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Screening and identification of oleaginous moulds for lipid production

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Abstract

Aim : The present study was carried out to isolate the moulds from garden soils of Lalbagh Botanical Garden and Cubbon Park Bangalore City, India and screen them for their oleaginicity.

Methodology : Several types of moulds were isolated randomly from garden soils and screened for their lipid production. Twenty five moulds were screened for their abilities to produce lipids. Potential lipid producers were identified by cultural method and confirmed by ITS region gene Sanger DNA sequencing. The fatty acid profile of two mould isolates was identified using GC-FID.

Results : Interestingly, two isolates such as GS7 and GS12 were found to be promising lipid producers among twenty five screened moulds, and maximum lipid contents found in GS7 and GS12 were 22 and 24.3%, respectively. The mould isolates were identified as *Penicillium citrinum* (GS7) and *Trichoderma asperellum* (GS12) using colony characteristics and Sanger Sequencing. Consensus gene sequences were submitted to NCBI genbank and Accession numbers (KX865284 and KY623504) were obtained. The fatty acid profile of *Pencillium citrinum* showed 56.85% of saturated fatty acid (SFA), 32.20% of monounsaturated fatty acids (MUFA) and 10.95% polyunsaturated fatty acids (PUFA), while *Trichoderma asperellum* showed 47.73% ofsaturated fatty acid (SFA), 25.12% of monounsaturated fatty acids (MUFA) and 27.09% polyunsaturated fatty acids (PUFA), respectively.

Interpretation : GS7 (*Penicillium citrinum*) and GS12 (*Trichoderma asperellum*) described in the study were identified as promising lipid producers. This study would help in better selection of moulds for producing lipids which are suitable for biodiesel production.

Isolation of moulds from garden soils to assess oleaginicity

Screening of isolate for lipid production

Detection of potential lipid producers (GS7 and GS12) using Cultural and ITS gene Sanger Sequencing

Assessment of lipid yield

Identification of fatty acid profile in *Penicillium citrinum* (GS7) and *Trichoderma asperellum* (GS12)

Introduction

Microorganisms have been receiving increased attention as source of novel lipids. Those that accumulate 20% and above are termed as oleaginous and their oils single cell oils (SCOs), unicellular oils or microbial oils (Akpinar-Bayizit, 2014). Microorganisms being ubiquitous have been recognized as potential sources of oils and fats. Oils and fats are both classed as lipids. Though bacteria, algae and yeasts are oleaginous they have some demerits and therefore, recent interest has centered upon moulds. Oleaginous moulds are single cell oil (SCO) producers that are capable of accumulating intracellular lipids under certain culture conditions.These moulds are an attractive source of lipids and show a greater diversity of fatty acids and higher proportion of polyunsaturated fatty acids (PUFA). The naturally isolated organisms are much variable in their characteristics. Screening of organisms for desired characteristics using selective medium is being adopted by various researchers (El-haj *et al*., 2015). Lipid accumulation in oleaginous moulds has been demonstrated to occur when a nutrient in the medium (e.g. the nitrogen or the phosphorous source) becomes limited and the carbon source is present in excess. The main focus of this study is on oleaginous moulds, as they can be easily grown in surface culture under static condition with cheap carbon sources, their life cycle is short and are not affected by space, light and climate, thus having several advantages comparatively to that of plants and algae (Pant *et al*., 2009; Yousuf *et al*., 2010; Khot *et al*., 2012). Molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al*., 2006). In view of the above, in the present study moulds were isolated from garden soils and screened for their oleaginicity and identified by classical and molecular methods.

Materials and Methods

Sample collection and isolation : Twenty five soil samples were collected from 5-15 cm below the surface of different garden soils of Lalbagh Botanical Garden and Cubbon Park, Bangalore city, in sterile plastic containers under aseptic conditions and stored at 4°C until further use. About 1 g of each soil sample was individually suspended in 1ml sterile distilled water, serially diluted to 10 fold and plated on Potato dextrose agar plates with pH 5.6. To suppress the bacterial growth, streptomycin (1 mg per 100 ml) was added to the medium.The plates were incubated at 28°C for 7 days in an incubator. Several mould colonies were obtained from different plates and were purified by picking small sample of hyphal spores, from each plate and transferred repeatedly to a new agar plate until pure cultures were confirmed. The pure cultures of moulds were maintained at 4°C by sub culturing at regular intervals.

