PREPARATIVE BIOCHEMISTRY AND BIOTECHNOLOGY
https://doi.org/10.1080/10826068.2018.1431783

Taylor & Francis
Taylor & Francis Group

Bioprospecting microbes for single-cell oil production from starchy wastes

Shivani Chaturvedi[a], Arti Kumari[a], Lata Nain[b], and Sunil K. Khare[a]

[a]Enzyme and Microbial Biochemistry Lab, Department of Chemistry, Indian institute of Technology, Delhi, India; [b]Division of Microbiology, ICAR- Indian Agricultural Research Institute, New Delhi, India

ABSTRACT

Production of lipid from oleaginous yeast using starch as a carbon source is not a common practice; therefore, the purpose of this investigation was to explore the capability of starch assimilating microbes to produce oil, which was determined in terms of biomass weight, productivity, and lipid yield. *Saccharomyces pastorianus, Rhodotorula mucilaginosa, Rhodotorula glutinis*, and fungal isolate *Ganoderma wiiroense* were screened for the key parameters. The optimization was also performed by one-factor-at-a-time approach. Considering the specific yield of lipid and cell dry weight yield, *R. glutinis* and *R. mucilaginosa* showed superiority over other strains. *G. wiiroense*, a new isolate, would also be a promising strain for starch waste utilization in terms of extracellular and intracellular specific yield of lipids. Extracellular specific yield of lipid was highest in *R. glutinis* culture (0.025 g g^{-1} of biomass) followed by *R. mucilaginosa* (0.022 g g^{-1} of biomass) and *G. wiiroense* (0.020 g g^{-1} of biomass). Intracellular lipid was again highest in *R. glutinis* (0.048 g g^{-1} of biomass). The most prominent fatty acid methyl esters among the lipid as detected by GC-MS were saturated lipids mainly octadecanoic acid, tetradecanoate, and hexadecanoate. Extracellular lipid produced on starch substrate waste would be a cost-effective alternative for energy-intensive extraction process in biodiesel industry.

KEYWORDS

Amylase; microbial oil; oleaginous yeast; starchy waste

Introduction

In general, biodiesel has characteristics quite similar to fossil fuel.[1] It is biodegradable, renewable, nontoxic, and safer to the environment.[2] Globally, renewable biofuels such as bioethanol and biodiesel can reduce consumption of traditional fuels.[3] At present, most of the biodiesel originate from plant oil, but microbial oil have additional benefits like being less labor intensive, climate resistant, and easier to scale up.[4]

There is also an increased focus on microbial oil due to their biotechnological applications such as production of bioplastics and utility as food ingredients.[5] Oleaginous microorganisms, mainly yeasts are more versatile due to their very simple cultural requirements as well as growth under aerobic conditions. The yeasts also can be grown on substrates with higher C/N ratio (>30) to initiate triglyceride accumulation within their cells.[6,7] Oleaginous yeasts were able to produce more than 20% of their biomass weight as triglycerides.[8] Under nitrogen-limiting conditions, the amount of lipids increased up to 70% of their biomass.[9]

The lipids so produced possess C16 and C18 fatty acids esterified in the form of triacylglycerol which are similar to the plant-originated biodiesel.[10] However, microbial oil cost is higher than plant and animal oil. Therefore, there is a need to reduce the production cost. One such cost-reducing alternative is to utilize the carbohydrate-rich biomass as substrates to grow yeast.[11] In general, food wastes contain high starch content and can be considered a cheap and cost-effective substrate for microbial oil production.

In the present work, commonly available starch sources were investigated as a substrate for growth of different oleaginous strains and their performance for the production of lipids was evaluated. Although, there is prior research demonstrating the presence of lipids in the cell of oleaginous yeasts, but there has been very limited comprehensive screening for oleaginous yeast producing lipids from real-life starch waste substrate like tapioca having C/N ratio 28.1 which is ideal for oleaginous microbes as reported by earlier researches.[6,7] The microbes were also evaluated to produce amylase enzymes which will hydrolyze the starch into monomers, thereby pretreatment step will be avoided. The microbes were screened to have consolidated system to preserve energy and resources of this critical step.

Materials and methods

p-Nitrophenyl palmitate (*p*NPP) and soluble starch were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and Merck Darmstadt Germany. All other chemicals used were of analytical grade.

Yeast strains used in the study

Saccharomyces pastorianus ATCC26602, *Rhodotorula mucilaginosa* Y-1, *Rhodotorula glutinis* NRRL Y-1091 were obtained from laboratory culture collection of Indian Institute of Technology, Delhi, whereas "Un" was isolated from

CONTACT Shivani Chaturvedi shivani.d123@gmail.com Enzyme and Microbial Biochemistry lab, Department of Chemistry, Indian institute of Technology, Delhi, India.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/lpbb.

discarded starchy substrate. The cultures were maintained on yeast, peptone and dextrose (YPD) agar slants at 4°C and were regularly subcultured at an interval of 1 month.

Identification of the isolated strain (Un) was performed on the basis of 18S rRNA gene sequence. Genomic DNA was isolated as per standard protocol and ~600 bp PCR product was obtained using high-fidelity PCR Taq polymerase. The sequence of PCR product was compared with NCBI database. Taxonomic positioning and construction of phylogenetic tree of the isolate were done by seq. scape v5.2 software (Weighbor).

