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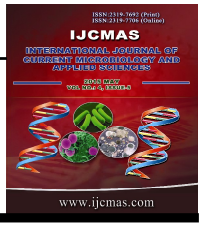

Original Research Article

Biodiversity of oleaginous microorganisms in the Lebanese environment

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Keywords

Oleaginous microorganisms, Single cell oils, Biodiversity, Lebanese environment

A B S T R A C T

Oleaginous microorganisms, including bacteria, yeasts and filamentous fungi, are single cell oil (SCO) producers which are capable of accumulating intracellular lipids under certain culture conditions. Few reports were evaluated on the biodiversity of the oleaginous microorganisms in different habitats. Lebanon is a mountainous country with variability in climatic conditions among seasons, soils and vegetation. In the present work a screening study was evaluated on the biodiversity of the oleaginous microorganisms in Lebanese habitats. Thirty-nine isolate SCO producers were selected from different habitats mainly: wetland, lawn, sand and farmland. The isolated microorganisms were identified as bacteria (*Escherichia coli*, *Arthrobacter* sp., *Pontoea* sp., *Agrobacterium* sp., *Chryseobacterium* sp.), yeasts and filamentous fungi (*Candida* sp., *Lipomyces* sp., *Cryptococcus* sp., *Yarrowia* sp., *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamari*, and *Aspergillus niger*). The diversity of the selected oleaginous microorganisms varies with habitats and was distributed as follows: 22(56.4%) isolate were found in farmland, 8(20.5%) in sand, 5 (12.8%) in lawn and 4 (10.3%) in wetland. The carbon utilization analysis indicated that most of the filamentous fungi can use xylose and carboxymethyl cellulose (CMC) as a sole carbon source for maximum lipid production and are considered as promising single cell oils producers on using agro-industrial waste materials.

Introduction

Biomass-based biofuel production represents a pivotal approach to face high energy prices and potential depletion of crude oils reservoirs, to reduce greenhouse gas emissions, and to enhance a sustainable economy (Zinoviev et al., 2010). Biodiesel production is based on animal fats and on plant oils, such as soybean, rapeseed, and palm oils that are produced on agricultural land, opening the debate on the impact of the expansion of bioenergy crop cultures, which displace land from food production

and restricts the large-scale development of biodiesel to some extent (Rossi et al., 2011). Recently, the development of processes to produce single cell oil (SCO) using heterotrophic oleaginous microorganisms has triggered significant attention in order to meet the increasing demand of biodiesel production (Azocar et al., 2010). These organisms accumulate lipids, mostly consisting of triacylglycerols (TAG) as reserve compounds which are widespread among all eukaryotic organisms such as fungi, plants and animals, whereas it has only rarely been described in bacteria (Meng et al., 2009).

The lipid content in oleaginous microorganisms including microalgae, yeasts and filamentous fungi is higher (70-90%) than that in bacteria (20-50%) (Xin et al., 2009). Biodiesel as an alternative fuel has been in the forefront of the liquid biofuel sector for the last two decades. The use of edible vegetable oils such as soybean, rapeseed and non-edible oils such as *Jatropha* in the United States, Europe and India, respectively, as oil feedstock for biodiesel production needs to be augmented with newer technologies.

To meet the demand of the biodiesel industry, alternative oil sources are being explored and developed. Recently, microbial lipids (single cell oils, SCOs) accumulated by oleaginous microorganisms e.g., microalgae and fungi, with 20% or more of their cell mass being composed of lipids, have emerged as a potential feedstock for biodiesel production (Meng et al.,2009 and Kosa & Ragauskas,2011). The applications of oleaginous fungi for biodiesel are very few although they have several advantages over conventional plant and algal resources as they can be easily grown in bioreactors, have short life cycles, display rapid growth rates, are unaffected by space, light or climatic variations, are easier to scale up and have the ability to utilize a wide range of inexpensive renewable carbon sources such as lignocellulosic biomass and agro-industrial residues. Many studies on oleaginous microorganisms focused on the discovery of new oleaginous microorganisms and the optimization of fermentation conditions (Chen et al., 2009; Li et al., 2007; and Loffhagen et al., 2006). However, there are very few reports on the biodiversity of the oleaginous microorganisms in different habitats (Li et al., 2009; Li et al., 2012). Lebanon is a highly mountainous country with extreme variability in climatic conditions, soils and vegetation. Up till now, there has been no report on the biodiversity of the oleaginous microorganisms in the Lebanese environment. Glucose is the carbon source most commonly employed for growth of oleaginous fungi and lipid production (Saxena et al., 2008; Zhao et al., 2008). However, the cost of the biodiesel produced by these microorganisms is so high that its sustainable development is limited (Li et al., 2012). As a result, several studies have reporting lipid accumulation by oleaginous yeasts and filamentous fungi on different renewable substrates such as glycerol, sewage water, whey, molasses and lignocellulosic wastes (Subramaniam et al., 2010).