Screening for oleaginous moulds : The spore suspension were prepared from well sporulated PDA slant cultures using 10 ml of sterile distilled water with a drop of Tween 80 and the spores were counted using haemocytometer then were used as standard inocula ($1x10^{6}$-10^{8} spores ml^{-1} medium) unless otherwise stated. One ml of inoculum was used to inoculate 50 ml of sterile CzapekDox liquid medium in 250 ml conical flasks.

The CzapekDox liquid medium consisted of $N_aH_2PO_4.2H_2O$; K_2SO_4; $M_gSO_4.7H_2O$; $ZnSO_4.7H_2O$; $FeCl_3.6H_2O$; NH_4NO_3 and Glucose with initial pH 6.8. Fifty ml of sterile medium was dispensed in 250 ml conical flask inoculated with one ml of seed culture ($1x10^{6}$-10^{8} spores ml^{-1}) and then incubated at 28°C under static condition for 8 days.

Biomass dry weight determination : After 8 days of incubation, the culture flasks were harvested by filtration using pre-weighed filter (Whatman No.1) and the mycelia mats were rinsed three times thoroughly with sterile distilled water to remove extraneous materials. The biomass was dried and the weight of dried biomass was calculated.

Residual glucose estimation : The residual glucose in the culture filtrate was estimated by using dinitrosalicylic acid method according to Miller (1959).

Lipid extraction : The dried biomasses were finely ground to obtain the homogenous product, 2-5 g of dry powder was then wrapped by a single thickness filter paper to extract in a Soxhlet extraction apparatus with light petroleum ether (40-60°C) as solvent for 10 hrs. The extracted lipid was dried at 60°C for 30 min, cooled and weighed.

Classical and molecular identification of most promising oleaginous mould isolates : Morphological characteristics (color, texture and diameter of the colonies) were studied by observing the growth on potato dextrose agar medium and microscopic characteristics were performed by staining with Lacto-phenol cotton blue to identify the mould isolates. The molecular identification of two promising oleaginous mould isolates GS7 and GS12 were indentified as *Penicilllium citrinum* and *Trichoderma asperellum*, respectively.

Preparation of genomic DNA : Genomic DNA was extracted using Macherey-Nagel Nucleospin food kit. Genomic DNA concentration was measured using Thermo Scientific Nano Drop 8000. and the average concentration for every sample obtained was about 40-50ng/µl.

Conventional PCR : To a MicroAmp® 96-well reaction plate (0.2 ml), 3µl buffer, 2µl DNTPS, 0.3µl Taq DNA polymerase (NEB, USA), 2µl 5M Betain, 2µl template, 2µl of 20 picomole primer forward, 2 µl primer reverse and 6.7 µl HPLC water was added and then sealed accordingly with the applicator. The ±600 bp product of ITS region gene was amplified by using primer set ITS1-5'CTTGGTCATTTAGAGGAAGTAA3' and ITS4-5'TCCTCCGCTTATTGATATGC3' (Eurofins Genomics India Pvt. Ltd.). Amplification was carried out in Applied BiosystemsVeriti 96 well Thermal cycler system. Amplification by Conventional PCR process was started with initial denaturation step at 94°C for 3

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min). Each cycle consisted of three steps (denaturation, annealing, and extension). Each PCR reaction consisted of 40 cycles of amplification (initial 10 cycles was denaturation at 94°C for 1 min, annealing at 50°C for 1 min and DNA chain extension at 72°C for 1 min, last 30 cycles was denatured at 94°C for 30 sec, annealing at 55°C for 10 sec and DNA chain extension at 72°C for 30 sec). A final extension cycle was performed at 72°C for 5 min (Applied BiosystemsVeriti Thermal Cycler).

PCR products were detected by using Agarose gel electrophoresis. Electrophoresis was performed with 2% Agarose gel (Himedia) prestained with 0.5 µl per 100ml of ethidium bromide (10 mg ml^{-1}). Gels were run at 80V using 1X TAE buffer and then photographed under UV illumination by using a Gel documentation system.

Sanger sequening : Amplified amplicons were purified using QIAquick PCR Purification kit (QIAGEN, Malaysia). The amplicons were sequenced automatically in both directions using BDT v3.1 chemistry, POP7 Polymer on 3730XL Genetic Analyzer. The thermal program was made up of an initial pre-denaturation step at 95°C for 2 min; followed by 25 cycles consisting of a denaturation step at 95°C for 10 seconds, annealing step at 50°C for 10 seconds and an extension step at 60°C for 4 min. Consensus sequences were generated from forward and reverse sequence data using aligner software.