Screening of yeast for oil production

All the four microbial strains were screened for lipid production by growing them in YPD medium[12]; and subsequently screened for oil production by Nile red staining to detect intracellular lipid droplets by fluorescence microscopy.[13,14] Nile red solution was prepared under reduced light by dissolving 0.1 mg of Nile red in 1 mL of acetone.

The broth culture was centrifuged (2000 rpm for 5 min) and supernatant was discarded. The cells were suspended in 1 mL of phosphate buffer solution (10 mM pH 7.4) and the process was repeated two to three times. The cells were than stained with 10 µL of Nile red solution. The cells were analyzed with fluorescence microscope (Nikon DS-F12, Japan) under 450–500 nm wave lengths.

Cultivation of microbial strains for bio-oil production

Preparation of inoculum

Inoculum was prepared by transferring loop full of culture into YPD broth (pH 7.0) containing gram per liter: yeast extract, 10; peptone, 20; dextrose, 20. Thereafter, the inoculated medium was incubated at 30°C, 120 rpm for 2 days in an incubator shaker.

Culture conditions for lipid production

Selected cultures were incubated for growth and lipid production both under solid-state fermentation (SSF) and submerged fermentation. In the case of SSF, the production was performed in basal medium having 0.5% ammonium sulfate, 0.05% magnesium sulfate, 0.01% sodium chloride, 0.01% calcium chloride supplemented with 10% (w/v) starch. Five kinds of starch (cationic starch, tapioca starch, soluble starch, high-viscosity and low-viscosity starch) were investigated for oil production.

For submerged fermentation, yeast phosphate-soluble starch (YPSs) medium was used.[15] This submerged production media contain 1.5% soluble starch, 0.4% yeast extract, 0.1% potassium monophosphate, 0.05% magnesium sulfate with 7 pH. The media were sterilized at 15 bps pressure for 15 min.

All the flasks were inoculated with individual yeast broth culture at 1% (v/v). These inoculated flasks were incubated at 130 rpm and 30°C temperature for 36 days. Samples were collected at a regular interval of time and intracellular product yield of lipid (YP/S)/enzymes were recovered by sonication followed by centrifugation at 4000 rpm for 12 min. The culture filtrate was used for assay of amylase, lipase using standard protocols. After incubation, both in submerged and SSF fermentation, media filtration was performed followed by separation of cell biomass for intracellular lipid extraction process. The filtrate of this process was kept for half an hour in the mixture of chloroform and methanol (2:1). Subsequently, the extracellular lipid extraction has been performed following the procedure similar to that of intracellular lipid extraction. The oil extraction for intracellular lipid from cell biomass was performed using liquid nitrogen drying followed by vigorous maceration and sonication of the culture sample at 40 pulses four times for duration of 3 min using Biologics inc., model 3000, ultrasonic homogenizer.[16]

Amylase assay in culture filtrate

Amylase was assayed by the method of Bernfeld,[17] using starch as a substrate. One milliliter of reaction mixture contained 500 µL of soluble starch (2%, w/v) in 50 mM sodium phosphate buffer (pH 7.0) containing 1% (w/v) sodium chloride and 500 µL culture filtrate. Reaction was performed for 20 min at 50°C temperature in water bath. The reducing sugars released were estimated using 3,5-dinitrosalicylic acid.[18] One unit of amylase activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute from soluble starch under assay condition.

Lipase assay in culture filtrate

Lipase activity was estimated by *p*NPP as a substrate following the method of Kilcawley et al.[19] Mixture of 1.8 mL of solution containing 0.15 M NaCl and 0.5% Triton X-100 in 0.1 M Tris–HCl buffer (pH 8.0) was preincubated at 40°C with 200 µL of cell-free culture supernatant. Substrate (50 mM *p*NPP in acetonitrile) of 20 µL was added to the reaction mixture and incubated at 40°C for 30 min. The liberated amount of *p*-nitrophenyl (*p*NP) was recorded at 400 nm. One unit of lipase activity was defined as the amount of enzyme liberating 1 µmol of *p*NP per minute under standard assay conditions.

Estimation of yeast biomass or cell dry weight

After incubation, the biomass was filtered with membrane filter (0.45 µm pore) through millipore filtration system. The biomass accumulated at membrane after filtration was dried and quantified by gravimetric method for cell dry weight.[16]

Disruption of cell wall for lipid quantification

Biomass was macerated in liquid nitrogen with the help of mortar and pestle. The resultant biomass was then suspended in distilled water and sonicated under cold condition for 3 min, 40 pulses (five times) using Ultrasonic homogenizer (model 3000, Biologics Inc. USA). The homogenized preparation was then added to 20 mL mixture of chloroform and methanol (2:1) and was kept for half an hour.[16]

Lipid extraction process

The crude extract after filtration was collected and diluted 0.2 times with distilled water. The diluted sample was vigorously mixed and was kept at rest for complete separation of the biphasic system. Without disturbing the interface, the upper phase was removed with the help of pipette. After the removal of upper phase, 10 mL of methanol was added to the lower phase, containing extracted lipid part. The lower phase was extracted repeatedly and collected in a preweight container. The container was then kept in an incubator (Shaker Lab therm Kuhner, Switzerland) at 37–40°C for complete evaporation of the solvent, and the amount was quantified by gravimetric method. The specific yield of lipid (YP/X) was represented as gram of lipid per gram of biomass.[20]

Qualitative detection of lipids in oil

The lipid composition of oil was determined by the method of Hartman and Lago by methylating the lipids into fatty acid methyl esters (FAME), which were detected by GC-MS (Agilent Technology 7889B gas chromatograph, coupled with mass spectrometer, USA).[21] The temperature of column was initially maintained at 80°C for 1.0 min, then raised to 220°C for 8 min at the rate of 8°C min^{-1}, followed by final increase to 280°C for 2.5 min at the rate of 15°C min^{-1}. The sample was operated in split mode. The carrier gas (helium) flow rate in column was 1.0 mL min^{-1}. Inbuilt National Institute of Standards and Technology standard mass spectral library was used for the identification of individual FAME in the bio-oil.