The present work is a screening study on the biodiversity of oleaginous microorganisms in the Lebanese environment that are economically relevant as a potential feedstock for biodiesel production from cheap renewable carbon sources.

Materials and Methods

Soil sample collection

Oleaginous microorganisms were isolated from soil samples (62) collected in sterile plastic containers under aseptic conditions

from 5-20 cm below the surface of different locations in wetland, sand, lawn and farmland in Lebanon, and were stored at 4°C until use. The geographical distribution of sampling sites was given in Figure 1.

Reagents and chemicals

All reference substances and chemicals were all purchased from Fluka. All other chemicals used were of analytical grade and were obtained from recognized chemical suppliers.

Enrichment of oleaginous microorganisms (Li et al., 2012)

One gram of each soil sample was added into a 250 ml Erlenmeyer flask containing 50 ml sterilized enrichment medium one at a time.

The enrichment medium contains (g/L) glucose, 100; yeast extract, 1; NH_4Cl, 1; KH_2PO_4, 2; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.01; $FeCl_3.6H_2O$, 0.01 and Na_2HPO_4, 1; the pH was adjusted to 6. The media were incubated at 28°C and 160 rpm for 48h for growth and lipid production initiation.

Screening and isolation of oleaginous microorganisms (Li et al., 2012)

One milliliter of the enriched samples was serially diluted with sterilized distilled water (1/10, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$) and then 0.1 ml of the diluents were plated on the screening medium that lacks carbon source and contained (g/L): (yeast extract, 1; NH_4Cl, 1; KH_2PO_4, 2; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.01; $FeCl_3.6H_2O$, 0.01 and Na_2HPO_4, 1 , pH of 6, then sterilized by autoclaving at 120°C for 20 min) and was incubated under static and shaken conditions (160 rpm) at 28°C for 1-4 days. The fasted growing colonies were picked for further study.

Maintenance of the stock culture

The bacterial and fungal isolates used throughout the present work was maintained on nutrient agar and Czapek Dox agar slants respectively and stored at 4°C with regular transfer at monthly intervals. For long preservation 25% glycerol was used.

Seed culture preparations and fermentation technique

Fungal seed culture (spore suspension)

The fungal spore suspensions were prepared from 96 hours old Czapek Dox agar slants cultures using sterile distilled water and the spores were counted using haemocytometer then were used as standard inocula ($1x10^6$-10^8 spores /ml medium) unless otherwise stated.

Preparation of bacterial seed culture

Seed cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml nutrient broth medium with 3 ml of bacterial suspension (prepared from 18 hrs old cultures) and then incubated at 30˚ C ± 2 on a rotary shaker (180 rpm) till reaches O.D 600 ≤ 1 then were used as standard inocula (3ml/flask) unless otherwise stated.

The fermentation medium used during all screening stages was carbon rich and nitrogen limited to induce lipid accumulation and contained (g/L) : (glucose, 60; yeast extract, 1; NH4Cl, 1; KH_2PO_4, 2; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.01; $FeCl_3.6H_2O$, 0.01; Na_2HPO_4, 1; pH 7.4.). Aliquots of 100ml medium was dispensed in 250 ml Erlenmeyer flasks, inoculated with

the selected pure SCO producers and then incubated at 28°C, under static and under shaken conditions (160 rpm) for 2 & 6 days (for bacteria and fungi respectively) , where triplicate samples were set up to determine dry weight and lipid content.

Determination of Biomass yield (dry weight) of oleaginous microorganisms

At the end of the incubation period the cells were harvested by filtration or centrifugation at 5000 rpm for 20 min, washed twice with distilled water and dried at 60˚C till constant weight whereby the weight of the dried biomass was calculated.

Extraction of lipid compounds

Lipids were extracted following the method of Bligh & Dyer (1959).

The obtained biomass (dry weight) was transferred to 50 ml centrifugal tube and 15 ml of HCl (4M) were added and the mixture was kept at room temperature for 30 min before it was kept at -80°C for 2 hours and subsequently in boiling water for 20 min.

This freezing/thawing process was repeated 3 times in order to break up the cells. Thirty milliliters chloroform/methanol (1:1) were added into the tube, shacked vigorously with a vortex and then centrifuged at 5000 rpm for 10 min.

The lipid containing chloroform layer (the lower layer) was dried using rotary evaporator before it was weighed for lipid content estimation.

Residual reducing sugars determination

Reducing sugars were determined using dinitrosalicylic acid method according to Miller (1959).

Iodine value determination of microbial lipids

The iodine value of the lipids was determined using the method adopted by Li et al.(2009).

Saponification value determination of microbial lipids

Lipids were mixed with excess amount of potassium hydroxide ethanol solution (0.5M) for saponification. With phenolphthalein as an indicator, hydrochloric acid standard solution (0.5M) was used for the titration of the remaining potassium hydroxide. Blank control was performed at the same time. The amount of potassium hydroxide (in milligrams) consumed in saponification marked the saponification value of the microorganism lipids.

