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Isolation Of Lipid Producing Yeast And Fungi From Secondary Sewage Sludge And Soil

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Abstract: High costs being the major obstacle for the commercialization of biodiesel, considerable research has been focused on the alternatives for biodiesel. Microbial lipids possess significant potential which can be explored for biodiesel production. In this research, novel strains of lipid producing yeast and fungi were isolated from secondary sludge of waste water treatment plants and soils based on their ability to grow on glycerol as sole carbon source. Sudan Black B staining revealed the presence of lipid inclusion bodies in the cells during cultivation under nitrogen-limiting conditions. Strains were identified as *Zygowiliopsis californica* and *Galactomyces geotrichium*. The predominant lipids accumulated by these microorganisms were $C_{12:0}$- $C_{20:2}$ as identified by GC-MS. Linoleic acid (C18:2) (28 was the main fatty acid in the lipids when yeast cells were grown under nitrogen limted medium followed by palmitic acid (C16:0) (19%) and oleic acid (C18:1) (18%). FAME anlaysis of fungal samples showed higher concentration of stearic (C18:0) (24 %) followed by palmitic acid (C16:0) 32% and oleic acid (C18:1) (16%). The fatty acid composition of the lipids accumulated by these strains is comparable to the fatty acid profile of vegetable oils and the lipids reported here are valuable for biodiesel production Further research directed to the utilization of various cheap carbon sources, such as wastes and waste water from food industry and sewage sludge as substrate of yeast and fungi and assessing their lipid potential hold high promise and could serve as sources for production of biodiesel.

Key words: Biodiesel, Oleaginous microorganisms, microbial lipid, Oleagnous yeast, oligeanous fungi, lipid extraction, fatty acid, lipid analysis

INTRODUCTION

The rising prices of petroleum products and environmental concerns associated with the emissions from combustion of these fuels, has led to an interest in the search for alternatives of petroleum products and one among them is biodiesel. Biodiesel, which is a mixture of fatty acid alkyl esters, can be used as a good substitute for petroleum. One of the conventional methods to produce biodiesel was to transesterify plant oils with methanol (Krawczyk, 1996; Ma and Hanna, 1999). It can be produced by the acid or base catlaysed transesterification of vegetable oils or animal fats, which yields fatty acid methyl esters. Microbial oils otherwise known as single cell oils can be considered as a potential alternative to conventional fuels, due to their renewability, fast growth rate and low cost. One of the major challenge for the commercialization of biodiesel production using vegetable or seed oils being their high price should be resolved and the major solution for this the dependence on alternative feedstocks which are cheaply and readily available. Municipal sewage sludge has been studied as a possible lipid feedstock for biodiesel production. There exists a small number of microorganisms which are capable of converting carbohydrates into oils and store these energy sources under appropriate conditions. The major lipids with long chain fatty acids the triacylglycerols in these microorganisms are comparable to plant oils (Ratledge and Wilkinson, 1989). Another strategy that can be implemented is the isolation of novel strains of microorganisms from environment and screening them for enhanced lipid production capacities. In this study an attempt has been made to isolate lipid producing strains from different environmental sources and also the feasibility of utilizing secondary sludge as a raw material for lipid production.

2. Experimental methods:

2.1 Chemicals:

All the chemicals and reagents used in the experiment were purchased from Fisher Scientific and Sigma Aldrich and were of analytical grade unless otherwise specified.

2.2. Sample Collection And Preparation:

Secondary sludge and soil samples were obtained for the isolation of oliganeous microorganisms. The secondary sludge samples were collected from a municipal waste water treatment plant located in Québec, Canada. Upon collection, the sludge samples were allowed to undergo gravity settling at 0^0C for 24h. The

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supernatant liquid was discarded and the concentrated samples were taken for the experiment. Separation of clarified water resulted in a sludge containing 35g/l suspended solids. Soil samples were collected from the upper 5-10 cm of a garden where flowering plants were cultivated in Quebec, Canada

2.3 Isolation Of Lipid Producing Microorganisms:

Isolation of lipid producing microorganisms was done by glycerol enrichment method following Rooney *et al*. (2005). 1 ml of sludge (from waste water treatment plants) and 1 g of soil (for soil samples) were respectively added to 250 ml of glycerol enriched medium, Glycerol mineral Salt medium (GMSM, p H 7) and incubated in an incubator shaker at 30^0C, with shaking at 200 rpm for 96h, so that the target microorganisms would be enriched to a great number.