Results and discussion

Identification and characterization of isolated strain

The isolated strain showed 99.9% similarity with *Ganoderma wiiroense* in 18S rDNA sequence analysis. The sequence was submitted to NCBI database with an Accession no. MF774620 NCBI. *Ganoderma* is a well-known mushroom with medicinal qualities[22] but is not being reported as an oil producer, as far as published literature is available.[23]

Growth and utilization of different starch by selected isolates

Five types of starch (namely, cationic starch, real-life tapioca or cassava starch substrate, soluble starch, high-viscosity and low-viscosity starch) were utilized by standard strain *S. pastorianus* and amylase activity was detected up to 36th day of incubation. Soluble starch was the best carbon source in terms of amylase activity (5.0 IU mL^{-1}) on the 9th day of incubation. Soluble and tapioca starch recorded decline in amylase activity on 26th day (Figure 1). While higher amylase activity was detected in the case of cationic, high-viscosity and low-viscosity starch turned up after the 26th day. It is known that most of the yeast cannot directly assimilate starch for the lipid production.[24] Starch need to be degraded with the help of extracellular amylases in sugars for assimilation.[25] Decline in amylase activity after 26th day is actually demonstrated by slowing down in yeast metabolism and initiation of lipid formation process. On the basis of amylase and reducing sugar pattern, soluble and real-life tapioca starch substrate were selected as a substrate for further SSF study. The optimum values for tapioca waste as real-life substrate 10% amount with temperature 30°C and pH 5 displayed for best lipid production by microbe.

FAME analysis of SSF products

Under SSF product yield (YP/S), 1.8 g lipid g^{-1} of tapioca starch substrate was produced by *G. wiiroense* followed by soluble starch product yield 1.3 g lipid g^{-1} substrate by the same strain. The oil were used for GC-MS analysis and identified as octadecanoic acid ($C_{20}H_{40}O_2$), ethyl 13 methyl tetra decanoate ($C_{17}H_{34}O_2$), hexadecanoic acid ($C_{18}H_{36}O_2$), and oleic acid ($C_{18}H_{34}O_2$). Earlier researchers have reported the presence of palmitic (C16:0) and oleic (C18:1) acids in fatty acid in *R. glutinis*.[26,27] In biodiesel, FAME component contributes toward the quality of oil and ultimately decides the specific gravity of oil. In our study, long incubation and presence of lipase might have caused the degradation of fats into fatty acid. Therefore, further experiments were performed under submerged condition where lipase activity was also quantified along with amylase.

Oil production under submerged fermentation with soluble starch

Since soluble starch was found to be the most suitable substrate for induction of amylase production within 9 days, further experiment was performed with soluble and tapioca starch as a carbon source under submerged production. To compare the lipid production, three yeast, namely, *S. pastorianus, R. glutinis*, and *R. mucilaginosa* and one fungal isolate *G. wiiroense* were used in this study. Assay of amylase and lipase revealed that enzyme activity was highest in *R. glutinis* and lowest in *G. wiiroense* strain (Figure 2). Highest coproduction of lipase (51.18 IU mL^{-1} min^{-1}) and amylase (0.29 IU mL^{-1} min^{-1}) was recorded in *R. glutinis* (Figure 2), while lowest activity of lipase (14.64 IU mL^{-1} min^{-1}) and amylase (0.3 IU mL^{-1} min^{-1}) was detected in *G. wiiroense*. Lower value of lipase reduces the chance of lipid degradation resulting in better accumulation of oil.

Estimation of microbial biomass or cell dry weight and product yield of lipid under submerged conditions

The cell dry weight (g L^{-1}) and product yield of lipid (YP/S; g lipid g^{-1} substrate) were quantified after 30 days of fermentation. Cell dry weight and product yield of lipid both had shown greater values at higher concentration during submerged conditions. This may be due to the easy availability of starch material in its monomeric forms. *G. wiiroense* with 20% tapioca starch substrate and another set 25% of soluble starch substrate displayed highest biomass or cell dry weight of 0.69 g L^{-1} or cell dry weight yield (YX/S; g biomass g^{-1} substrate) 0.79 g biomass g^{-1} substrate and 0.71 g L^{-1} or cell dry weight yield 0.50 g biomass g^{-1} substrate, respectively.