Carbon utilization of oleaginous microorganisms

In order to test the ability of the organism to utilize a certain carbon source, the following medium that contained (g/L): NH_4Cl, 5; KH_2PO_4, 2; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.01; $FeCl_3.6H_2O$, 0.01; Na_2HPO_4, 1 and agar 15; pH 7.4, was supplemented with different carbon sources: CMC, xylose, soluble starch and sucrose one at a time at the final concentration of 10 g/L. The medium was sterilized, inoculated and incubated as previously mentioned.

Identification of oleaginous microorganisms

The oleaginous fungi were identified based on morphological characteristics and with the help of an identification key (Webster & Weber, 2007), the yeast isolates were identified using SIM key for yeast

identification (Deak, 2007) and the Gram positive bacterial isolates were identified according to Bergey's Manuel of Determinative Bacteriology (Holt, 1994) and Gram negative isolates using API 20E Kit (Biomerieux, France).

Molecular identification of the most promising oleaginous fungi

Five fungal isolates (S5, S7, S13, S26, S32(1)) were chosen to be identified by using molecular techniques. The genomic DNA of each fungal strain was isolated by using the standard protocol (Sambrook & Russell, 2001). Partial region of small subunit (SSU) rDNA was amplified by PCR using universal fungal primers, NS1 (5' GTAGTCATATGCTTGTCTC-3') and NS4 (5'- CTTCCGTCAATTCCTTTAAG-3') of 1,100 bp (White et al., 1990). PCR products were purified by using gel extraction kit (GeNei, Bangalore, India) and sequenced using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer's protocol followed by purification using Big Dye X-Terminator Purification kit (Applied Biosystems, USA) and analyzed in a DNA Analyzer (3730 DNA Analyzer, Applied Biosystems, USA). Sequence data were analysed using Sequence analysis software. The 18 S sequence obtained was aligned using BLAST algorithm to find matches within the non redundant database at NCBI (National Centre for Biotechnology Information; http://blast.ncbi. nlm.nih.gov/ Blast.cgi) (Zheng et al., 2000). Sequence data were submitted to GenBank through submission tool BankIt of NCBI.

Statistical analysis

All the experiments were performed in triplicates and the data were analyzed by SPSS17 (statistical package software) (SPSS Inc., Chicago, IL, USA) and using Paired-t-test for comparison of means. Differences with $p<0.05$ were considered statistically significant.

Results and Discussion

Species-sample curve

The soil samples were taken randomly from the Lebanese environment. In a species-sample curve, the cumulative number of species is plotted against the cumulative number of physical samples to demonstrate if the number of samples taken is representative to study the biodiversity of oleaginous microorganisms in the Lebanese environment (figure 2). The species-sample curve was used for evaluating both the number of replicates and the size of physical sample (Brower et al., 1998) in ecological study of biodiversity. Figure 2 showed that after about 52 samples taken the curve reached a plateau, which indicated that the number of soil samples taken was sufficient and representative to study the biodiversity of oleaginous microorganisms in the Lebanese environment.

Isolation of oleaginous microorganisms

Data in table 1 revealed that out of 200 isolated bacterial and fungal isolates 39 strains were proved to be oleaginous microorganism that produce and accumulate more than 20% lipid content, 6 out of 39 were identified as *Escherichia coli*, *Arthrobacter* sp., *Pontoea sp*, *Agrobacterium* sp., *Arthrobacter* sp., *Chryseobacterium meningosepticum*; 9 out of 39 isolates were different yeast strains namely: *Candida* sp., *Lipomyces* sp., *Cryptococcus* sp., *Yarrowia sp.*; and 24 out of 39 isolates were filamentous fungi and were identified as *Fusarium* sp., *Mucor* sp., *Penecillium* sp., *Rhizopus* sp., *Aspergillus* sp.

A great variation was noticed in the biodiversity of SCOs producers among Lebanese habitats, it was showed that in lawn soil few oleaginous microorganisms were detected namely: *Escherichia coli*, *Candida* sp. , *Mucor* sp. *Aspergillus* sp. *and Fusarium* sp. with 29.59±1.16, 30.98±1.14, 63.17±1.80, 71.43±1.56 and 67.64±0.85% lipid content with a noticeable variations in the biomass, residual sugar and lipid characteristics . On the other hand the richest soil with oleaginous microorganisms was the farmland soil where about 22 out of 39 oleaginous microorganisms were detected and identified as bacteria, yeast and filamentous fungi with lipid contents ranged from 22.57±3.94 to 30.55±5.33, from 38.48±2.23 to 82.79±0.93 and from 36.52±0.19 to72.07±8.41 % respectively, with a noticeable variations in the biomass, residual sugar and lipid characteristics. In wetland soil 4 strains were detected namely: *Arthrobacter* sp., *Cryptococcus* sp., *Candida* sp., and *Mucor* sp. with 21.01±4.01, 36.10±1.36, 40.43±0.73 and 70.06±1.03% respectively, whereas in sand soil about 8 out of 39 oleaginous microorganisms were detected and identified as bacteria, yeast and filamentous fungi with lipid contents ranged from 25.71±4.61 to 44.39±2.91, from 23.33±6.52 to 56.19±0.93, and from 39.98±1.56 to 60.82±2.01% respectively, showing a moderate richness in oleaginous microorganisms.

