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Single Cell Oil Production from Petroleum Sludge by Native Yeast Strains

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ABSTRACT

In this research, 11 yeast strains with ability to grow on petroleum sludge were isolated from effluent of a petroleum refinery. Based on growth on mineral media contaminated petroleum sludge, two isolates were selected as the super strains. Meanwhile, results based on biochemical and morphological experiments on the strains indicated that the two selected isolates belonged to Candida and Prototheca genus. Optimization with Taguchi Statistical Method (TSM) indicated that appropriate conditions for both isolates considering sludge concentrations, nitrogen source, pH, temperature and shaking rate (rpm) are equal to 10%, 2 g/l sodium nitrate, 6.5, 25°C and 190 rpm, respectively. Biomass production in optimal growing conditions for Candida and Prototheca were 1.54 g/l and 2.3 g/l, respectively. Gas Chromatography analyses of extracted fatty acids from supernatants and surface portions after methyl-esterifies with methanol: KOH solvents, indicated that content quantity of fatty acids on the surface was more than other portions and mainly in the forms of 16 and 18 saturated carbons and in the forms of palmitic acid and stearic acid. Therefore, these isolates can be used for recycling of petroleum sludge in production of yeast biomass and cell oil.

1. INTRODUCTION

Nowadays, nutritional and medicinal importance of fatty acids and their applications in health products, has found a special altitude [1]. Since, plant sources of fatty acids and production of oily grains always has been faced with many problems such as climate changings, fluctuation of agronomy soil, water shortage and entire removal of products, therefore microbial oils are now considered as promising feedstock because of their similar fatty acid composition to that of vegetable oils [2,3]. In 1980, Verkooyen and Rietema reported that 40 to 50% of member of Candida genus were able to utilize n-Alkanes as a carbon and energy source [4]. Candida sp. LEB-M3 yeast was isolated from a Brazilian biome which showed capacity to accumulate up to 55% lipids (w/w) and convert about 43% of glycerol into lipids. Recently in industry, yeasts strains in the form of Saccharomyces cerevisiae, Cryptococcus curvatus, and Hansenula kioechera have been utilized in order to produce stearic acid, ergosterol and precursor of steroid, respectively [5]. Some oleaginous yeast strain such as Rhodosporidium sp. can accumulate intracellular lipids to level exceeding 70% of their biomass under nutrient limitation condition [6, 7]. Tanimura et al. used oleaginous yeasts with high lipid productivity for biodiesel production [8]. Other studies demonstrated that yeasts strains if growing on Alkanes, are able to produce fatty acids of 12 to 18 carbons depending on different ambient conditions and available amounts of food sources [9]. The utilization of microbial lipids for biodiesel production is economically feasible only if oleaginous microbes are cultured with inexpensive substrates (e.g. the byproducts or wastes of other processes) in processes yielding high lipid and biomass concentration and exhibiting adequate yield and productivity. The costs of lipid production currently is relatively high and therefore, importantly, searching inexpensive carbon sources, which are nutritionally rich enough to support the growth of the yeast as well as the production of lipid is quite necessary. In this research, the role of petroleum sludge as an inexpensive substrate and waste in production of lipid by native strain of yeast was studied. Eventually, the TSM as an optimizing method was used for optimization of the nutrient inhibitor and booster conditions.

2. MATERIAS AND METHODS

2.1. Materials and Microorganisms All needed materials for microorganisms culturing were purchased

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from Merck (Germany). Mineral salts with high purity were supplied from Sigma Aldrich (St. Louis, Mo, USA) and used without any purification. Yeast strain Yarrowia lipolytica ATCC 20255 for comparison of experiments was prepared from the Microbiology Lab, Faculty of Medical Science, Tarbiat Modares University in Tehran. In addition, petroleum sludge sample was prepared from oil Refinery of Shazand-Iran.

