Electronic Journal of Biotechnology 44 (2020) 60–68

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Original Research Article

Single cell oil production by a novel yeast *Trichosporon mycotoxinivorans* for complete and ecofriendly valorization of paddy straw

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ARTICLE INFO

Article history:
Received 30 October 2019
Accepted 30 January 2020
Available online 4 February 2020

Keywords:
Biodiesel
Fatty acids
Lignocellulosic biomass
Lignocellulosic hydrolysate
Lipid production
Lipogenesis
Oleaginous yeast
Paddy straw
Single cell oil
Trichosporon mycotoxinivorans
Yeast

ABSTRACT

Background: Oleaginous yeasts can be grown on different carbon sources, including lignocellulosic hydrolysate containing a mixture of glucose and xylose. However, not all yeast strains can utilize both the sugars for lipogenesis. Therefore, in this study, efforts were made to isolate dual sugar-utilizing oleaginous yeasts from different sources.

Results: A total of eleven isolates were obtained, which were screened for their ability to utilize various carbohydrates for lipogenesis. One promising yeast isolate *Trichosporon mycotoxinivorans* S2 was selected based on its capability to use a mixture of glucose and xylose and produce 44.86 ± 4.03% lipids, as well as its tolerance to fermentation inhibitors. In order to identify an inexpensive source of sugars, nondetoxified paddy straw hydrolysate (saccharified with cellulase), supplemented with 0.05% yeast extract, 0.18% peptone, and 0.04% $MgSO_4$ was used for growth of the yeast, resulting in a yield of 5.17 g L^{-1} lipids with conversion productivity of 0.06 g L^{-1} h^{-1}. Optimization of the levels of yeast extract, peptone, and $MgSO_4$ for maximizing lipid production using Box–Behnken design led to an increase in lipid yield by 41.59%. FAME analysis of single cell oil revealed oleic acid (30.84%), palmitic acid (18.28%), and stearic acid (17.64%) as the major fatty acids.

Conclusion: The fatty acid profile illustrates the potential of *T. mycotoxinivorans S2* to produce single cell oil as a feedstock for biodiesel. Therefore, the present study also indicated the potential of selected yeast to develop a zero-waste process for the complete valorization of paddy straw hydrolysate without detoxification.

How to cite: Sagia S, Sharma A, Singh S, et al. Single cell oil production by a novel yeast Trichosporon mycotoxinivorans for complete and ecofriendly valorization of paddy straw. Electronic Journal of Biotechnology 2020;44. https://doi.org/10.1016/j.ejbt.2020.01.009.

1. Introduction

Oils, fats, and lipids, present in our food are an important source of energy and have various industrial applications, including biodiesel production. Vegetable oils, nonedible oils, and waste cooking oils serve as potential feedstocks for biodiesel production. At world exhibition of Paris in 1898, Sir Rudolf Diesel demonstrated first compression ignition engine using peanut oil as a fuel [1]. Triglycerides from vegetable oils (Soybean, palm oil seeds, and rapeseed) are usually considered as prominent feedstocks for biodiesel production. The use of vegetable oils as feedstock for biodiesel has led to "food or fuel" controversy and prompted the use of nonedible oil resources like jatropha, jojoba and waste cooking oil, grease and animal fats. But these oils are not abundant to meet the global energy needs and animal fats perform poorly in cold weather. Moreover, if vegetable oil or animal fat is used for biodiesel production, the cost of substrate will be 70–85% of the total expenses, thus making it unsuitable to compete with fossil fuels. These limitations in the use of vegetable and animal fats paved the way for the development of microbial oil; so-called single cell oil (SCO) which represents a fascinating feedstock for the development of biodiesel. The term "SCO" was extended to all fatty acid-containing lipids from microorganisms including algae, bacteria, yeasts and filamentous fungi. Lipids are synthesized by all microorganisms for their essential functions, but there are certain oleaginous microorganisms that can accumulate more than 20% of their dry cell weight as lipids. The

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2020.01.009

profiles of fatty acid in these organisms varies according to microbial genera [2].