In the present study, the soil samples were enriched with high C/N medium (carbon rich and nitrogen limiting conditions) which permits the oleaginous microorganisms to accumulate a certain amount of lipids; the selection of strains was based on the growth rate on medium without carbon. Results in table 1 showed that the initial lipid content was 20-82%, which indicated that the isolation method was reliable (Li et al. ,2012). Oleaginous fungi accumulate high levels of lipids when carbon is in excess and a key nutrient such as nitrogen or phosphorous is limiting (Ratledge & Wynn, 2002). The accumulated lipids or SCOs get deposited as intracellular lipid bodies (LBs) using this approach.

The fermentation process was carried under shaken and static conditions, the statistical analysis of data (table 1) showed that the dry weight and the lipid yield obtained with oleaginous microorganisms cultivated under shaken conditions were higher than that obtained under static conditions, which can be explained by the high degree of aeration (more dissolved oxygen in the medium), the more availability of nutrients and homogenization of the medium that can be obtained during shacking. Also the residual reducing sugars were at their minimum levels under shaking conditions. However the statistical analysis of the lipid content percentage under both conditions indicated no significant difference among static and shaken conditions, which implies that the lipid content is more related to environmental conditions in which the microorganisms have been cultivated (high C/N ratio).

Ratledge & Wynn (2002) showed that many species of microorganisms can accumulate lipids efficiently, such as *Schizochytriu*m sp., *Arthrobacter* sp., *Bacillus* sp., *Candida* sp., *Cryptococcus* sp., *Aspergillus* sp., etc. Li et al., (2009 & 2012) isolated 13&15 species of oleaginous microorganisms respectively with 7 newly discovered species. In the present study, isolated oleaginous microorganisms belong to 15 different genera, the majority of them had been reported to be oleaginous, 10 out of 15 isolate showed high ability to accumulate lipids (up to 40%) and they will be promising in single cell oil production. The obtained data indicated that the biodiversity of oleaginous microorganisms in the Lebanese environment is extremely rich.