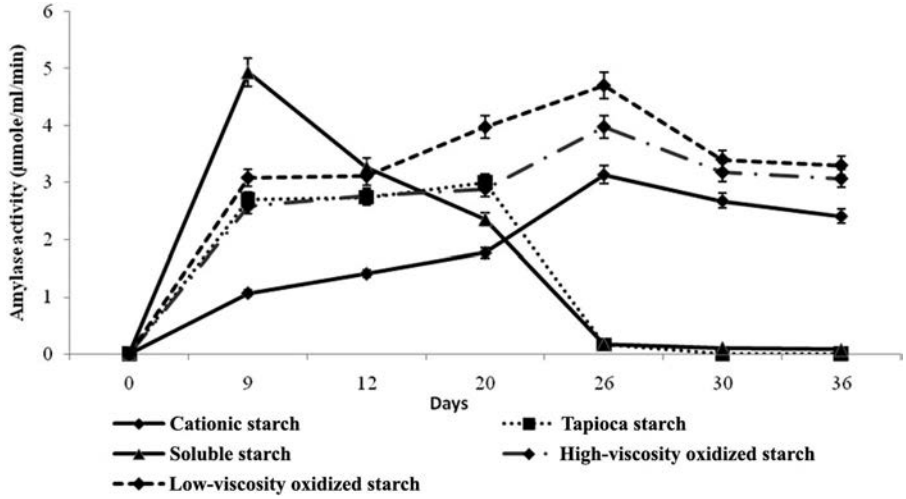

Figure 1. Amylase activity production in *Saccharomyces Pastorianus* during solid-state fermentation of different kinds of starch. *S. Pastorianus* was inoculated (2%) to different starch substrates (10%) and were inCubated for 36 days at 30°C and 120 rpm. During the course of inCubation, amylase activities were estimated in different starchy substrates.

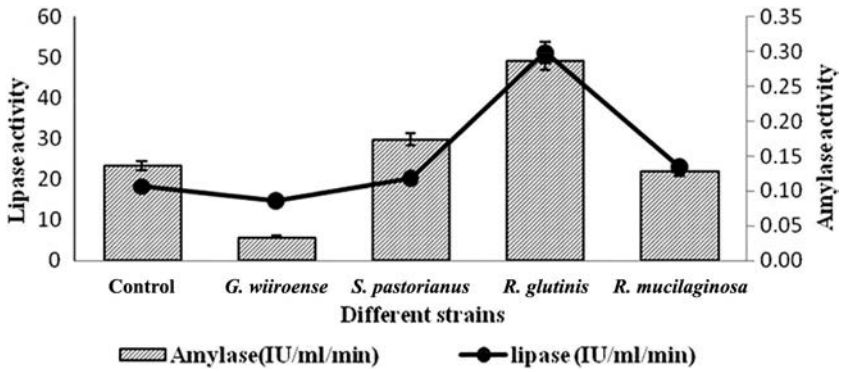

Figure 2. Amylase and lipase activity in microbial strain under submerged fermentation of soluble starch. Soluble starch (10%) containing medium was inoculated with 2% of overnight-grown different microbial cultures followed by inCubation at 30°C and 120 rpm. Lipase and amylase activities were determined after 30 days of inCubation.

The product yield of lipid also follows the same trend; 0.79 g g^{-1} of substrate, 0.28 g g^{-1} of substrate highest in *G. wiiroense* with 20% tapioca, and 25% of soluble starch substrate concentration (Figure 3).

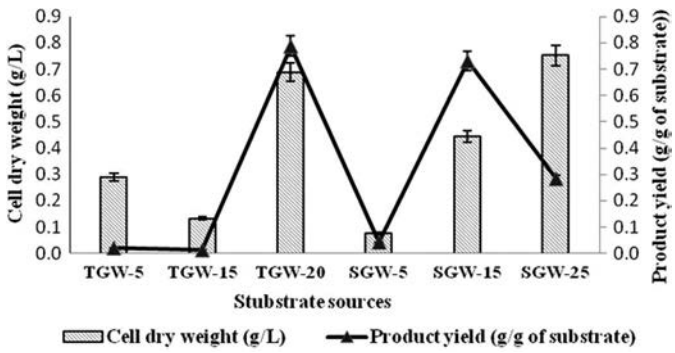

Figure 3. Biomass and weight of lipid under the submerged condition with the help of *Ganoderma wiiroense*, strain with tapioca and soluble starch. TGW: tapioca starch with strain *G. Wiiroense*; SGW: soluble starch with strain *G. Wiiroense*; 5, 15, 20, and 25: percentage of particular starch used *G. Wiiroense*, strain was grown in media containing tapioca and soluble starch for 30 days of inCubation. Thereafter, weight of lipid and biomass content were quantified.

High product yield of lipid and cell dry weight yield were displayed in higher concentration with soluble and tapioca starch in the presence of *G. wiiroense* (in 20% tapioca starch substrate product yield of lipid is 0.09 g lipid g^{-1} of substrate, in 15% soluble starch substrate production is 0.07 g lipid g^{-1} of substrate). With 20% tapioca 0.05 g lipid g^{-1} of biomass yield has been recorded and at par with this value 15% soluble starch substrate-specific yield of lipid 0.048 g lipid g^{-1} of biomass has been detected (Figure 4). Poontawee et al.,[28] Knot and Ghosh,[29] and Johnravi et al.[30] have determined lipid product yield of 0.01–0.17 g lipid g^{-1} of substrate. Same authors also estimated growth yield coefficient or cell dry weight (YX/S) in the range of 0.09–0.29 g biomass g^{-1} of substrate. Optimization of conditions will further increase the lipid product yield and specific yield (YP/X; g lipid g^{-1} biomass).

Cell dry weight, extracellular and intracellular lipid product yields were estimated in all four strains *G. wiiroense, S. pastorianus, R. glutinis*, and *R. mucilaginosa* with soluble starch substrate as a source of carbon. Highest cell dry weight 0.18 g L^{-1} was recorded in *R. mucilaginosa* followed by *G. wiiroense* (0.11 g L^{-1}) and *R. glutinis* (0.11 g L^{-1}).