2.2. Enrichment and Isolation Conditions

To enrichment and isolate yeast strains, petroleum sludge was added by 10% w/v to 500 ml Erlenmeyer flask containing 90 ml mineral salty media with compositions of 1.94 g/l NH4Cl, 0.24 g/l NaHPO4, 0.01 g/l CaCl2, 0.01 g/l FeSO4, 0.05 g/l KCL, 0.2 g/l MgSO4, 0.3 g/l NaCl, 1 ml trace elements(0.4g CuSO4 5H2O, 0.4g ZnCl2,0.14g MnCl2 4H2O, 100 mg FeCl3 6H2O, 60mg H3BO3, 40 mg Na2MoO4 2H2O in1litter of deionised water) and 10 g/l sterilized glucose (pass through a 0.22um filter), placed in a shaker-incubator at 30°C and 150 rpm for 2 weeks according to Chunli reports [10]. In order to isolate yeast strains, 1 ml from the above media was cultured in surface of SDA (Sabouraud Dextrose Agar) plate containing 50 µg/ml chloramphenicol antibiotic and incubated at 30 °C for 48 h. To select super strain based on biomass formation, at first, the isolates were inoculated in mineral salty media culture containing petroleum sludge without glucose at similar conditions and incubated in shaker-incubator at 30 °C for 48 h. Then, the produced biomass was separated by centrifuging 8000 g for 10 min using Universal 16R-MSE model, and dried (under vacuum at 45 °C for 12h) and weighted by digital balance Sartorius Mettler AC100 model.

2.3. Conditions of Fatty Acids Production

In order to achieve producing conditions of fatty acids by two super yeast strains selected, two loop full of colony of each purified isolate were inoculated in 100 ml Erlenmeyer flasks containing 20 ml mineral media culture and 5 ml sterilized petroleum sludge (sterilized in an oven at 180 °C for 2 h). The Flasks comprising pre-cultures media with petroleum sludge re-degraded placed in the shaker-incubator device at 30°C with 150 rpm for 24h. To produce desired fatty acids, 10 ml of 24h inoculated yeast strains in the pre-culturing media was added to 500 ml Erlenmeyer flasks containing 100 ml same pre-culturing media and 10% sterilized petroleum sludge, and was grown at 30 °C in an incubator shaker at a shaking speed 150 rpm for 48h. Then, the separation of solid phase from liquid phase was carried out by discharging of the flasks contents after 48h incubation and then centrifuging with 8000 g for 10 min. Finally, the produced fatty acids were truly determined using Gas Chromatography [11].

2.4. Extraction of Surface Cellular Fatty Acids

To remove petroleum sludge and other compounds in media culture from surface of yeast cells, the biomass was washed three times with physiology serum and then placed into methanol: hexane (1:1) organic solvent at room temperature for 24h until both organic and liquid phases was completely separated from the other constituents. The organic phase was used to methylate fatty acid to make sure the death of cells without being degraded. Importantly, the prepared suspension in organic solvent has undergone the microscopic experiments and culturing in SDA medium simultaneously [12].

2.5. Extraction of Inter Cellular Fatty Acids

After three times washing the biomass produced from culturing with physiology serum, 10 ml ammonium chloride (0.25M) was added to 6 gram of extracted biomass and then kept in70 °C for 15 min. After cooling, 60 ml chloroform: methanol (2:1) mixture was added to the biomass at room temperature and ultimately the organic phase was used for methylation. Meanwhile, the methanol: KOH solution was used to convert fatty acids to their methylated derivative.

2.6. Extraction and Gas Chromatography of the Fatty Acids Methyl Ester To separate methylated fatty acids from other impurities, 2ml n-hexane solvent was added to the obtained solution in methylation process and vibrated (vortex) for 2 min until two phases were separated from each other. Also, for complete removal of the methyl ester fatty acids from organic phase (hexane), separation process was repeated three times. The sampled organic phase was initially dehydrated with anhydrous sodium sulphate and passed through the filter paper having 0.45 µm diameter pores. In the following, for identification, 1 µl of resulted organic phase was injected to the column of gas chromatography (GC-17A Shimadzu) with a column type of CBP5 with 25m height and 0.22mm diameter. The Injection temperature, detector temperature (FID), primary and secondary temperature were selected as 200, 50, 250, 250°C respectively. Moreover, other parameters including, rate of flow, column pressure and linear velocity content were adjusted to 20 cm/s, 74 kpa and 16 ml/min respectively. The type of obtained fatty acids from outer surface of the biomass was determined using GC-mass.

2.7. Identification of Isolates Relative identification of the isolates was performed based on their morphological and biochemical characteristics including its microscopic shape, filamentous growing cells products, ability to grow at 37 °C, growth in high concentrations of D-glucose, growth in the presence of

cycloheximide. Other features of the resulted isolates were identified by Rap ID Yeast Plus System kit [13].