Table.1 Biodiversity of Oleaginous Microorganisms in Lebanese Habitats

Isolate number	Soil type	Name	Dry weight (g/L)		Lipid yield (g/L)		Lipid content(%)		Residual Reducing sugars(g/100ml)		Iodine value	Saponification value
			static	shaken	static	shaken	static	shaken	static	shaken		
S1-1	Lawn	*Escherichia coli*	1.26±0.05*	1.53±0.09*	0.35±0.04	0.45±0.045	28.05±4.10	29.59±1.16	5.21±0.19	4.73±0.24	70.50±4.45	155±17.14
S3-1	Wetland	*Arthrobacter* sp.	1.19±0.07*	1.72±0.10*	0.25±0.05	0.35±0.04	21.01±4.01	20.32±1.73	5.18±0.45	5.12±0.36	69.38±4.41	247.33±11.45
S5-1	Farmland	*Pontoea sp.*	1.35±0.12	1.66±0.08	0.29±0.05	0.38±0.08	21.20±1.52	22.57±3.94	5.85±0.27	5.28±0.26	62.9±7.32	167.58±9.42
S6-2	Farmland	*Agrobacterium* sp.	1.31±0.06	1.52±0.06	0.27±0.05	0.46±0.08	20.48±2.96	30.55±5.33	5.75±0.48	5.17±0.34	66.97±8.14	231.87±7.47
S9-2	Sand	*Arthrobacter* sp.	1.30±0.07*	1.53±0.05*	0.57±0.07	0.65±0.03	44.39±2.91	42.60±1.06	5.23±0.42	5.48±0.24	60.63±5.47	120.68±6.35
S12-1	Sand	*Chryseobacterium meningosepticum*	1.25±0.04	1.33±0.10	0.26±0.05	0.34±0.09	20.72±3.90	25.71±4.61	5.02±0.32	4.94±0.18	64.67±3.89	197.04±8.24
S1-2	Lawn	*Candida* sp.	4.63±0.16*	6.08±0.31*	1.43±0.10	1.74±0.10	30.98±1.14	28.62±0.20	0.35±0.13	0.15±0.12	47.72±1.24	238.26±8.78
S2-2	Sand	*Lipomyces* sp.	2.89±0.20*	6.22±0.29*	1.44±0.10*	3.46±0.15*	49.89±1.34*	55.72±0.80*	0.47±0.21	0.12±0.08	20.13±2.36	255.80±11.89
S2-3	Sand	*Cryptococcus* sp.	7.53±0.31*	9.20±0.20*	2.71±0.15	3.27±0.17	36.01±1.39	35.60±1.13	3.75±0.49	3.32±0.25	21.43±2.45	215.40±7.47
S3-2	Wetland	*Cryptococcus* sp.	7.12±0.13*	5.04±0.12*	2.20±0.12*	1.87±0.11*	30.84±1.13*	36.10±1.36*	1.01±0.25*	0.21±0.11*	32.40±1.63	168.80±6.47
S3-3	Wetland	*Candida* sp.	5.04±0.21*	6.36±0.11*	2.04±0.12*	2.50±0.10*	40.43±0.73	39.16±1.83	1.45±0.24*	0.25±0.11*	33.78±2.85	191.80±4.74
S4-2	Farmland	*Yarrowia sp.*	3.45±0.12*	6.24±0.10*	2.77±0.11*	5.16±0.11*	80.29±2.65	82.79±0.93	1.89±0.14*	0.23±0.14*	50.89±1.47	269.80±9.68
S4-3	Farmland	*Candida* sp.	1.68±0.10*	2.54±0.10*	0.52±0.12	0.98±0.10	30.74±5.58	38.48±2.23	0.56±0.17	0.21±0.10	64.80±4.63	151.08±4.98
S7-4	Sand	*Candida* sp.	1.39±0.07*	1.86±0.10*	0.33±0.11	0.41±0.06	23.33±6.52	21.84±1.93	5.45±0.75*	4.31±0.27*	71.41±8.47	175.00±8.74
S7-6	Sand	*Lipomyces* sp.	6.39±0.22*	11.23±0.16*	2.97±0.10*	6.31±0.19*	46.51±0.41*	56.19±0.93*	3.24±0.36	2.82±0.32	49.77±1.47	246.25±7.86
S5	Farmland	***Fusarium oxysporum***	13.33±0.13*	23.20±0.30*	4.70±0.09*	10.01±0.11*	35.27±0.51*	43.16±0.10*	3.51±0.45	2.56±0.24	62.81±2.23	160.17±7.45
S6	Farmland	*Mucor* sp.	15.48±0.61*	18.41±0.52*	5.13±0.42*	6.72±0.21*	33.08±1.45	36.52±0.19	3.11±0.32	2.68±0.24	20.67±2.45	143.70±9.89
S6f	Farmland	*Fusarium* sp.	16.96±0.66*	19.22±0.21*	6.88±0.20*	7.55±0.31*	40.57±0.74	39.25±1.41	3.57±0.54	2.47±0.23	11.83±1.24	117.09±6.78
S7	Sand	*Mucor hiemalis*	16.43±1.41*	21.47±0.65*	6.35±0.52*	8.58±0.36*	38.65±1.05	39.98±1.56	2.27±0.24	1.51±0.15	48.51±1.42	192.78±8.78
S8	Farmland	*Fusarium* sp.	15.08±1.24*	11.24±0.36*	6.76±0.41*	3.98±0.16*	44.87±1.11*	35.39±1.55*	3.09±0.21	2.78±0.21	27.47±2.58	117.32±7.98
S13	Farmland	***Penicillium citrinum***	17.89±0.35*	17.13±0.29*	9.57±0.41*	11.10±0.26*	53.46±1.28*	64.77±0.48*	3.26±0.24	2.47±0.21	27.89±3.47	229.84±11.34
S14	Wetland	*Mucor* sp.	2.58±0.17*	5.10±0.22*	1.25±0.05*	4.18±0.10*	48.69±3.13*	70.06±1.03*	2.00±0.29*	0.74±0.26*	23.94±2.27	160.31±10.36
S15	Farmland	*Mucor* sp.	2.54±0.14*	3.18±0.11*	1.18±0.07*	1.37±0.12*	46.46±0.50	43.04±2.64	2.78±0.52	2.74±0.39	54.95±3.32	191.4±9.25
S16	Lawn	*Mucor* sp.	2.60±0.71*	5.08±0.44*	0.88±0.32*	3.21±0.22*	33.13±4.62*	63.17±1.80*	1.17±0.34	0.22±0.11	32.74±1.89	343.85±12.40