Oleaginous yeasts are unicellular fungi which accumulate lipids mostly in the form of triacyl and diacyl glycerol. The most common fatty acids of such oleaginous yeasts are C_{16} or C_{18}- palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), and oleic acid ($C_{18:1}$), resembling closely those of rapeseed and sunflower oil, which are commonly used as feedstock for biodiesel production [3]. The advantages of yeasts are their ability to grow at low pH, thereby reducing the risk of contamination, and their larger cell size makes harvesting easier than that for bacteria. SCO production using yeasts is unaffected by climate and light intensity, unlike plants and phototrophic algae. Moreover, yeasts exhibit higher tolerance to low oxygen availability than fungi [4]. Lipid accumulation in oleaginous yeasts occurs in late log/stationary phase with starvation of nutrients like nitrogen and excess of carbon source. Lipid accumulation is usually optimal at molar C:N ratio exceeding 65 and near 100 [5]. Typical oleaginous yeasts belong to genera *Rhodotorula, Yarrowia, Candida, Cryptococcus, Rhodosporidium, Trichosporon, Rhizopus*, and *Lipomyces*.

Use of low cost and abundant substrates makes SCO production economically feasible and sustainable. Among low cost substrates, lignocellulosic biomass is the most promising, abundant, inexpensive and bio renewable, particularly for the production of biofuels and other value added products. Lignocellulose is composed of three major polymers — cellulose, hemicelluloses, and lignin with glucose and xylose being the predominant monomers. Several oleaginous yeasts can grow aerobically on glucose/xylose individually, but lack the ability to co-metabolize both hexose and pentose sugars. Therefore, it is important to identify oleaginous yeast strains that can utilize glucose and xylose simultaneously or sequentially leading to the effective utilization of lignocellulosic hydrolysates. Pretreatment of lignocellulosic biomass also leads to the generation of lignin degradation compounds (furfual, hydroxymethyl furfural, syringaldehyde, acetic acid, 4-hydroxybenzaldehyde, vanillin, formic acid, levulinic acid) depending on the method of pretreatment used. These degradation compounds can be inhibitory to the yeasts carrying out the subsequent fermentation. However, oleaginous yeasts like *Trichosporoncutaneum, Trichosporonfermentas, Rhodosporidiumtoruloides,* and *Lipomycesstarkeyi* are reported to be tolerant to lignocellulosic degradation compounds [6]. Utilization of low cost and abundant lignocellulosic biomass makes SCO production from oleaginous yeasts more attractive. Therefore, the present study was carried out to isolate yeasts capable of utilizing both glucose and xylose for lipid production by using alkali pretreated paddy straw hydrolysate (without detoxification of inhibitors generated during saccharification) as a feedstock.