2.8. Optimization with Different Parameter and Taguchi Method Taguchi method for optimization of the parameters was followed. The selection of the factors is based on the most critical parameters that can strongly affect the production of yeast biomass for two selected isolates (temperature from 23 to 32 °C, pH in range 2.0 to 7.0, sodium nitrate in range 1 to 2.5 g/l, shaking: 90 to 200 rpm, petroleum sludge concentrations: 0 to 50% g/l). For each factor, the experiments were carried out in triplicates. Means were compared and analysed by t-test. Differences were considered statistically significant for $p<0.05$. After designing experiments in the mentioned ranges and interpreting of results with QT4 software, the optimum production conditions for each isolate was determined.

3. RESULTS AND DISCUSION

3.1. Microorganisms Selection Following the sampling, purification and culturing in SDA medium containing chloramphenicol antibiotic, the eleven effective yeast isolates were drawn out from petroleum sludge of the Shazand-Iran oil refinery and were completely purified. Since the necessity of extraction of the cell surface fatty acids is to achieve greater biomass, thus two isolates under the names PM1 and PM2 with the highest biomass were selected as super strains in the production of fatty acids among the eleven isolates. So, all identification experiments, optimization of the medium conditions and extraction of the desired fatty acids were only carried out hereafter on the selected two super strains. Initial identification based on biochemical experiments and according to instructions of Rap ID Yeast Plus System kit indicated that two effective isolates PM1 and PM2 belonged to genus of Candida and Prototheca respectively (Table 1).

3.2. Effect of Different Parameters on the Biomass Production The effect of some effective parameters on the growth rate of the desired isolates was successfully investigated and the results are shown in Figure 1(a-f). At first, the effect of temperature on the growth rate of isolates was surveyed in the range of 23 to 32 °C. As can be clearly seen in Figure 1-a. maximum growth rate was observed at 25 °C and decrease by 50% at 30 °C. Therefore, 25 °C would be the best temperature for growth of isolates with the highest rate. In addition, the optimal pH effective in the growth rate was quested in the range of 2.0 to 7.0 and the results indicated that increasing pH to 6 causes the growth rate to increase. However, with more increasing of the pH, undesirable outcomes are achieved (Figure 1-b). Papanikolaou et al. reported that lipid accumulation during primary anabolic growth in Yarrowia lipolytica was critically influenced by the medium pH and the incubation temperature [14].

TABLE 1. Identification experiments carried out by kit of Rap ID Yeast Plus System and other biochemical experiments.

Exp. No.	Experiments Name	PM1	PM2
1	GLU(Glucose)	+	+
2	MAL(Maltose)	+	–
3	SUC(Sucrose)	+	–
4	TRE(Trehalose)	–	–
5	RAF (Raffinose)	+	–
6	LIP(Fatty acid ester)	–	–
7	NAGA(p- Nitrophenyl-N-acetyl-B,D-galactosaminide)	–	–
8	αGLU9(p-Nitrophenyl-α,D-glucoside)	–	–
9	βGLU(p-Nitrophenyl-β,D-glucoside)	–	–
10	ONPG(p-Nitrophenyl-β,D-galactoside)	–	–
11	αGAL(p-Nitrophenyl-α,D-galactoside)	–	–
12	βFUC(p-Nitrophenyl-β,D-fucoside)	–	–
13	PHS (p- Nitrophenyl phosphate)	+	–
14	PCHO (p-Nitrophenyl phosphorylcholine)	–	–
15	URE (Urea)	–	–
16	PRO (Praline-β-naphthyamide)	–	–
17	HIST(Histidine β-naphthylamide)	–	+
18	LGY(leucyl-glycine β-naphthylamide)	–	–
19	Production of filamentous growing cells	+	+
20	Ability to growth at 37 °C	+	+
21	Growth in high levels of D-glucose	+	+
22	Growth in presence of cycloheximide	+	+

Between utilized inorganic and organic nitrogen sources which included nitrate, ammonium, peptone and urea, the isolates PM1 and PM2 showed higher growth rate in the presence of 2 g/l peptone and sodium nitrate as

indicated in Figure 1-c. but sodium nitrate was selected as the optimum nitrogen source because of the low cost and its availability compared to other materials. It is known that lipid production requires a medium with an excess of sugars and limited other nutrients, usually nitrogen [15].