For isolation 1ml of pre cultured samples were added to 9ml of saline water (.85%) and 10- fold serial dilutions were made. Portions of 0.1ml from each dilution ranging from 10^{-1} to10^{-9} were spread on to GMSM agar plates and single colonies were streaked and maintained as pure cultures for further study.

2.4 Screening For Oleaginous Microorganisms:

The isolated single colonies of bacteria, yeast and fungi were further screened for their lipid producing capabilities by qualitative analysis with Sudan Black B Staining technique following the method of Burdon (1946). The strains which showed positive for Sudan Black B staining were maintained in cryovials and stored under refrigerated conditions.

2.5 Determination Of Microbial Dry Biomass:

The strains were transferred to 50 ml of inoculation media (GMSM) in 250 ml Erlenmeyer flask and incubated in an incubator shaker, at 28^0C at 200 rpm for 48 hours. After 48 hours, 5ml of culture was transferred to 45 ml of nitrogen limited fermentation media containing (in g/L) Glucose 40g, $(NH_4)_2SO_4$- 2g, KH_2PO_4.7g, NaH_2PO_4-2g, Mg $SO_4.7H_2O$- 1.5g, Yeast extract- 1g) and incubated at 30^0 C, at 200 rpm for 120h. 50ml of this culture was harvested by centrifugation at 5000rpm for 10 minutes at 4^0C. Harvested biomass was washed twice with water and dried at 60^0C to constant mass. The biomass was then determined gravimetrically.

2.6 Extraction Of Lipids From Isolated Strains:

The estimation of lipid content from the isolated strains was done following the method of Bligh and Dyer with modifications. The strains were transferred to 50 ml of inoculation media (GMSM) in 250ml Erlenmeyer flask and incubated in an incubator shaker, at 28^oC at 200 rpm for 48 hours. After 48 hours, 5ml of culture was transferred to 45 ml of nitrogen limited fermentation media (Glucose 40g, $(NH_4)_2SO_4$- 2g, KH_2PO_4.7g, NaH_2PO_4-2g, Mg $SO_4.7H_2O$- 1.5g, Yeast extract- 1g in 1L) and incubated at 30^oC, at 200 rpm for 120h. From this, 50ml of culture was harvested by centrifugation at 5000g for 5 minutes. Harvested biomass was washed twice with water. Followingly, 10 ml of 4M Hcl was added and incubated at 60^oC for 2 h. The acid hydrolysed mass was stirred with 20ml of chloroform: methanol mixture (1:1) at room temperature for 3h followed by centrifugation at 2000g for 5 minutes at room temperature to separate aqueous upper phase and organic lower phase. Followingly, lower phase containing lipids were recovered and evaporated using nitrogen gas and dry lipids were weighed. The yield of extracted material was determined and expressed as grams of extractable lipid per gram of dry solid.

2.7 Transmethylation Of Total Lipids:

Two strains which showed positive response with Sudan Black B staining, one yeast strain (isolated from sludge) and fungal strain (isolated from soil) which exhibited maximum lipid accumulation were selected for further studies, which included transmethylation and analysis of fatty acid profiling by GC-MS. Conversion of lipids to fatty acid methyl esters was carried out by the method of Lewis *et al*. (2000). Total lipids extracted from the strains were transmethylated using 5% methanolic Hcl (Choo Woong and Mo Jun, 1999) and analysed with standards using using gas chromatography.thyl Fatty acid methyl esters were extracted from transmethylated samples by adding 1 ml of water followed by 6 ml of hexane: chloroform (4:1) for extraction (Lewis *et al*., 2000)

2.8 FAME (Fatty acid methyl ester) analysis:

The FAMEs in hexane: chloroform were analyzed using an Clarus 500 GC/MS with autosampler (Perkin Elmer, Massachussetts, USA). The column used was a 30 m × 0.25 mm × 0.25m Rxi -17 capillary column (Restek, Bellefonte, PA, USA) with crossbond 50% diphenyl / 50% dimethylpolysiloxane as the stationary phase. The column temperature was programmed to increase from 60 °C to 150 °C at 10 °C/min; then ramped from 150 °C to 300 °C at 5 °C/min; and finally maintained at 300 °C for 10 min. The injector port was set at 280 °C. The transfer line was set at 280 °C and the source at 230 °C. The carrier gas used was helium 1 mL/min, while the sample injection volume was 1.0 µl with a split/splitless mode, ratio of 40:1. The internal standard

used was 1, 3-dichlorobenzene (Fisher Scientific, Pittsburgh, PA, USA). Calibration curves between peak area and concentration were established by injecting reference FAME samples of known concentrations into the GC/MS. The acquisition method is in selected ion monitoring.

