6 ± 1,50 g dry mass/L) (mL)	Amount of SBP (g)	BMM (mL)
Suspended State Fermentation (SuSF)	1	5	-	10	80
	2	10	-	10	80
	3	-	5	10	80
	4	-	10	10	80
Controls	5	-	-	10	80
	6	5	-	-	80
	7	-	5	-	80
	8	-	-	-	80

2.5. Lipid extraction

At the end of the incubation, SuSF experiment flasks were filtered and SBP were separated from the fungi. All SBP obtained from experimental flasks was washed twice with distilled water and dried at 80^0C for 24 h and equilibrated to room temperature in a desiccator before weighing to determine dried biomass yield.

The extraction was performed with 1.0 g dried biomass in a glass vessel containing methanol and toluene at a 1:1 (v/v) ratio [16]. The extraction was assisted by a vortex in three steps of 10 min each. After vortexing, further extraction with methanol and chloroform at a 2:1 (v/v) ratio and 1.0 g of dried biomass was filtered through Whatman No 1 filter paper. The total lipids extracted by this technique were measured gravimetrically after the complete removal of the organic solvents by evaporation at 60 ^{0}C in a water bath.

3. Results

3.1. Fungal biomass calculation

At the end of the 24 h, homogenized *M. circinelloides* and *A. oryzae* equilibrated in a desiccator to room temperature and measured gravimetrically**.** The mycelium yield of *M. circinelloides* and *A. oryzae* was 10,3± 1,50 g and 6,6± 1,50 g dry-weight mass, respectively.

3.2. Fungal growth

During the incubation period, *M. circinelloides* and *A. oryzae* grown on SBP was used to cover the surface of all SuSF (Figure 2). It indicated that SBP was used as a carbon source by the *M. circinelloides* and *A. oryzae*.

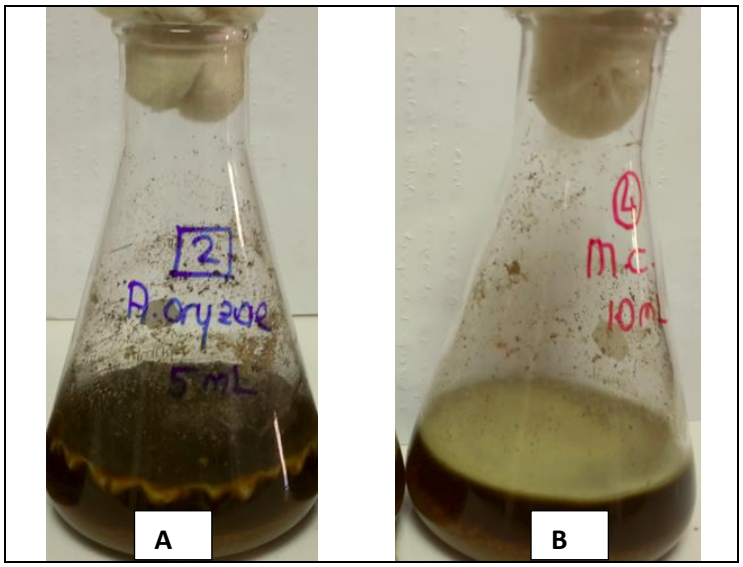

Figure 2. (A) Growing *A. oryzae* on SBP (Flask no 3); (B) Growing *M. circinelloides* on SBP (Flask no 2)

During the incubation period, *M. circinelloides* and *A. oryzae* didn't grow in control flasks 6 and 7, respectively (Figure 3). It supported that BMM didn't contain any carbon source.

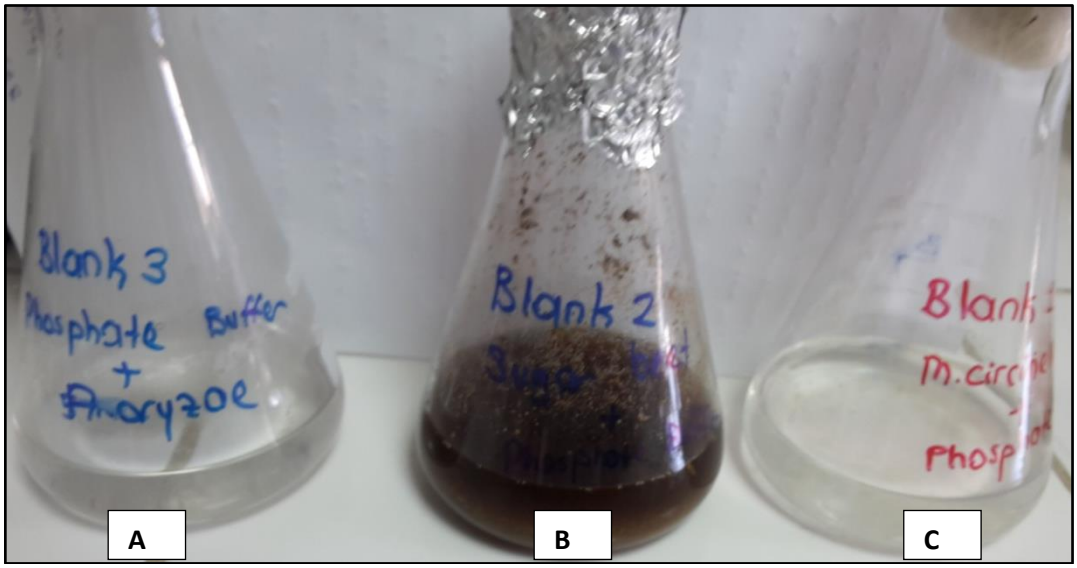

Figure 3. (A) *A. oryza*e in control flask no 7; (B) Control flask no 8; (C) *M. circinelloides* in control flask no 6

3.3. Lipid extraction

For the lipid extraction, separated *M. circinelloides* and *A. oryzae* from the SBP are shown in Figure 4. Also, dried fungal biomass of *M. circinelloides* and *A. oryzae* are shown in Figure 5.