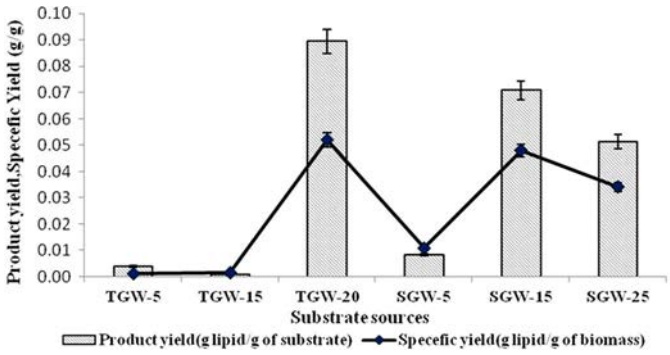

Figure 4. Productivity and yield of lipid by *Ganoderma wiiroense*, strain under submerged fermentation of tapioca and soluble starch. TGW: tapioca starch with strain *G. Wiiroense*; SGW: soluble starch with strain *G. Wiiroense*; 5, 15, 20, & 25: percentage of particular starch used *G. Wiiroense*, strain was grown in submerged media containing tapioca and soluble starch for 30 days of inCubation. Thereafter, yield of lipid and productivity were observed.

Extracellular lipid product yield was highest in *R. glutinis* (0.025 g lipid g^{-1} of substrate) followed by *R. mucilaginosa* (0.021 g lipid g^{-1} of substrate) and *G. wiiroense* (0.020 g lipid g^{-1} of substrate). Intracellular lipid product yield was observed highest in *R. glutinis* (0.048 g lipid g^{-1} of substrate), whereas *R. mucilaginosa* (0.027 g lipid g^{-1} of substrate), *S. pastorianus* (0.014 g lipid g^{-1} of substrate), and *G. wiiroense* displayed comparable amount (Figure 5). Poli et al.[16] recorded dry biomass of range 0.17–0.41 and total lipid of range 0.01–0.09 g using *Yarrowia lipolytica* QU21.

Identification of FAME in submerged fermentation

GC-MS results displayed a typical trend among four strains (Table 1). *G. wiiroense* and *S. pastorianus* lack extracellular lipids, whereas they were rich in intracellular lipid content. Since intracellular lipid will need disruption of cell, selection of a strain capable of producing extracellular lipid will be more beneficial and economical.

R. glutinis and *R. mucilaginosa* produced only extracellular lipids and no intracellular lipids were detected. This needed less energy and efforts to obtain lipid from medium. Bhosale and Gadre[31] reported cell growth and carotenoid production on starch using *R. glutinis*. Schneider et al.,[32] observed significant reduction of starch assuming that *R. glutinis* develops the enzymatic system with time for utilization of starch.

The important FAME produced under submerged condition as intracellular lipids by *G. wiiroense* and *S. pastorianus* were methyl tetra decanoate ($C_{15}H_{30}O_2$), methyl 13 methyltetradecanoate ($C_{16}H_{32}O_2$), pentadecanoic acid ($C_{15}H_{30}O_2$), hexadecanoic acid ($C_{34}H_{68}O_2$), and dodecanoic acid ($C_{14}H_{28}O_2$). *R. glutinis* and *R. mucilaginosa* under the same kind of submerged condition produced extracellular lipid in the form of FAME. The key FAME were methyl 13 methyltetradecanoate ($C_{16}H_{32}O_2$), cyclopentane undecanoic acid methyl ester ($C_{17}H_{32}O_2$), methyl 18 fluro octadecanoate ($C_{19}H_{37}FO_2$), and 9 hexadecanoic acid methyl ester ($C_{17}H_{32}O_2$). All these standard fatty acids may result in better specific gravity of oil similar to vegetable oil/biodiesel.[33,34] Ali et al.[33] screened the lipid profile of a fungus *Penicillium brevicompactum* NRC829 and found a wide range of fatty acid profile. In the present study, many similar types of fatty acids were detected. Asghar et al.[34] did GC-MS of *Iris germanica*, the compound identified by them also matched with the present findings. According to Khot and Ghosh[35] *R. mucilaginosa* IIPL32 yeast produced lipid, which was found to be rich in monounsaturated fatty acid (MUFA), similarly in the present work MUFA was recorded in high percentage. The fatty acid composition of vegetable oil reported by Ramos et al.[36] has similarity with present findings, they also found myristic, palmitic, palmitoleic, stearic, and behnic acids. This type of fatty acid profile is suitable for the ideal biodiesel feedstock as reported by Khot and Ghosh,[35] *R. mucilaginosa* proved to be a great potential condition as a biofuel producer having better oxidative stability, cetane number, and cold flow properties on the basis of its high MUFA, with balanced saturated and poly unsaturated (PUFA) fatty acid esters.