2.9 Genomic Identification Of Yeast And Fungal Strains:

The total DNA from yeast and fungal cultures were extracted following the protocol of Harju *et al*. (2004)**.** Genomic identification of the isolated yeast and fungal strains would be done by sequencing their 18S rDNA. PCR amplifications were perfomed with 1U of Taq DNA polymerase, 200µM PCR master mix, 1.0 µM each of forward and reverse oligonucleotide primers and 50-100ng of template DNA. The universal oligonucleotide primers NS1 and NS6 were used to amplify and sequence 18S r DNA gene. All PCR reactions were performed for 35 cycles, each consisting of 30s denaturation at 94^{o}C, a 30s annealing at 54^{o}C and 1 min extension step at 72^{o}C. The amplified products were subjected to sequencing, by sequencing the forward and reverse strands of each fragment (The sequences are submitted in GenBank, awaiting accession numbers.)

RESULTS AND DISCUSSION

In the preliminary study, seven colonies with the morphology typical of bacteria, yeast and fungi were isolated from the secondary sludge, (soil and apple pomace waste (samples that can utilize glucose or glycerol as the sole carbon source. Out of these, microorganisms, two microorganisms, one yeast and fungi which exhibited more than 15% lipid accumulation was selected for analysis of fatty acid profile by GC-MS. The details of the strains isolated from different sources and their lipid accumulation percentage are given in Table 1.

Table 1: Details of isolates obtained from different sludge and soil

Name	Dry biomass (g/L)	Lipid yield (g/L)	Lipid (%)	Organism identified
SLY	8.4	1.738	20.6	*Zygowilliopsis californica*
SOF	2.6	0.352	17	*Galactomyces geotrichium*

3.1 Screening And Characterization Of The Isolates For Lipid Accumulation:

The lipid accumulation capacity of the isolated colonies was revealed by Sudan Black B staining of the cultures grown in nitrogen limited media. The colonies were further characterized for certain parameters like dry biomass, lipid yield and lipid content. The dry biomass and lipid yield of the isolated strains are given in the Table 2.

3.2 Genomic Characterization Of Isolates:

Genomic identification studies by PCR with 18S forward and 18S reverse primers yielded an amplicon of around 600-700 bp, for both the isolates. Results from the sequencing confirm their genetic differences, supporting their identification. The yeast strain isolated from sludge and fungal strain isolated from soil sample which exhibited maximum lipid accumulation which accounted to 20.6 and 17 % respectively were selected for analysis of fatty acid profile by GC-MS. The fatty acid ester were analysed by GC-MS. The results revealed the composition of different fatty acids as follows:

Table 2: Fatty acid profile by GC and GC/MS analysis

	Percentage of principal fatty acids (%)																
	$C_{6:0}$	$C_{8:0}$	$C_{9:0}$	$C_{10:0}$	$C_{11:0}$	$C_{12:0}$	$C_{13:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{20:1}$	$C_{20:2}$
SLY		0.12	-	0.04	-	0.13	-	0.67	1.40	19.24	2.75	-	18.0	28.27	0.18	0.35	0.34
SOF	0.46	0.53		0.39	0.34	0.76	0.62	1.60	2.30	32.49	2.06	24.89	11.68	16.91	0.87	2.83	1.27

The fatty acid composition of lipids from yeast and fungal strains was inferred from the FAME analysis of the biodiesel produced by transesterification of samples. Analysis of biodiesel samples from yeast showed the occurrence of significant amounts of the methyl esters of palmitic acid (C16:0) (19%),linoleic acid (C18:2) (28%), and oleic acid (C18:1) (18%) (Fig 1.), whereas FAME anlaysis of fungal samples showed significant amount of palmitic acid (C16:0) 32%, oleic acid (C18:1) (16%) and stearic (C18:0) (24 %)(Fig 2).

Discussion:

It had been well studied that lipid production for microorgnaisms requires a medium that is rich or excesss in sugars or polysaccharides and with little of other nutrients, especially nitrogen. The activity of Nicotinamide Adenine dinucleotide isocitrate dehydrogenase (NAD-IDH) lowers due to low nitrogen in the media, which results in the repression of TCA cycle, wherein protein synthesis is stopped and induces lipid accumulation (Evans *et al*, 1981, Botham *et al*., 1979, Palmieri *et al*., 1996). Glycerol as the sole carbon source in the medium

has been found to enrich the growth of oleaginous microorganisms (Pan *et al*., 2009). Ability of microorganisms for lipid production in nitrogen limited media had been supported by earlier scientists (Gema *et al*., 2002).