2. Materials and methods

2.1. Isolation of xylose and glucose utilizing yeasts and screening for carbohydrate utilization

Yeast strains were isolated from field soil and rotten fruits (pear, pomegranate, and lemon) using glycerol enrichment media [7]. Each sample (1 g) was added to 50 ml of glycerol enrichment broth (yeast extract 1.00 g L^{-1}, $NaNO_3$ 4.00 g L^{-1}, KH_2PO_4 2.00 g L^{-1}, K_2HPO_4 2.00 g L^{-1}, $MgSO_4.7H_2O$ 0.40 g L^{-1}, $CaCl_2.2H_2O$ 2.00 g L^{-1}, $FeSO_4.7H_2O$ 0.04 g L^{-1}, Nicotinic acid 0.02 g L^{-1}, Glycerol 20 ml and 1 ml of trace element solution — $MnSO_4.H_2O$ 2 m g L^{-1}, $CuCl_2.2H_2O$ 0.5 m g L^{-1}, $ZnSO_4.7H_2O$ 0.5 m g L^{-1}, Na_2MoO_4 5 μ g L^{-1}, $NiSO_4.6H_2O$ 2 μ g L^{-1}, $CoCl_2.6H_2O$ 2 μ g L^{-1}, $Na_2B_4O_7.10H_2O$ 1 μ g L^{-1}) and incubated at 28°C for 48 h. After incubation, appropriate dilutions from glycerol enrichment broth were plated onto YM xylose media (Xylose 20.00 g L^{-1}, Yeast extract 5.00 g L^{-1}, $(NH_4)_2SO_4$0.10 g L^{-1}, $KH_2PO_4$0.25 g L^{-1}, $K_2HPO_4$0.05 g L^{-1}, $MgSO_4$0.10 g L^{-1}, $FeSO_4$0.01 g L^{-1}, Chloramphenicol 0.20 g L^{-1}, Sodium propionate 2.50 g L^{-1}, Agar 18.00 g L^{-1}, pH 6) and incubated at 28°C for 24–48 h. Prominent morphotypes from YM xylose plates were purified by streaking on MGY Pagarslants (Malt extract 3.00 g L^{-1}, Glucose 10.00 g L^{-1}, Yeast extract 3.00 g L^{-1}, Peptone 5.00 g L^{-1}, Agar 18.00 g L^{-1}). Further, these isolated strains were preserved in agar slants made of MGYP medium and stored at 4°C. Yeast isolates are screened for glucose and xylose utilization with MGYP, MGXYP and MXYP (Glucose replaced with xylose) broth at 28°C for 24 h and using HiCarbo™ kit — Part A, B and C, containing 35 sugars (Hi Media Laboratories, Mumbai, India).

2.2. Identification of xylose/glucose utilizing yeast isolates

Selected yeast isolates were identified by amplified rDNAgene sequencing. The DNA isolated from the isolates was amplified in a Biorad Thermocycler by using primers homologous to the conserved sequences of ITS1, 5.8 s rDNA and ITS (ITS1 - 5′ TCCGTAGGTGAACCTG CGG 3′) and ITS4 -5′ TCCTCCGCTTATTGATATGC 3′) [8]. Gene sequences of the amplified DNA were subjected to BLASTn (Basic Local Alignment Search Tool) for homology identification in the National Center for Biotechnology Information (NCBI) database and subsequently submitted to NCBI GenBank to obtain accession numbers.

2.3. Screening for lipid production

2.3.1. Qualitative screening

Yeast isolates found to utilize both glucose and xylose were further screened for lipid production qualitatively by staining and microscopy as well as quantitatively by gravimetric extraction. The yeast colonies on the Petri plate were directly stained with 0.3% Sudan Black B for 30 min, and then the colonies were rinsed with 70% ethanol. Oleaginous yeast strains retained the blue color after rinsing with ethanol.

The colonies stained blue were further confirmed for lipid bodies by microscopy using Sudan black B and Nile red staining. A smear of culture was made on the glass slide and was heat fixed. The entire slide was flooded with Sudan Black B solution (0.3%), and the slide was left undisturbed for 5–15 min at room temperature. Excess stain was drained off with 70% alcohol. The slide was counterstained with safranin (0.5%) for 30 s, washed with water, air dried and observed under light microscope. The lipid bodies were stained blue [9].

For Nile red fluorescence staining, cell suspension was prepared in sodium phosphate buffer, and a smear was prepared by spreading 10 μl of cell suspension on glass slide. The slide was air dried and stained with 10 μl of Nile red solution (0.01%). Excess Nile red was washed with sodium phosphate buffer after 5 min. The slide was observed under a fluorescence microscope with emission wavelength of about 450–500 nm [10].