In a certain study by knot et al.,[29] *Aspergillus terreus* IBB M1 was found to produce FAME (C12-C18) by fermentation of copra cake under optimal conditions. The yeast/fungi can produce up to 30% (w/w) lipid[35,37] with sugary substrate, e.g., glucose or molasses within 80–120 hr.[36,38] Since starch

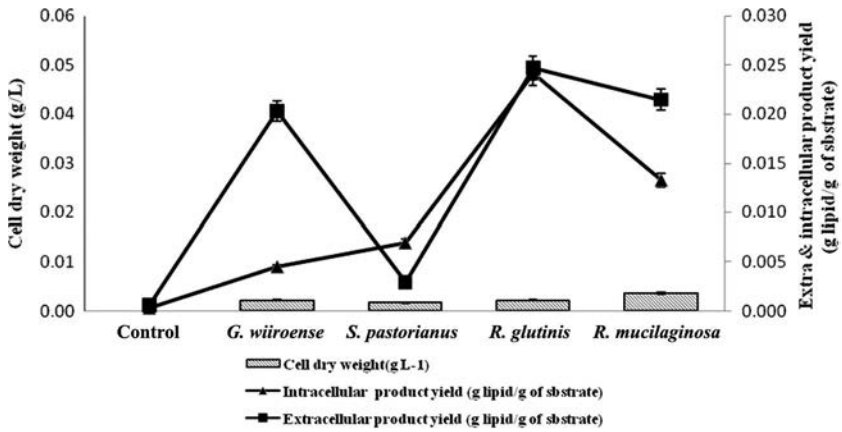

Figure 5. Extracellular and intracellular production of lipid by *Ganoderma wiiroense, Saccharomyces pastorianus, Rhodotorula glutinis*, and *Rhodotorula mucilaginosa* strains under submerged condition with soluble starch as a carbon source Different microbial cultures were grown under submerged condition using soluble starch as a carbon source for 30 days at 30°C and 120 rpm. Weight of biomass, and extracellular and intracellular lipids were recorded at the end of inCubation.

Table 1. Identification of FAME formation produced by microbial strain under submerged fermentation.

Common name	Systematic name	Molecular formula	Microorganisms	Lipid produced in	Molecular weight (g/mol)	Lipid number	Percentage of each fatty acid
–	–	–	Ganoderma *wiiroense*	Extracellular	–	–	–
Octadecanoic acid	Methyl 18 fluro octadecanoate	$C_{19}H_{37}FO_2$		Intracellular	316.50	C18:0	0.738
Tribehnic acid	Docosanoic acid 1,2,3propanetriyl ester	$C_{69}H_{134}O_6$			1058.82	C22:0	
Myristic acid	Methyl tetra decanoate	$C_{15}H_{30}O_2$			242.40	C14:0	1.162
Octacatrienoic acid	9,12,15 octadecatrienoic acid	$C_{25}H_{40}O_6$			436.58		3.400
Tetradecanoic acid	Methyl 13 methyltetradecanoate	$C_{16}H_{32}O_2$			256.43	C14:0	
Pentadecylic acid	Pentadecanoic acid	$C_{15}H_{30}O_2$			242.40	C15:0	3.942
–	–	–	Saccharomyces *pastorianus*	Extracellular	–	–	–
Myristic acid	Dodecanoic acid	$C_{14}H_{28}O_2$		Intracellular	228.37	C14:0	1.684
Tridecylic acid	Tridecanoic acid	$C_{13}H_{26}O_2$			214.34	C13:0	1.488
Geddic acid	Hexadecanoic acid	$C_{34}H_{68}O_2$			508.91	C34:0	2.235
Tetradecanoic acid	Methyl13 methyltetra decanoate	$C_{16}H_{32}O_2$			256.43	C14:0	1.957
Myristic acid	Dodecanoic acid	$C_{14}H_{28}O_2$	Rhodotorula *glutinis*	Extracellular	228.37	C14:0	24.528
Tetradecanoic acid	Methyl 13 methyltetradecanoate	$C_{16}H_{32}O_2$			256.43	C14:0	24.914
–	–	–		Intracellular	–	–	–
Tetradecanoic acid	Methyl 9 methyltetradecanoate	$C_{16}H_{32}O_2$	Rhodotorula *mucilaginosa*	Extracellular	256.43	C14:0	10.258
Palmitelaidic acid	Cyclopentane undecanoic acid methyl ester	$C_{17}H_{32}O_2$			268.43	C16:0	5.024
Octadecanoic acid	Methyl 18 fluro octadecanoate	$C_{19}H_{37}FO_2$			316.50	C18:0	3.648
Palmitelaidic acid	9hexadecanoic acid methyl ester	$C_{17}H_{32}O_2$			268.43	C16:0	26.108
–	–	–		Intracellular	–		

is used for the production of oil in this study, further optimization is needed to increase the productivity of lipid.

In the present study, oleaginous yeast showed significant amount of extracellular fatty acids by directly assimilating starch and no separate step was needed for enzymatic hydrolysis of substrate which validates the cost-effectiveness of starch as a cheap source of carbon. Moreover, pelleted growth of fungal strain will facilitate easier downstream processing. The presence of extracellular lipid also reduces the cost and energy consumption of long tedious extraction process for breaking the cell wall of microbes. Novelty to this research work is the report of *G. wiiroense* basidomycetes for intracellular lipid production. As per our knowledge, this is the first report of single-cell oil (SCO) production by basidomycetes using starch as a carbon source.

Conflicts of interest

All authors declared that they have no conflicts of interest.

Funding

The author (Shivani Chaturvedi) is thankful for financial support from University grant commission Post-doctoral fellowship program, UGC award letter number F.15-1/2017/PDFWM-2017-18-DEL-3915(SA-II) from UGC, India.

References

[1] International Energy Agency. *Renewable Energy Out-look, World Energy Outlook 2012*; IEA Publications: Paris, 2012; p 211.

[2] Sharma, Y. C.; Singh, B. Development of Biodiesel: Current Scenario. *Renewable Sustainable Energy Rev.* **2008**, *13*, 1646–1651. DOI: 10.1016/j.rser.2008.08.009.