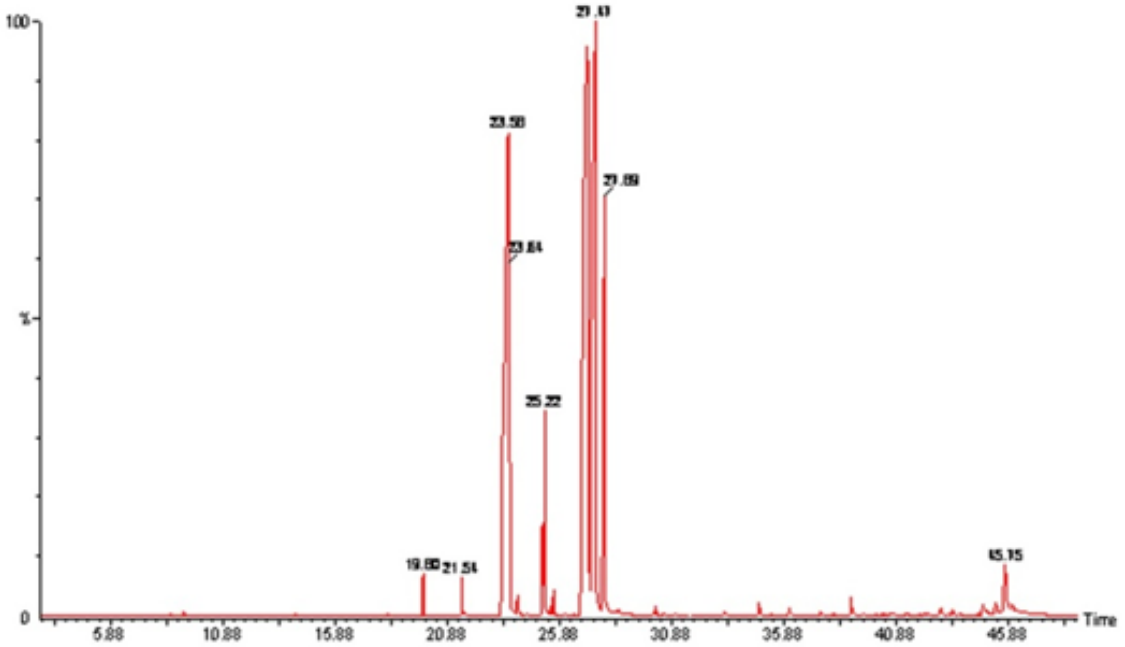

Fig. 1: Fatty acid profile of yeast by GC-MS analysis

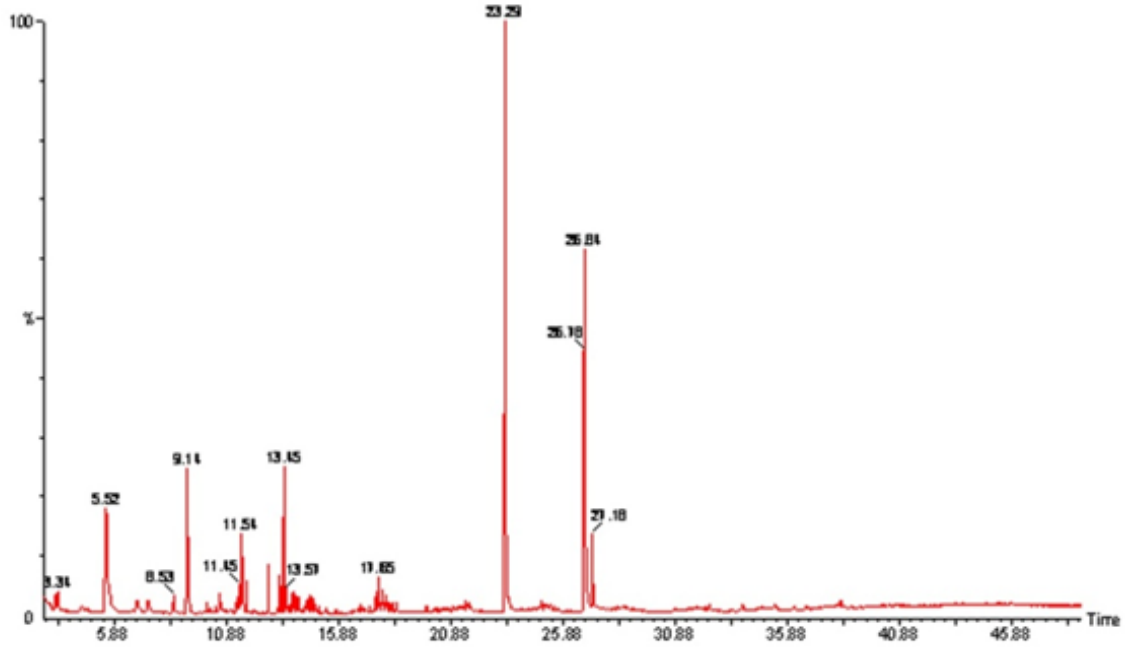

Fig. 2: Fatty acid profile of fungi by GC-MS analysis

Among the two strains isolated which showed positive response to Sudan Black Bstaining, the yeast strain isolated from municipal sludge showed lipid accumulation potential of 20%. Lipid accumulation potential of yeasts strains isolated from soil samples have been shown in the range of 17- 38 % in the studies by Pan *et al*. (2009), utilizing xylose as the carbon source, which had been attributed to posses the potential for industrial production of biodiesel or cocoa butter substitute. Many yeast species had been reported to accumulate oils under different cultivation conditions (Li *et al*., 2008, Liang *et al*., 2006). Yeast oils could be used as feedstocks for biodiesel production with catalysis either by lipase or chemical catalyst (Li *et al*., 2007). Lignocellulosic

biomass such as cornstalk, tree leaves and rice straw can be used as a promising alternative energy source for our limited crude oil. *Rhodotorula glutinus* has been reported as a potential strain which can convert lignocellulosic hydrolyzates into raw material for biodiesel production (Dai *et al*., 2007).

The lipid accumulation potential of fungus isolated from the soil accounted to 17 %. Although some fungi have the ability to produce oils, fungi are mainly explored for the production of special kind of lipids such as DHA, GLA, EPA and ERA spell out (Ma, 2006; Du *et al*., 2007; Yan and Chen, 2003; Seramphim *et al*., 2004). The lipid accumulation potential of these fungal and yeast strains have never been studied before and hence this paper reports the lipid production capabilities of *Zygowilliopsis californica* and *Galactomyces geotrichium* for the first time.

The extent of lipid accumulation in microorganisms is determined by their genetic constitution and the maximum lipid content attainable varies among species and even among individual strains. Oleaginous yeasts and fungi have been reported to be the potential alternative oil resources for biodiesel production (Meng *et al*., 2009). Lipid accumulation in microorganisms is induced by the exhaustion of a nutrient in the medium (especially nitrogen) with an excess of carbon source in the medium, which is assimilated by the cells and converted to triacylglycerols. The limitation of nitrogen in the