2.3.2. Quantitative screening

The xylose/glucose-utilizing yeast isolates that stained positive for lipid production were further screened quantitatively based on gravimetric method by lipid extraction from yeast grown in glucose/xylose or a mixture of both xylose and glucose. Yeast isolates were inoculated into lipid production media (Glucose/xylose 100 g L^{-1} or glucose + xylose 50 g L^{-1} each, Yeast extract 0.50 g L^{-1}, Peptone 1.80 g L^{-1}, $MgSO_4.7H_2O$ 0.40 g L^{-1}, K_2HPO_4 2.00 g L^{-1}, $MnSO_4.H_2O$ 0.003 g L^{-1}, $CuSO_4.5H_2O$ 0.0001 g L^{-1}) and incubated for 6 d at 30°C, 150 rpm in incubator shaker (Kuhner, Germany). The fermented broth was centrifuged at 8000 rpm for 10 min. The fermentate was collected and stored at −20°C. The pelleted biomass was macerated with liquid nitrogen and sonicated under ice-cold condition at 40 pulses (5 times) for 3 min using an ultrasonic homogenizer (Model 3000, Biologics Inc. USA). Solvent mixture (20 ml) of chloroform and methanol (2:1) was added to the homogenized biomass. The mixture was left undisturbed for about 30 min. The upper phase was removed without disturbing

the interface. Ten milliliters of solvent mixture was added and the lower chloroform layer containing the lipids was removed into a pre-weighed container. The pre-weighed container with the lower layer was kept in oven at 40–50°C for the evaporation of solvent. After complete evaporation of the solvent, the container was weighed again and lipid yield was expressed as g of lipid g^{-1} of biomass [11].

2.4. Determination of co-metabolites

The fermentate was also tested for the production of co-metabolites like organic acids and ethanol. The fermentate collected after centrifugation was filtered through 0.45-μ disposable membrane filters before injection into HPLC. Analysis of organic acids and ethanol was performed by HPLC (Waters pump 515 model) with 5 mM H_2SO_4 as mobile phase and Aminex HPX-87H column at a flow rate of 0.6 ml/min with refractive index (RI) detector at 60°C.

2.5. Screening of the isolates for tolerance to lignin degradation compounds

The yeast isolates were tested for their tolerance to commonly found inhibitors in the lignocellulosic hydrolysates: furfural and 5-hydroxymehtyl furfural. For this purpose, 10× Yeast Nitrogen Base medium (YNB) containing 1% glucose broth was supplemented with different concentrations of furfural (0.5 g L^{-1}, 1 g L^{-1}, 1.5 g L^{-1}) and 5-hydroxy methyl furfural (1.5 g L^{-1}, 2 g L^{-1}, 2.5 g L^{-1}). The medium was inoculated with yeast isolates and incubated at 30°C in an incubator shaker (150 rpm) for 24 h. After incubation, optical density (OD) at 660 nm was recorded to test the tolerance of yeast isolates to inhibitors. Percent decrease in growth was calculated from growth in control tubes without inhibitor.

2.6. Lipid production from lignocellulosic hydrolysate

The chopped and sun-dried paddy straw was pretreated using 1% NaOH in the ratio of 1:10 (NaOH:Paddy straw) for 1 h. The treated biomass was washed with water to bring its pH to neutral. Pretreated wet biomass was saccharified with commercial hollocellulase enzyme (Cat no C2730, Sigma, USA) consisting of exo & endoglucanase (FPase 1024.87 IU/ml; CMCase 13,230.75 IU/ml), β-glucosidase (12,508.016 IU/ml), and xylanase (20,002.2 IU/ml) at 50°C for 48 h with 8% (*w*/*v*) substrate loading and 15 FPU/gds enzyme loading. The total amount of reducing sugars released during saccharification of alkali-pretreated paddy straw was estimated using HPLC and spectrophotometrically with the DNSA method [12]. The amount of monosaccharides released upon enzymatic hydrolysis was also quantified by HPLC. The lignocellulosic hydrolysates was concentrated five times by a rotary evaporator (Heidolph Laborota 4001, efficient) at 40°C to desired concentration of sugars (35 mg/ml) and supplemented with yeast extract 0.5 g L^{-1}, peptone 1.8 g L^{-1} and $MgSO_4$ 0.4 g L^{-1}. The selected yeast isolate identified to have higher oil production capacity was grown in the hydrolysate by incubating at 30°C for 6 d at 150 rpm. After incubation, the lipids were extracted from biomass by the solvent extraction method as discussed in Section 2.3.2 and estimated gravimetrically.