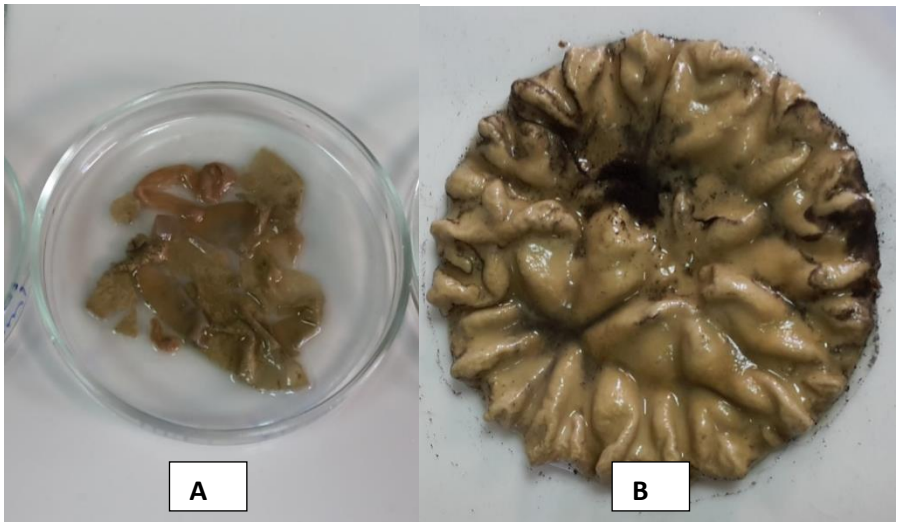

Figure 4. (A) Separated *M. circinelloides*; (B) Separated *A. oryzae* from SBP

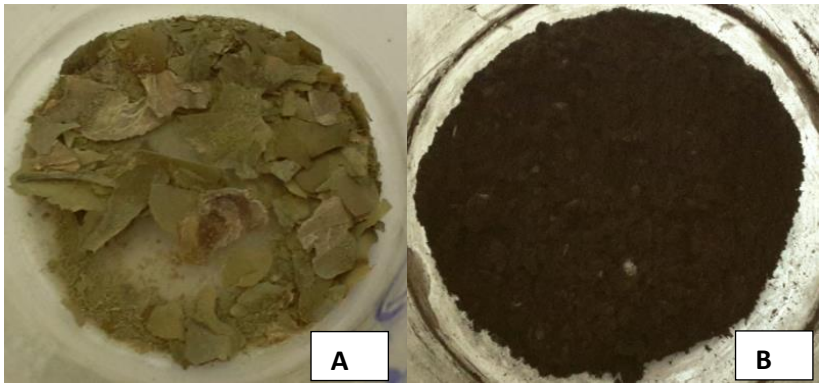

Figure 5. Dried fungal biomass of (A) *M. circinelloides*; (B) *A. oryzae*

The oil content in the dried *M. circinelloides* and *A. oryzae* fungal biomass are shown in Table 2. As expected, no lipid was detected by the extraction method on the control flasks.

Table 2. Oil content of dried fungal biomass

	Flask No	g oil / 100 g fungal biomass	Content of fungal biomass
Suspended State Fermentation (SuSF)	1	25,6	5 mL *M. circinelloides*
	2	24,3	10 mL *M. circinelloides*
	3	4,52	5 mL *A. oryzae*
	4	3,67	10 mL *A. oryzae*

4. Discussion

The total oil obtained was about 25% of dried biomass of *M. circinelloides* and about 4% of dried biomass of *A. oryzae* after 13 days of incubation. This study shows *M. circinelloides* and *A. oryzae* accumulated lipids within 13 days of fermentation, indicating that SBP can be utilized to produce single cell oils. Also, when grown on SBP under the same culture conditions, the oil content of *M. circinelloides* dried fungal biomass was higher than *A. oryzae*. Also, different amounts of fungal inoculum did not lead to any significant increase/decrease in the oil content.

Conclusion

M. circinelloides and *A. oryzae* were investigated as a potential feedstock for fungal oil production in suspended solid-state fermentation of SBP. SBP could be a potential alternative raw material for microbial lipid production using *M. circinelloides*. The biodiesel quality is dependent on the fatty acid profile of the oil used as feedstock for its production. Therefore, for the production of biodiesel, the lipid obtained should be analyzed and compared with standard specifications for renewable diesel. This strategy for suspended solid-state fermentation would be a useful tool for direct conversion of lignocellulosic materials into valuable products including fungal lipids. This would also greatly contribute toward the economics of biofuel production from lignocellulosic biomass.

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