Fatty acid composition and FAME analysis by GC-FID : The fatty acid profile of lipid sample was determined by converting fatty acids in lipid to fatty acid methyl esters (FAME). Lipid was transesterified (obtained after extraction) with 5ml of methylated mixture of methanol:sulfuricacid:toluene (2:1:1) for 8 hrs in a water bath at 50°C. Then FAME was recovered with 3 ml of diethyl ether by vortex and the upper phase was passed through sodium sulphate anhydrous to eliminate the water. The sample was filtered in 0.2 µm nylon filter and recovered. Fatty acid profile was analyzed by using gas chromatography (Agilent 6890N) with flame ionization detector (GC-FID). The column used was DB WAX (30 m x 0.25 mm ID). The chromatographic condition was as follows. The initial temperature of column was 50°C, held for 2 min, then 10°C min^{-1} ramp to 230°C and held for 15 min. The carrier gas was hydrogen at a flow rate of 2 ml min^{-1}. Fatty acid identification was made by comparing with the relative retention time of FAME peaks from sample with standards. The results were recorded and processed using software Chemstation and expressed in relative % of each fatty acid.

Results and Discussion

Accession numbers for 2 isolates *Penicillium citrinum* (KX865284) and *Trichoderma asperellum* (KY623504), based on 100% Sanger Sequence Similarity, was successfully obtained from NCBI Genbank.

The results of screening tests of the moulds for lipid accumulation ability is presented in Table 1. Out of 25 mould isolates screened, only two strains were found to be oleaginous that produced and accumulated more than 20% lipid of their dry weight. GS12 *Trichoderma asperellum* showed highest (24.3%) ability to accumulate lipids followed by GS7 *Penicillium citrinum* (22% of their dry weight). While other mould isolates were able to accumulate lipid less than the standard expected and were considered as non-oleaginous moulds.

In general, those microorganisms which utilized high sugar showed good growth and high lipid accumulation. All microorganisms cannot be considered as source of oils and fats. Organisms which accumulate 20% or more of their biomass as lipid have been termed as Oleaginous (Akpinar-Bayizit, 2014). Nitrogen limitation is the most efficient condition for inducing lipogenesis. During growth phase, nitrogen is necessary for synthesis of proteins and nucleic acids, while carbon flux is distributed among energetic and anabolic processes yielding carbohydrates, lipids, nucleic acids and proteins.

Table 1: Screening of different moulds for their oleaginicity

Mould isolates	Biomass dry weight (gl^{-1})	Total lipids dry weight (gl^{-1})	Lipid percentage to biomass dry weight (%)
GS1	5.4	1.03	19
GS2	5.1	0.56	10.9
GS3	6.2	0.51	8.2
GS4	5.6	0.5	8.9
GS5	6.2	0.86	13.8
GS6	3.74	0.46	12.2
GS7 (Penicillium citrinum)	**4.3**	**0.95**	**22**
GS8	3.56	0.36	10.1
GS9	4.68	0.49	10.4
GS10	5.6	0.72	12.8
GS11	1.17	0.1	8.5
***GS12* (*Trichoderma asperellum*)**	**3.16**	**0.77**	**24.3**
GS13	3.8	0.74	19.4
GS14	3.6	0.25	6.9
GS15	5.6	0.31	5.5
GS16	4.46	0.33	7.3
GS17	5.2	0.8	15.3
GS18	5.02	0.19	3.7
GS19	5.26	0.45	8.5
GS20	4.14	0.22	5.3
GS21	5.5	0.89	16.1
GS22	5.3	0.72	13.5
GS23	4.1	0.64	15.6
GS24	4.68	0.49	10.4
GS25	4.1	0.58	14.1

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Table 2 : Fatty acid methyl ester composition in moulds