2.7. Optimization of lipid production by response surface methodology — Box–Behnken design

SCO production by the selected yeast isolate in lignocellulosic hydrolysate was optimized by the Box–Behnken design experiment (Design expert, Stat-Ease Inc., Minneapolis, USA). Three factors, (A) Yeast extract (B) Peptone, and (C) $MgSO_4$, that affect the lipid production were selected to find the optimum condition as well as the interaction between the selected factors for SCO production.

A total of 15 experiments including three center points were performed along with selected range of variables in paddy straw hydrolysate with the C:N ratio of 83.8:1 at 30°C, 150 rpm for 6 d (Table 1). Each experiment was run in triplicates. Variables A, B and C are independent, whereas response Y (SCO production) is dependent on the independent variables. The lipids were extracted by solvent extraction (chloroform and methanol in 2:1 ratio) and estimated gravimetrically. The complete experimental design with coded variables for the optimization of SCO is shown in Table S1.

Table 1
Experimental range and coded values of variable used in response surface methodology using Box Behnken design.

	Process variable	Range and level		
		-1	0	+1
A	Yeast extract (g L^{-1})	0.5	0.75	1
B	Peptone (g L^{-1})	1	1.5	2
C	$MgSO_4$ (g L^{-1})	0.25	0.375	0.5

2.8. Statistical optimization of process parameters to maximize lipid yield

The interactions of independent variables on the dependent variable were evaluated and studied using statistical analysis with Design expert 8.0.7.1. All experiments were carried out in triplicate and were expressed as average values. The effect of independent variables on response (single cell oil production) was shown in three-dimensional surface plots.

2.9. Quantitative analysis of SCO by fatty acid methyl ester (FAME)

About 0.25 g of freeze-dried yeast biomass was transferred to a completely dried test tube and subjected to methanolysis. Methanol (5 ml) was added to the dried biomass followed by the addition of 100 μl of conc.H_2SO_4 and incubated at 65°C in a water bath for 1 h. The tubes were then cooled to room temperature, and 2 ml of hexane was added to the mixture and shaken well in a vortex. About 1–1.5 ml of the hexane layer was removed and transferred into a 2-ml screw capped vial. One microliter of this aliquot was analyzed by a gas liquid chromatography (Perkin Elmer Claurus 500) fitted with a megabore column (30-meter-long and 0.53 μm diameter packed with OV-101, a polymer of methyl silicone) using flame ionization detector (FID). The column temperature was maintained at 150–270°C, with injector and detector temperature at 250°C. The ramp rate was 10°C/min and the final temperature was maintained at 270°C. The individual fatty acids were quantified according to standards and their respective retention time.

3. Results

3.1. Isolation and identification of glucose/xylose-utilizing yeasts

A total of 11 isolates (S1 and S2 from soil; Pe1, Pe2 and Pe3 from rotten pear; Po1, Po2 and Po3 from rotten Pomegranate; L1, L2 and L3 from rotten lemon) based on colony morphology were selected and purified. Eight isolates (S1, S2, Pe1, Pe2, Pe3, Po2, L2 and L3) out of eleven were found to grow in both glucose and xylose media and were thus selected for further studies. These eight isolates were identified on the basis of amplification of ITS1 region. PCR-based identification revealed that the eight isolates belong to three genera — *Candida tropicalis* S1, Pe1, Po2, L2 and L3; *Candida dubliniensis* Pe2; *Meyerozyma caribbica* Pe3; and *Trichosporon mycotoxinivorans* S2. The sequences of all the isolates were submitted to NCBI with the accession numbers listed in Table S2. A phylogenetic tree was constructed to depict the relationship of identified yeast isolates with nearest phylogenetic relative (Fig. 1).