Higher lipid yield and cellular lipid content were observed when inorganic nitrogen sources were used compared with organic [16]. The lipid content increased from 18% (w/w) with NH+4-grown cells to 52% (w/w) after 90 h growth of Rhodosporidium toruloides CBS [17]. It was found from the results that to isolates PM1 and PM2, the optimum concentration of the sodium nitrate was 2 gL−1, a concentration in which the growth rate of the isolates will be maximum (Figure 1-d). Also, a comparison of aeration results indicated that aeration by shaking at 190 rpm had the highest effect on growth rate and this effect for the both isolates was the same (Figure 1-e). In addition, investigation of effect of petroleum sludge concentrations from 0 to 50% by volume of culture medium indicated that, maximum biomass production of isolates PM1 and PM2 were observed in 10% and 20% respectively (Figure 1-f).

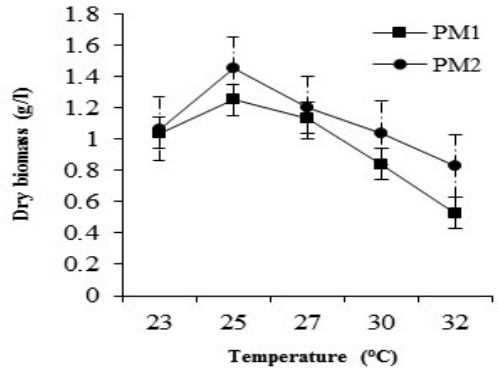

Figure 1-a. Influence of different temperature on dry biomass production of strainsPM1 and PM2 in mineral media containing sludge after 48h incubation in 25 °C. Error Bar indicates the significant difference of P<0.05.

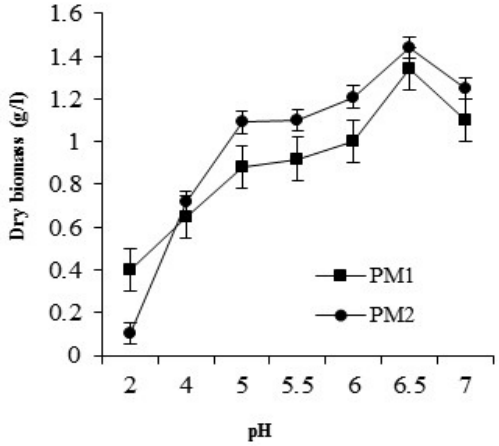

Figure 1-b. Influence of pH of media on dry biomass production of strainsPM1 and PM2 in mineral media containing sludge after 48h incubation in 25 °C.

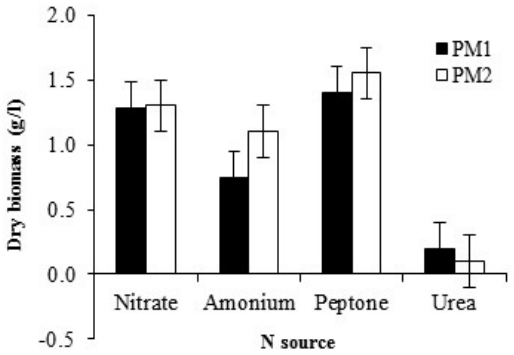

Figure 1-c. Influence of different source of nitrogen on dry biomass production of strainsPM1 and PM2 in mineral media containing sludge after 48h incubation in 25 °C.

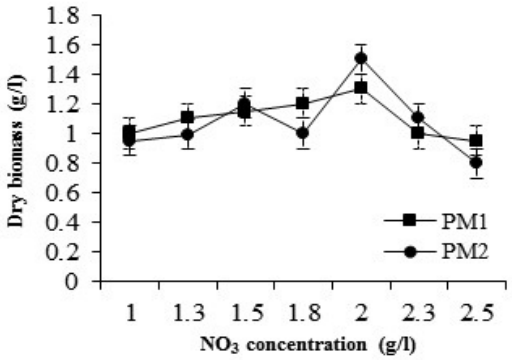

Figure 1-d. Influence of different concentration of sodium nitrate on dry biomass production of strains PM1 and PM2 in mineral media culture containing sludge after 48h incubation in 25 °C.