[3] Fischer, C. R.; Klein-Marcuschamer, D.; Stephanopoulos, G. Selection and Optimization of Microbial Hosts for Biofuels Production. *Metab. Eng.* **2008**, *10*, 295–304. DOI: 10.1016/j.ymben.2008.06.009.

[4] Liang, M. H.; Jiang, J. G. Advancing Oleaginous Microorganisms to Produce Lipid Via Metabolic Engineering Technology. *Prog. Lipid Res.* **2013**, *52*, 395–408. DOI: 10.1016/j.plipres.2013.05.002.

[5] Amaretti, A.; Raimondi, S.; Sala, M.; Roncaglia, L.; Lucia, M. D. E.; Leonardi, A.; Rossi, M. Single Cell Oils of the Cold-Adapted Oleaginous Yeast Rhodotorula glacialis DBVPG 4785. *Microb. Cell Fact.* **2010**, *9*, 1–6. DOI: 10.1186/1475-2859-9-73.

[6] Możejko-Ciesielska, J.; Kiewisz, R. Bacterial Polyhydroxyalkanoates: Still Fabulous? *Microbiol. Res.* **2016**, *192*, 271–282. DOI: 10.1016/j.micres.2016.07.010.

[7] Dasguptaab, D.; Ghoshab, D.; Bandhu, S.; Adhikaria, D. K. Lignocellulosic Sugar Management for Xylitol and Ethanol Fermentation with Multiple Cell Recycling by *Kluyveromyces marxianus* IIPE453. *Microbiol. Res.* **2017**, *200*, 64–72. DOI: 10.1016/j.micres.2017.04.002.

[8] Papanikolaou, S.; Aggelis, G. Lipids of Oleaginous Yeasts. Part I: Biochemistry of Single Cell Oil Production. *Eur. J. Lipid Sci. Technol.* **2011a**, *113*, 1031–1051. DOI: 10.1002/ejlt.201100014.

[9] Papanikolaou, S.; Aggelis, G. Lipids of Oleaginous Yeasts. Part II: Technology and Potential Applications. *Eur. J. Lipid Sci. Technol.* **2011b**, *113*, 1052–1073. DOI: 10.1002/ejlt.201100015.

[10] Li, Q.; Du, W.; Liu, D. Perspectives of Microbial Oils for Biodiesel Production. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 749–756. DOI: 10.1007/s00253-008-1625-9.

[11] Vicente, G.; Bautista, L. F.; Rodriguez, R.; Gutiérrez, F. J.; Sadaba, I.; Ruiz-Vazquez, R. M. Biodiesel Production from Biomass of An Oleaginous Fungus. *Biochem. Eng. J.* **2009**, *48*, 22–27. DOI: 10.1016/j.bej.2009.07.014.

[12] Kricka, W.; Fitzpatrick, J.; Bond, U. Metabolic Engineering of Yeasts by Heterologous Enzyme Production for Degradation of Cellulose and Hemicellulose from Biomass: A Perspective. *Front. Microbiol.* **2014**, *5*, 174. DOI: 10.3389/fmicb.2014.00174.

[13] Sherman, F. Getting started with yeast. *Methods Enzymol.* **1991**, *194*, 3–21.

[14] Greenspan, P.; Mayer, E. P.; Fowler, S. D. Nile Red: A Selective Fluorescent Stain for Intracellular Lipid Droplets. *J. Cell Biol.* **1985**, *100*, 965–973. DOI: 10.1083/jcb.100.3.965.

[15] Kimura, K.; Yamaoka, M.; Kamisaka, Y. Rapid Estimation of Lipids in Oleaginous Fungi and Yeasts Using Nile Red Fluorescence. *J. Microbiol. Methods* **2004**, *56*, 331–338. DOI: 10.1016/j.mimet.2003.10.018.