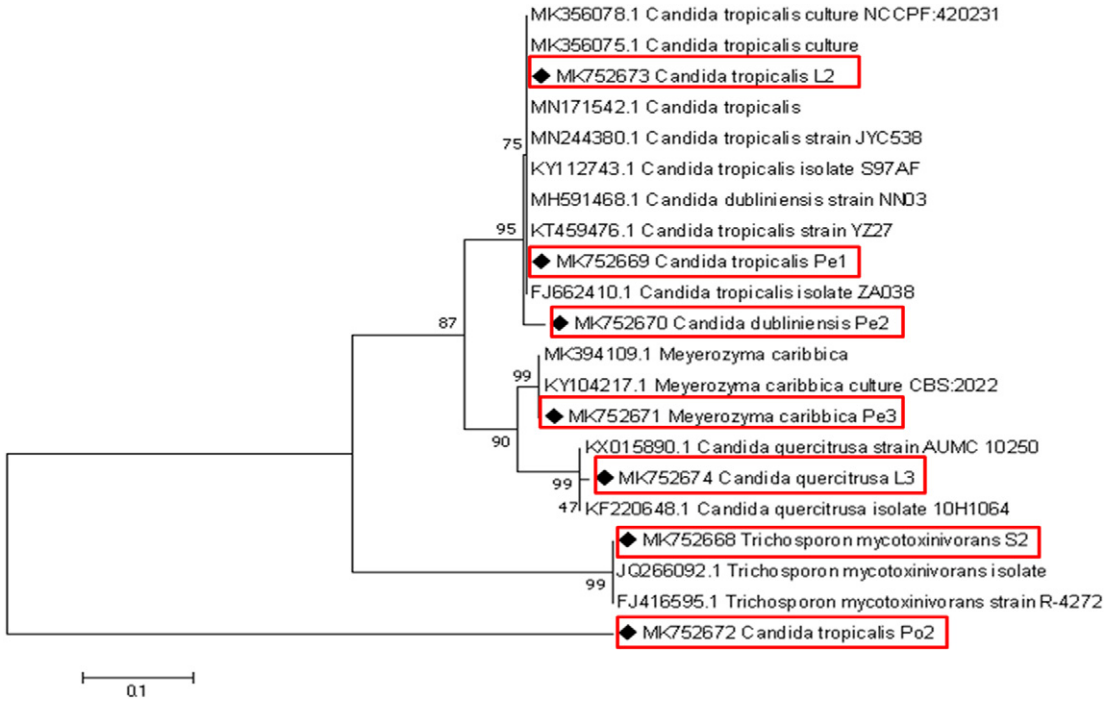

Fig. 1. Phylogenetic tree based on 18s rDNA gene sequence showing the relationship of yeast isolates with the nearest phylogenetic relative.

3.2. Biochemical profiling of yeast isolates

The eight isolates *Candida tropicalis* S1, Pe1, Po2, L2 and L3; *C. dubliniensis* Pe; *M. caribbica* Pe3; and *T. mycotoxinivorans* S2 were biochemically profiled using Hicarbo™ kit (Himedia) with 35 sugars. The sugar utilization patterns are presented in Table S3. It was observed that xylose, dextrose, and galactose were utilized by all isolates. Sucrose, maltose, fructose, inulin, and sugar alcohols like sorbitol, mannitol, adonitol, and melezitose were found to be utilized by all isolates except *T. mycotoxinivorans* S2. Glycerol which is commonly used as a low cost substrate for SCO production was found to be utilized by all isolates except *C. dubliniensis* Pe2 and *C. quercitrusa* L3. *T. mycotoxinivorans* S2 was found to utilize lactose and also possess β-galactosidase activity. These results suggest that cellobiose, a common disaccharide produced during enzymatic hydrolysis of lignocellulosic biomass, could be utilized by *T. mycotoxinivorans* S2, *C. dubliniensis* Pe2, *M. caribbica* Pe3, and *C. tropicalis* L3.