medium prevents cell proliferation and the already formed lipids are stored in the cells, which leads to lipid accumulation. Two enzymes, malate enzyme and ATP;citrate lyase have been found to effect lipid accumulation. In scenario of biodiesel production by microorganisms, microorganisms with higher content of oils ranging from C_{13} to C_{22} or higher are considered as potential targets of interest, since these lipids accumulated by microoranisms are capable of mimicking properties of higher value oils, improve oxidative stability and possess more potential adaptability in industrial production of biodiesel (Meng *et al*., 2009).

Conclusion:

To reduce the cost of microbial oils, then need for exploring cheap carbon sources such as glycerol instead of glucose is of paramount significance for industrial biodiesel production. Industrial waste being rich in simple sugars, serves as a growth medium which can be effectively utilized by a wide variety of microorganisms. Several yeast species have been reported to accumulate oils, sweet potato starch processing waste and starch hydrolysate as carbon source and hence cell growth was found to be better than utilizing glucose as the carbon source. Utilization of crude glycerol for yeast oil production, which is a byproduct of biodiesel industry could be an interesting and promosing area of research. Biodiesel production had been demonstrated through the in situ transesterification of municipal primary and secondary sludges (Mondala *et al*., 2010).

Hence the effect of media composition, cultivation conditions such as C/N ratio, nitrogen resources, temperature, pH, oxygen, concentration of trace elements and inorganic salt on the growth, and influence on lipid accumulation of microorganisms should be investigated. This can be studied by using different carbon sources and estimating the lipid conversion in comparison with the substrate consumed. The fermentation conditions for the strains need to be optimized along with improving the potential of yeast for using cheap carbon sources for studying the maximum lipid accumulation from the strains. Exploration of new sources for isolation of potential lipid producing strains should be focused. The advancement of molecular biology and genetic engineering hold much promise for the genetic manipulation of these microorganisms, so as to improve their performance for producing oils.

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