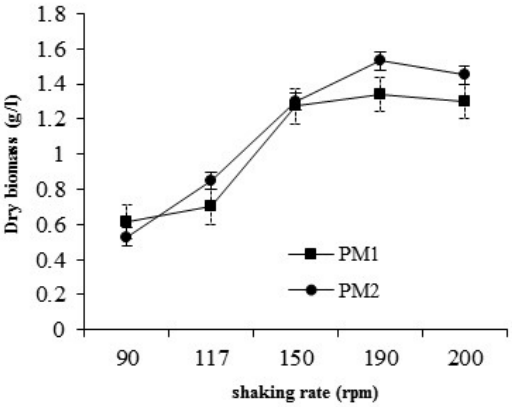

Figure 1-e. Influence of shaking rate on dry biomass production of Strain PM1 and PM2 in mineral media culture containing sludge after 48h incubation in 25 °C.

The growth conditions of mineral medium containing petroleum sludge and sludge-free medium containing glucose after 48 h incubation, for both isolate PM1and PM1 were 1.54 g/l and 2.3 g/l respectively. Similar results were also observed in biomass of T. globosa YU5/2 when several types of agro carbon source tested in nitrogen – limiting medium with pH 5.0 at 30 °C for 10 days incubation [17].

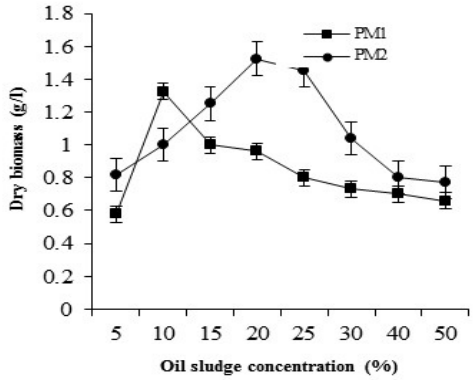

Figure 1-f. Influence of oil sludge concentration on dry biomass production of Strain PM1 and PM2 in mineral media culture after 48h incubation in 25 °C.

3.3. Optimization Results The suggested conditions by the QT4 software for maximum production efficiency achievement are 2 and 1.5 g/l sodium nitrate as a nitrogen source, 10% and 20% petroleum sludge, pH of 6 and 5.5 for isolate PM1 and PM2 respectively. Hartwig et al. reported thatculture conditions are important on lipid production in Candida sp. LEB-M3 when glycerol was used as carbon source [18]. After fulfilment of the optimum conditions suggested by the Taguchi method, the content of produced biomass by isolate PM1and PM2 was 1.54 g L- and 2.3 g L- respectively.

3.4. Growth Curve The growth curves concern to isolates PM1, PM2 was indicated in Figure 2. and 3. The results indicated that, there are a negligible difference between growth of yeast isolates based on petroleum sludge and glucose (data not shown).

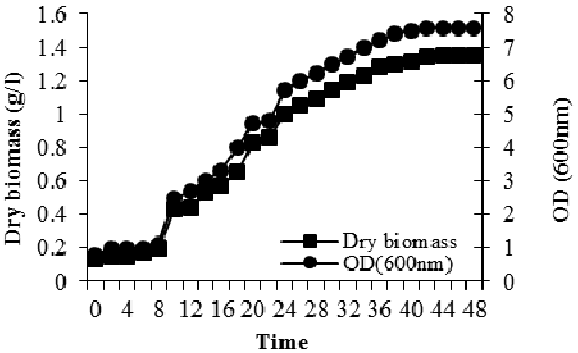

Figure 2. Obtained Growth curve of the isolate *PM1* based on optical density (OD) and cellular dry weight in mineral media culture containing petroleum sludge.

3.5. Fatty Acids Analysis The chromatography results of concentrated solution of each isolate in specific neutral polar and non-polar fatty acid solvents in comparison with control sample movement indicated that, neither fatty acids are secreted out from chromatography column nor identification peak has appeared on the TLC paper.

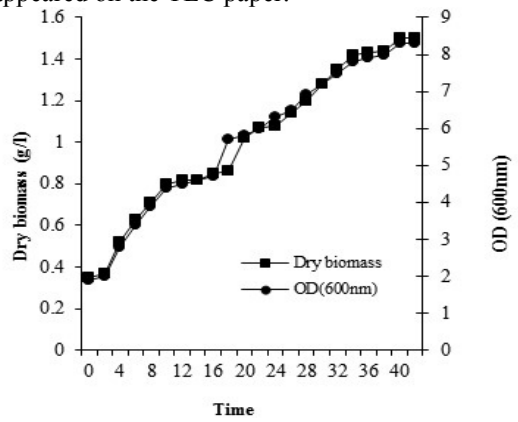

Figure 3. Obtained Growth curve of the isolate PM2 based on optical density (OD) and cellular dry weight in mineral media culture containing petroleum sludge.