3.3. Screening for lipid production and determination of co-metabolites

The colonies of *T. mycotoxinivorans* S2 and *M. caribbica* Pe3 stained blue and were further confirmed for the presence of lipid bodies using Sudan Black B and Nile Red staining through microscopy (Fig. S1).

The results from quantitative screening for lipid production indicated that *T. mycotoxinivorans* S2 was able to accumulate higher amount of oil,

Table 2
Results for quantitative screening of lipid production by xylose/glucose-utilizing yeast isolates.

Isolates	Substrates	Biomass ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	Lipid (% of dry weight biomass)
C. tropicalis S1	Glucose	10.50 ± 0.04	2.20 ± 0.14	20.95 ± 1.25
	Glucose + xylose	18.14 ± 0.10	2.35 ± 0.25	12.50 ± 1.31
	Xylose	19.68 ± 0.50	1.79 ± 0.01	9.10 ± 0.18
T. mycotoxinivorans S2	Glucose	22.18 ± 0.98	3.20 ± 0.04	14.45 ± 0.82
	Glucose + xylose	21.94 ± 1.56	9.78 ± 0.62	44.86 ± 4.03
	Xylose	17.66 ± 1.76	5.68 ± 0.62	32.61 ± 3.79
C. tropicalis Pe1	Glucose	6.30 ± 0.26	1.44 ± 0.02	22.89 ± 1.26
	Glucose + xylose	9.87 ± 0.45	1.19 ± 0.05	12.06 ± 0.04
	Xylose	12.52 ± 0.44	1.28 ± 0.04	10.22 ± 0.04
C. dubiliniensis Pe2	Glucose	10.36 ± 3.98	0.99 ± 0.07	10.46 ± 3.60
	Glucose + xylose	10.61 ± 0.07	1.05 ± 0.09	9.89 ± 0.78
	Xylose	13.94 ± 1.32	0.99 ± 0.33	6.99 ± 1.71
M. caribbica Pe3	Glucose	4.43 ± 0.83	0.50 ± 0.16	11.09 ± 1.56
	Glucose + xylose	4.54 ± 0.96	0.49 ± 0.090	10.84 ± 0.32
	Xylose	5.46 ± 0.04	1.37 ± 0.17	25.11 ± 3.30
C. tropicalis Po2	Glucose	7.28 ± 1.44	0.76 ± 0.28	10.20 ± 1.87
	Glucose + xylose	10.67 ± 1.29	1.11 ± 0.63	10.99 ± 7.29
	Xylose	10.58 ± 2.78	0.96 ± 0.24	9.95 ± 5.05
C. tropicalis L2	Glucose	8.22 ± 0.10	0.19 ± 0.17	2.29 ± 2.04
	Glucose + xylose	11.63 ± 0.53	0.20 ± 0.16	1.76 ± 1.46
	Xylose	14.24 ± 1.18	0.21 ± 0.17	1.55 ± 1.33
C. quercitrusa L3	Glucose	5.94 ± 0.10	0.13 ± 0.09	2.17 ± 1.48
	Glucose + xylose	8.59 ± 0.001	0.52 ± 0.12	6.05 ± 1.39
	Xylose	10.08 ± 0.14	0.26 ± 0.24	2.62 ± 2.42

Table 3
Determination of co-metabolite production by oleaginous and nonoleaginous yeast isolate.

S. No	*T. mycotoxinivorans* S2	*Meyerozyma caribbica* Pe3	*Candida tropicalis* L2	*C. quercitrusa* L3
Succinic acid (mg/ml)	0.429	0.442	1.091	1.111
Lactic acid (mg/ml)	ND	ND	28.172	71.624
Malic acid (mg/ml)	ND	ND	0.139	0.079
Citric acid (mg/ml)	ND	ND	0.428	0.059
Ethanol (mg/ml)	0.429	0.757	19.222	11.267

ND — Not detected.