[16] Harrigan, W. F.; McCance, M. E. *Laboratory Methods in Microbiology*; Academic Press: London, UK, 1966; p 342.
[17] Poli, J. S.; Dallé, P.; Senter, L.; Mendes, S.; Ramirez, M.; Vainstein, M.; Valente, P. Fatty Acid Methyl Ester Produced by Oleaginous Yeast Yarrowia Lipolytica QU21: An Alternative for Vegetable Oils. *Revista Brasileira de Biociências* **2013**, *11*, 203–208.
[18] Bernfeld, P. *Amylase, α and β, in Methods in Enzymology*; Colowick, S. P., Kaplan, N. O. Eds.; Methods in enzymology. Academic Press: New York, NY, USA, 1955; pp 149–158.
[19] Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. DOI: 10.1021/ac60147a030.
[20] Kilcawley, K. N.; Wilkinson, M. G.; Fox, P. F. Determination of Key Enzyme Activities in Commercial Peptidase and Lipase Preparations from Microbial or Animal Sources. *Enzyme Microb. Techno.* **2002**, *31*, 310–320. DOI: 10.1016/s0141-0229(02)00136-9.
[21] Folch, J.; Lees, M.; Stanley, G. H. S. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. *J. Biol. Chem.* **1957**, *226*, 497–509.
[22] Hartman, L.; Lago, R. C. Rapid Preparation of Fatty Acid Methyl Esters from Lipids. *Lab. Pract.* **1973**, *22*, 475–476.
[23] Ferreira, I.C. F.R.; Heleno, S. A.; Reis, F. S.; Stojkovic, D.; Queiroz, M.J. R.P.; Vasconcelos, M. H.; Sokovic, M. Chemical Features of Ganoderma Polysaccharides with Antioxidant, Antitumor and Antimicrobial Activities. *Phytochemistry* **2015**, *114*, 38–55. DOI: 10.1016/j.phytochem.2014.10.011.
[24] Obodai, M.; Mensah, D. L. N.; Fernandes, A.; Kortei, N. K.; Dzomeku, M.; Teegarden, M.; Schwartz, S. J.; Barros, L.; Prempeh, J.; Takli, R. K.; et al. Chemical Characterization and Antioxidant Potential of Wild Ganoderma Species from Ghana, *Molecules* **2017**, *22*, 196. DOI: 10.3390/molecules22020196.
[25] Spencer-Martins, I.; Vanuden, N. Yields of Yeast Growth on Starch. *Eur. J. Appl. Microbiol.* **1977**, *4*, 29–35. DOI: 10.1007/bf01390667.
[26] Tanimura, A.; Takashima, M.; Sugita, T.; Endoh, R.; Kikukawa, M.; Yamaguchi, S.; Sakuradani, E.; Ogawa, J.; Ohkuma, M.; Shima, J. *Cryptococcus terricola* is a Promising Oleaginous Yeast for Biodiesel Production from Starch through Consolidated Bioprocessing. *Sci. Rep.* **2014**, *4*, 4776. DOI: 10.1038/srep04776.
[27] Saenge, C.; Cheirslip, B.; Suksaroge, T. T.; Bour-Toom, T. Efficient Concomitant Production of Lipids and Carotenoids by Oleaginous Red Yeast *Rhodotorula glutinis* Cultured in Palm Oil Mill Effluent and Application of Lipids for Biodiesel Production. *Biotechnol. Bioprocess Eng.* **2011**, *16*, 23–33. DOI: 10.1007/s12257-010-0083-2.
[28] Poontawee, R.; Yongmanitchai, W.; Limtong, S. Efficient Oleaginous Yeasts for Lipid Production from Lignocellulosic Sugars and Effects of Lignocellulose Degradation Compounds on Growth and Lipid Production. *Process Biochem.* **2017**, *53*, 44–60. DOI: 10.1016/j.procbio.2016.11.013.
[29] Knot, M.; Ghosh, D. Lipids of *Rhodotorula mucilaginosa* IIPL32 with Biodiesel Potential: Oil Yield, Fatty Acid Profile, Fuel Properties. *J. Basic Microbiol.* **2017**, *9999*, 1–8. DOI: 10.1002/jobm.201600618.
[30] Johnravindar, D.; Karthikeyan, O. P.; Selvam, A.; Murugesan, K.; Wonga, J. W. C. Lipid Accumulation Potential of Oleaginous Yeasts: A Comparative Evaluation using Food Waste Leachate as a Substrate. *Bioresour. Technol.* **2017**, *248*, 221–228. DOI: 10.1016/j.biortech.2017.06.151.
[31] Bhosale, P. B.; Gadre, R. V. Production of β-Carotene by a Mutant of *Rhodotorula glutinis*. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 423–427. DOI: 10.1007/s002530000570.
[32] Schneider, T.; Rempp, T.; Graeff-Hönninger, S.; French, W. T.; Hernandez, R. W. C. Utilization of Soluble Starch by Oleaginous Red Yeast *Rhodotorula glutinis*. *Sustainable Bioenergy Syst.* **2013**, *3*, 57–63.
[33] Ali, T. H.; Mamdouh, S. E. G.; Dina, H. E. G.; Ghada, E. A.; Amir, K. T. Improvement of Lipid Production from an Oil-Producing Filamentous Fungus *Penicillium brevicompactum* NRC829 through Central Composite Statistical Design. *Ann. Microbial.* **2017**, *67*, 601–613. DOI: 10.1007/s13213-017-1287-x.
[34] Asghar, S. F.; Rehman, H. U.; Choudahry, M. I.; Rahman, A. U. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Petroleum Ether Extract (Oil) and Bio Assays of Crude Extract of *Iris germanica*. *Int. J. Mol. Genet.* **2011**, *3*(7), 95–100.
[35] Knot, M.; Gupta, R.; Barve, K.; Zinjarde, S.; Govindwar, S.; Ravikumar, A. Fungal Production of Single Cell Oil using Untreated Copra Cake and Evaluation of Its Fuel Properties for Biodiesel. *J. Microbiol. Biotechnol.* **2015**, *25*, 459–463. DOI: 10.4014/jmb.1407.07074.
[36] Ramos, M. J.; Fernández, C. M.; Casas, A.; Rodríguez, L.; Pérez, Á. Influence of Fatty Acid Composition of Raw Materials on Biodiesel Properties. *Bioresour. Technol.* **2009**, *100*, 261–268. DOI: 10.1016/j.biortech.2008.06.039.
[37] Wang, G. Y.; Chi, Z.; Song, B.; Wang, Z. P.; Chi, Z. M. High Level Production by Novel Inulinase-Producing Yeast *Pichia guilliermondii* Pcla22. *Bioresour. Technol.* **2012**, *124*, 77–82. DOI: 10.1016/j.biortech.2012.08.024.
[38] Vinarta, S. C.; Angelicola, M. V.; Barros, J. M.; Fernandez, P. M.; Cormak, W. M.; Aybar, M. J.; Figueroa, L. I. C. Oleaginous Yeasts from Antarctica: Screening and Preliminary Approach on Lipid Accumulation. *J. Basic Microbiol.* **2016**, *56*, 1360–1368. DOI: 10.1002/jobm.201600099.