S17	Farmland	*Rhizopus* sp.	3.26±0.95	4.74±0.79	1.02±0.64*	2.49±0.45*	29.29±11.19	52.41±0.69	0.82±0.21	0.14±0.08	24.71±4.47	239.35±11.32
S24(2)	Farmland	*Rhizopus* sp.	2.87±0.35*	7.03±0.75*	1.82±0.12*	4.85±0.36*	63.76±3.77	69.12±2.06	1.39±0.27*	0.21±0.09*	50.19±1.21	139.18±7.54
S25	Farmland	*Rhizopus* sp.	3.37±1.19*	5.57±0.80*	1.25±0.20*	3.27±0.16*	39.52±11.09*	59.17±5.42*	1.54±0.23	0.79±0.12	40.32±4.36	201.51±11.14
S26	Farmland	***Aspergillus tamari***	9.79±0.40*	15.57±0.90*	6.25±0.15*	10.67±0.51*	63.90±1.11	68.57±2.65	2.93±0.48	2.15±0.24	46.25±1.24	237.10±12.23
S28	Farmland	*Mucor* sp.	4.93±0.25	5.40±0.51	3.42±0.19	3.86±0.13	69.49±1.73	72.07±8.41	4.02±0.36	2.78±0.32	37.58±3.63	300.23±13.01
S29(1)	Farmland	*Aspergillus* sp.	21.44±1.28	24.75±1.64	14.53±0.55	17.36±1.41	67.85±2.33	70.11±1.03	2.24±0.32	1.72±0.21	44.41±4.24	246.15±7.42
S30	Farmland	*Rhizopus* sp.	4.57±0.33	5.34±1.14	2.64±0.19	3.44±0.47	57.85±0.62	65.11±5.07	2.73±0.34	2.08±0.39	61.65±4.78	288.89±11.47
S32(1)	Lawn	***Aspergillus niger***	17.01±0.39*	25.48±1.16*	8.59±0.35*	18.19±0.65*	50.51±1.74*	71.43±1.56*	2.17±0.31	1.97±0.24	67.86±1.69	233.60±9.32
S32(2)	Lawn	*Fusarium* sp.	4.33±0.41*	6.90±0.34*	2.84±0.18*	4.66±0.18*	65.81±2.49	67.64±0.85	3.81±0.45	2.68±0.34	45.68±4.47	352.63±18.42
S35	Farmland	*Mucor* sp.	3.22±1.01	5.41±0.72	0.86±0.37*	3.06±0.16*	26.14±3.36*	56.96±4.46*	0.90±0.24	0.59±0.21	15.36±2.21	331.20±14.20
S43	Farmland	*Rhizopus* sp.	2.82±0.28*	4.89±0.35*	0.92±0.12*	2.98±0.21*	32.57±0.94*	60.91±0.87*	0.82±0.23	0.41±0.11	33.39±3.32	269.76±6.45
S45	Farmland	*Mucor* sp.	4.86±0.39	3.95±0.26	2.55±0.41	2.68±0.18	52.17±4.40*	67.70±0.77*	3.88±0.42	2.77±0.23	50.60±5.57	254.72±8.21
S47	Sand	*Fusarium* sp.	5.79±0.46*	9.70±0.50*	2.72±0.14*	5.89±0.14*	46.99±1.39*	60.82±2.01*	3.73±0.56	2.69±0.32	32.19±2.24	349.56±17.69
S50	Farmland	*Fusarium* sp.	3.48±1.26*	6.29±0.90*	1.18±0.319*	2.71±0.54*	34.80±3.42	42.79±3.02	4.06±0.32*	2.62±0.25*	24.06±1.87	269.76±12.78
S59	Farmland	*Rhizopus* sp.	4.22±0.36	4.53±0.75	2.08±0.12	2.51±0.42	49.33±1.48*	55.35±0.92*	2.68±0.24	1.95±0.23	72.76±4.65	357.14±14.47

*There is a significant difference between the means of shaken and static conditions (alpha=0.05), by using the statistical paired-t-test for comparison of means

Table.2 Carbon utilization of oleaginous microorganisms

Isolate number	Sucrose	Starch	CMC	Xylose
S1-1	++	+	+	++
S3-1	+++	+	+	+++
S5-1	+++	+	+	++
S6-2	+++	-	+	+
S9-2	++	+	+	-
S12-1	+++	+	+	+++
S1-2	+++++	+	+	+
S2-2	+++++	+	+	++++
S2-3	+++++	+	+	++++
S3-2	+	+	+	+
S3-3	+++++	+++	+	+
S4-2	+++++	+++	+	++++
S4-3	+++++	++++	++	++++
S7-4	+	+	+	+
S7-6	+++++	++++	++	+++++
S5	++	++	+++	++
S6	++++	+++++	+++++	+++
S6f	++	+	++	++
S7	++++	++++	+++++	++++
S8	+++	++++	++++	++
S13	+++	+	++	++
S14	++++	+	+++++	+++++
S15	++++	+++++	+++	++++
S16	+++++	+++++	+++++	+++++
S17	++++	+++++	++	++++
S24(2)	+++++	+++++	-	++++++
S25	++++	+++++	+++	+++++
S26	+++++	+++++	+++++	++++
S28	++++	+++++	++	++++
S29(1)	+++++	+++++	++++	+++++
S30	+++++	+++++	+++	+++++
S32(1)	+++++	+++++	++++	++++
S32(2)	++++	+++++	++++	++++
S35	+++++	+++++	+++++	+++
S43	+++++	+++++	+++++	++++
S45	+++++	+++++	+++	+++++
S47	+++++	+++++	+++++	++++
S50	++++	+++++	++++	++++
S59	+++++	+++++	++++	+++++

(+) indicates that the strain could utilize the substrate, the more + indicates that the utilization is better, the growth is faster; (-) indicates that the strain could not utilize the substrate.