Moreover, GC analysis of leaching solution resulted from cellular surface of isolates PM1and PM2 showed that saturated 16 and 18 carbon fatty acids exist on the extra surface of cellular membrane (Figure 4a,b). Yarrowia lipolytica, when cultivated on mixtures of free fatty acid substrates was found to remove C12:0, C14:0, (Delta9) C18:1, (Delta9,12) C18:2 and (Delta9,12,15) C18:3 at significantly higher rates than C16:0 and C18:0, regardless of fatty acid composition of the initial substrate [14]. Studies on lipid accumulation during primary anabolic growth indicated that this was influenced by the medium pH and the incubation temperature, was independent of the nitrogen concentration in the culture medium, but was favored at a high carbon substrate level and at a low aeration rate [19]. Also, the results showed that both growth rate and fatty acids production on mineral medium containing petroleum sludge were more than medium containing glucose, indicating the significance of petroleum waste as renewable resource in these types of bioconversions.

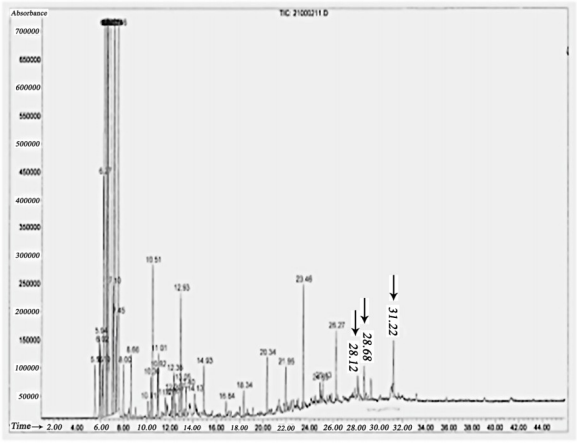

Figure 4 a. GC spectrums of extra surface fatty acids isolate PM1. Denoted peaks by inverted arrows indicate extra surface fatty acids.

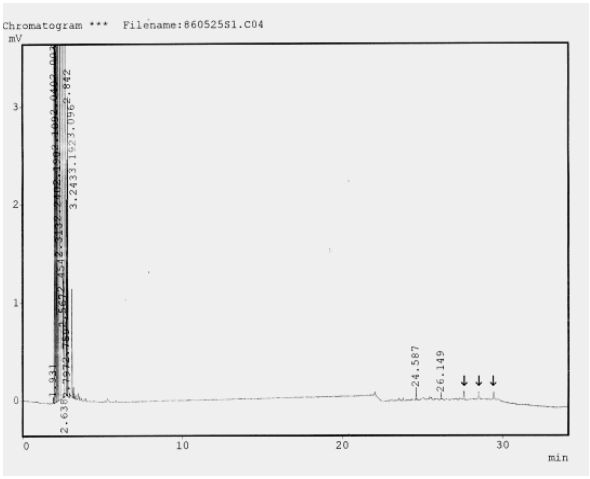

Figure 4 b. GC spectrums of extra surface fatty acids isolatePM2. Denoted peaks by inverted arrows indicate extra surface fatty acids.

4. CONCLUSION

Biochemical and morphological experiments indicated that the two selected isolates belonged to Candida and Prototheca genus. Optimization with Taguchi statistical method indicated that appropriate conditions for both isolates considering sludge concentrations, nitrogen source, pH, temperature and shaking rate (rpm) are equal to 10%, 2 g/l sodium nitrate, 6.5, 25 °C and 190 rpm, respectively. Content of biomass production in optimal growth conditions 1) in mineral media containing sludge and 2) sludge-free media containing glucose, were similar after 48 h incubation. These values for PM1 and PM2 were 1.54 g/l and 2.3 g/l respectively. Gas Chromatography analyses of extracted fatty acids from surface and intracellular portions indicated that content of fatty acids in surface section was more than other sections and mainly are in the forms of 16 (Palmitic acid) and 18 saturated (Stearic acid). As a conclusion, saturated acids could be alternatively produced from petroleum sludge.

5. ACKNOWLEDGMENT

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