Methyl esters of fatty acid		*Pencillium citrinum (%)*	*Trichoderma asperellum (%)*
Total saturated fatty acids (SFA)			
Caproic acid	(C6:0)	0.42	Nil
Caproic acid	(C6:0)	0.42	Nil
Caprillic acid	(C8:0)	0.25	Nil
Capric acid	(C10:0)	Nil	Nil
Lauric acid	(C12:0)	Nil	0.26
Myristic acid	(C14:0)	0.75	0.62
Palmitic acid	(C16:0)	34.99	36.71
Strearic acid	(C18:0)	20.44	10.14
Total manounsaturated fatty acids (MUFA)			
Oleic acid	(C18:1 n9)	32.2	24.47
Erucic acid	(C22:1w9)	Nil	0.72
Total polyunsaturated fatty acids (PUFA)			
Linoleic acid	(C18:2n6c)	Nil	0.42
Linolenic acid	(C18:3n3)	9.92	25.41
Arachidonic acid	(C20:1c)	1.03	0.63
Behenic acid	(C22:0)	Nil	Nil
Lignoceric acid	(C24:0)	Nil	0.63
Recenoleic acid	(C18:0)	Nil	Nil
SFA		56.85	47.73
MUFA		32.2	25.12
PUFA		10.95	27.09

Several reseachers have reported that when nitrogen gets limited, the growth rate slows down and synthesis of proteins and nucleic acids tends to cease, but excess of carbon continues to be metabolized to lipid (Wynn *et al*., 2001; Beopoulos *et al*., 2009). In oleaginous species, the excess carbon is channeled towards lipid synthesis, leading to accumulation of triacylglycerol within intracellular lipid bodies (Ratledge and Wynn, 2002; Akpinar-Bayizit, 2014). Triacylglycerols consist of three fatty acids linked to three carbon with varying properties from fats to oils (Christie, 2003). However, lipid accumulation by microorganisms varies depending on strain specificity of the organisms and various cultural conditions.

Oleaginicity is related to lipid producing ability of moulds.The lipids of moulds (filamentous fungi) show diversity in fatty acids.The lipids of oleaginous microorganisms contain good fraction of saturated fatty acids than unsaturated fatty acids (Papanikolaou *et al*., 2004; Wahlen *et al*., 2011; Venkata Mohan *et al*., 2011).Saturated fatty acids do not have double bonds and unsaturated fatty acids with one double bond are known as monounsaturated fatty acids (MUFA) and with more double bonds are known as polyunsaturated fatty acids (PUFA) (Gurr *et al*., 2008). Thus, the profile of lipids from *Penicillium citrinum* and *Trichoderma asperellum* was studied to find the type of fatty acids present.

The FAME analysis of lipids produced by both *Penicillium citrinum* and *Trichoderma asperellum* is presented in Table 2. *Pencillium citrinum* showed 56.85% SFA, 32.20% MUFA, 10.95% PUFA, while *Trichoderma asperellum* showed 47.73% SFA, 25.12% MUFA and 27.09% PUFA. GC-FID study revealed that the fraction of saturated fatty acids consisted of 34.99% palmitic acid (C16:0), 20.44% strearic acid (C18:0) and MUFA, 32.20% oleic acid (C18:1 n9) and 9.92% linolenic acid (C18:3n3) PUFA in *Pencillium citrinum*. In *Trichoderma asperellum*, 36.71% palmitic acid (C16:0), 10.14% strearic acid (C18:0) SFA, 24.47% oleic acid (C18:1n9) MUFA and 25.41% linolenic acid (C18:3n3) PUFA was noted. A small percentage of fatty acids mainly C6, C8, C14 (SFA), C20 (PUFA) in *Pencillium citrinum* and C12 and C14 (SFA), C22 (MUFA), PUFA C18:2n6c and C20:1c in case of *Trichoderma asperellum* were found in lipid samples. The result of the present study is in agreement with Xie *et al*. (2013) who reported that lipid extract of *Fusarium* sp. ML-GEN.1 mainly contained oleic acid (41.66%), palmitic acid (23.26%) and linoleic acid (19.18%). Similarly, Ziino *et al*. (1999) found that stearic acid and hexadecanoic acid was the most abundant fatty acid isolated from *Geotrichum*. In addition, Thanaa *et al*. (2014) showed that the fatty acids palmitic, linoleic and linolenic acid were predominant in the lipid sample of *Trichoderma viridie* NRC 314.

Palmitic (C16), stearic (C18), oleic (C18) and linolenic (C18) acid in lipid samples tend to give more favorable properties of biodiesel and hence, can be useful for biodiesel production. However, for optimized biodiesel both long-chain saturated and polyunsaturated fatty acids should be present (Wynn *et al*., 2001; Gadallah *et al*., 2014).

In conclusion, several fungal isolates were screened for their lipid production ability. Two of them GS7 (*Penicillium citrinum*) and GS12 (*Trichoderma asperellum*) were identified as promising lipid producers with maximum lipid yield and favorable for biodiesel production.

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