Figure.1 Lebanon map showing the geographical distribution of 62 soil samples

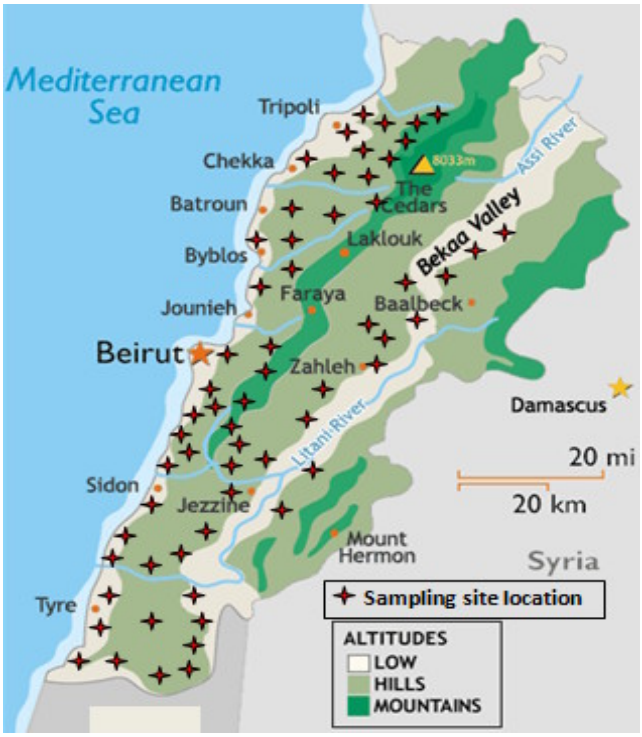
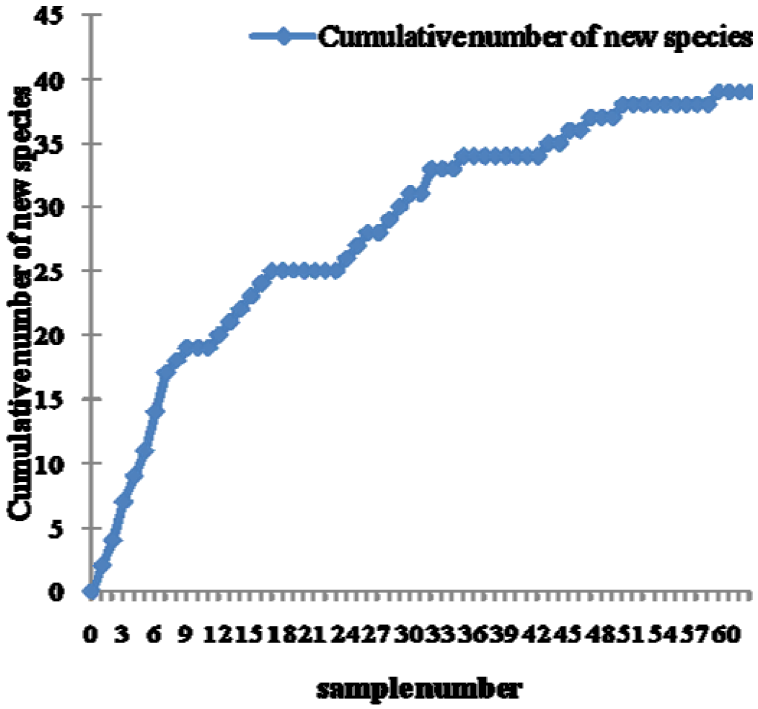


Figure.2 Species-sample curve



The iodine value (table 1) was ranging from 60.63±5.47 to 70.50±4.45 and from 20.13±2.36 to 71.41±8.47 for bacterial and yeast lipids respectively. For lipids extracted from filamentous fungi, the iodine value was ranging from 11.83±1.24 to 67.86±1.69. Concerning the saponification value (table 1), it was ranging from 155±17.14 to 247.33±11.45, from 151.08±4.98 to 269.80±9.68, and from 117.09±6.78 to 352.63±18.42 for bacterial, yeast and fungal isolates respectively. Moreover, the results of iodine value and saponification value determination indicate that the compounds of microbial lipids are similar to those of vegetable oils.

Molecular identification of the most promising oleaginous fungi

The molecular identification of the five fungal isolates S5, S7, S13, S26 and S32(1) indicated that belong to *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamari*, and *Aspergillus niger* respectively.

Carbon utilization of oleaginous microorganisms

As shown in Table 2, nearly all the isolated microorganisms had the ability to utilize xylose, CMC, starch and sucrose as a sole carbon source including bacteria, yeasts and filamentous fungi, but the growth rate on the different carbon sources varies among the isolated microorganisms where the filamentous fungi showed the strongest ability. On the other hand it was noticed that S6-2, S9-2 and S24(2) isolates could not utilized starch, xylose and CMC respectively. Most of the oleaginous microorganisms reported utilize glucose to produce lipids (Li et al., 2012). However, the use of glucose in microbial lipid production will increase the cost greatly, which limits its application. The substrate utilization of 39 strains indicates that most of the isolated microorganisms can utilize xylose and CMC, which can be grown on agricultural waste for microbial lipid production. The obtained results constitute a solid foundation for using the promising strains in producing microbial lipid from agro-industrial waste products.

In the present study, the SCOs of fungal cell mass from Lebanese soil will be explored as biodiesel feedstock.

References

Azocar, L.; Ciudad, G.; Heipieper, H. and Navia, R. (2010). Biotechnological processes for biodiesel production using alternative oils. Applied Microbiology and Biotechnology, 88: 621-636.

Bligh, E. and Dyer, W. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Physiology and Pharmacology, 37: 911–917.

Brower, J.; Zar, J. and von End, C. (1998). Collecting, analyzing, and reporting ecological data. In: Field and Laboratory Methods For General Ecology, 4th edition,WCB/Mc-Graw-Hill.

Chen, X.; Li, Z.; Zhang, X.; Hu, F.; Dewey, D. and Bao, J. (2009).Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. Applied Biochemistry and Biotechnology, 159:591-604.

Deak, T. (2007). Yeast identification. In: Handbook of food spoilage yeasts, second edition, CRC Press, p. 221-251.

Holt, J. (1994). Gram positive rods bacteria. In: Bergey's manual of determinative bacteriology, LWW, ninth edition, p.210-250.

Kosa, M and Ragauskas, A. (2011). Lipids from heterotrophic microbes: advances in metabolism research. Trends Biotechnology**,** 29:53-61.

Li, S.; Lin, Q.; Li, X.; Xu, H.; Yang, Y; Qiao, D. and Cao, Y. (2012). Biodiversity of the oleaginous microorganisms in Tibetan plateau. Brazilian Journal of Microbiology, p.627-634.

Li, X.; Deng, F.; Li, S.; Wei, L.; Gui, G. and Zhi, Q. (2009). Isolation of the oleaginous yeasts from the soil and studies of their lipid-producing capacities. Food Technology and Biotechnology, 47: 215-220.

Li, Y.; Zhao, Z. and Bai, F. (2007). High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. Enzyme Microbiology and Technology, 41: 312–317.

Loffhagen, N.; Hartig , C. and Harms, H. (2006). Impact of membrane fatty acid composition on the uncoupling sensitivity of the energy conservation of Comamonas testosterone ATCC 17454. Applied Microbiology and Biotechnology, 70: 618-624.

Meng, X.; Yang, J.; Xu, X.; Zhang, L.; Nie, Q. and Xian, M. (2009). Biodiesel production from oleaginous microorganisms. Renewable Energy, 34: 1-5.

Meng, X.; Yang, J.; Xu, X.; Zhang, L.; Nie, Q. and Xian, M.(2009). Biodiesel production from oleaginous microorganisms. Renewable Energy**,** 34:**1-5.**

Miller, G. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31: 426–428.

Ratledge, C. and Wynn, J. (2002). The biochemistry and biotechnology of lipid accumulation in oleaginous microorganisms. Advanced Applied Microbiology**,** 51:1-51.

Ratledge, C. and Wynn, J. (2002). The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Advances in Applied Microbiology, 51: 1-44.

Rossi, M.; Amaretti, A.; Raimondi, S. and Leonardi, A. (2011). Getting lipids for biodiesel production from oleaginous fungi, Biodiesel-Feedstocks and Processing Technologies, 1: 72-74.

Sambrook, J and Russell, D. (2001). Molecular cloning, a laboratory manual. In: Cold Spring Harbor Laboratory, 3rd edition. New York, CSHL Press.

Saxena, V.; Sharma, C.; Bhagat, S.; Saini, V. and Adhikari, D. (2008). Lipid and fatty acid biosynthesis by *Rhodotorula minuta*. Journal of the American Oil Chemists Society,75: 501–505.

Subramaniam, R.; Dufreche, S.; Zappi, M. and Bajpai, R. (2010). Microbial lipids from renewable resources: production and characterization. Journal of Industrial Microbiology and Biotechnology, 37:1271–1287.

Webster, J. and Weber, R. (2007). In: Introduction to fungi, third edition, Cambridge University Press, p.126-670.

White, T.; Bruns, T.; Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR-Protocols and applications- a laboratory manual. New York, Academic, p.315–322.

Xin, M.; Yang, J.M.; Xu, X.; Zhang, L.; Nie, Q. and Xian, M. (2009). Biodiesel production from oleaginous microorganisms. Renewable Energies, 34: 1-5.

Zhao, X.; Kong, X.; Hua, Y.; Feng, B. and Zhao, Z. (2008). Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. European Journal of Lipid Science and Technology, 110: 405-412.

Zheng, Z.; Schwartz, S.; Wagner, L. and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. Journal of Computational Biology, 7:203–214.

Zinoviev, S.; Müller-Langer, F.; Das, P.; Bertero, N.; Fornasiero, P.; Kaltschmitt, M.; Centi, G. and Miertus, S. (2010). Next-generation biofuels: Survey of emerging technologies and sustainability issues. Chemistry & sustainability, energy & materials, 3: 1106-1133.