32.61% from xylose and 44.86% from both glucose and xylose. *C. tropicalis* S1 and *C. tropicalis* Pe1 accumulated about 20.95% and 22.89% oil, respectively, from glucose. *M. caribbica* Pe3 was found to accumulate 25.11% oil in xylose media with least oil accumulation in glucose and xylose media (Table 2). Because the highest oil production capacity was recorded in *T. mycotoxinivorans* S2 in both glucose and xylose-based media, it was selected for SCO production from lignocellulosic biomass (paddy straw).

The co-metabolites were also profiled by HPLC, and the results revealed that isolates with low lipid accumulation coproduced higher levels of ethanol *C. tropicalis* L2 (19.22 mg/ml) and *C. quercitrusa* L3 (11.267 mg/ml) when compared to the isolates with high oil accumulating capacity *T. mycotoxinivorans* S2 (0.429 mg/ml) and *M. caribbica* Pe3 (0.757mg/ml). Further organic acids like succinic acid, malic acid, citric acid and lactic acid were also detected in the culture filtrate of *C. tropicalis* L2 and *C. quercitrusa* L3, whereas only succinic acid was detected in the case of *T. mycotoxinivorans* S2 and *M. caribbica* Pe3 (Table 3).

3.4. Screening of the yeast isolates for tolerance to fermentation inhibitor compounds

A decrease in growth was observed from 0.42 to 43% with furfural at the concentration of 0.5 g L^{-1} with least reduction (0.42%) occurred in the isolate *M. caribbica* Pe3 and 43% growth reduction occurred in the case of *C. tropicalis* Po2. A significant reduction in growth of about 23 to 99% occurred at higher concentration of furfural (1.5 g L^{-1}). Least tolerance to furfural at 1.5 g L^{-1} was shown by *C. tropicalis* Pe2 whereas higher tolerance was exhibited by *C. tropicalis* S1. On the other hand, all the eight isolates showed better tolerance to 5-HMF with 0.5–26% reduction in growth. Moreover, the isolates were more tolerant to 5-HMF at higher concentration of 2.5 g L^{-1} than furfural. Growth reduction occurred in the range of 11–59% with 5-HMF at 2.5 g L^{-1}. Highest tolerance to 5-HMF was shown by *C. tropicalis* S1, whereas *C. tropicalis* Po2 shown least tolerance to 5-HMF at 2.5 g L^{-1} (Fig. 2).

3.5. SCO production from alkali-pretreated paddy straw hydrolysate with T. mycotoxinivorans S2

The paddy straw hydrolysate contained glucose (5.91 mg/ml) and xylose (0.48 mg/ml), which was concentrated fivefold and finally had 35 g L^{-1} of total sugars. The lipid accumulation was found to be about 35% of dry weight biomass with lipid and biomass yield of about 5.17 g L^{-1} and 14 g L^{-1}, respectively, in the case of paddy straw hydrolysate. Lipid productivity was decreased by 50% to about 0.03 g L^{-1} h^{-1} when compared with synthetic media. The results indicated the necessity for optimization of culture conditions for higher lipid productivity from nondetoxified paddy straw hydrolysate. In our experiment, no residual sugars were detectable in the hydrolysate at the end of fermentation as detected by HPLC.

3.6. Process optimization of lipid production with T. mycotoxinivorans S2 using RSM with Box–Behnken design

Three variables, namely Yeast extract (0.5–1 g L^{-1}), peptone (1–2 g L^{-1}) and $MgSO_4$ (0.25–0.5 g L^{-1}) that affect the lipid production from yeast were selected for the optimization of single cell oil production with *T. mycotoxinivorans* S2 from paddy straw hydrolysate using RSM of Box–Behnken design. The actual and predicted lipid yield for 15 different combinations of experiments is listed in Table S4, and the correlation between actual and predicted values of lipid production is shown in the Fig. S2. The predicted ethanol yield was calculated using the binomial equation:

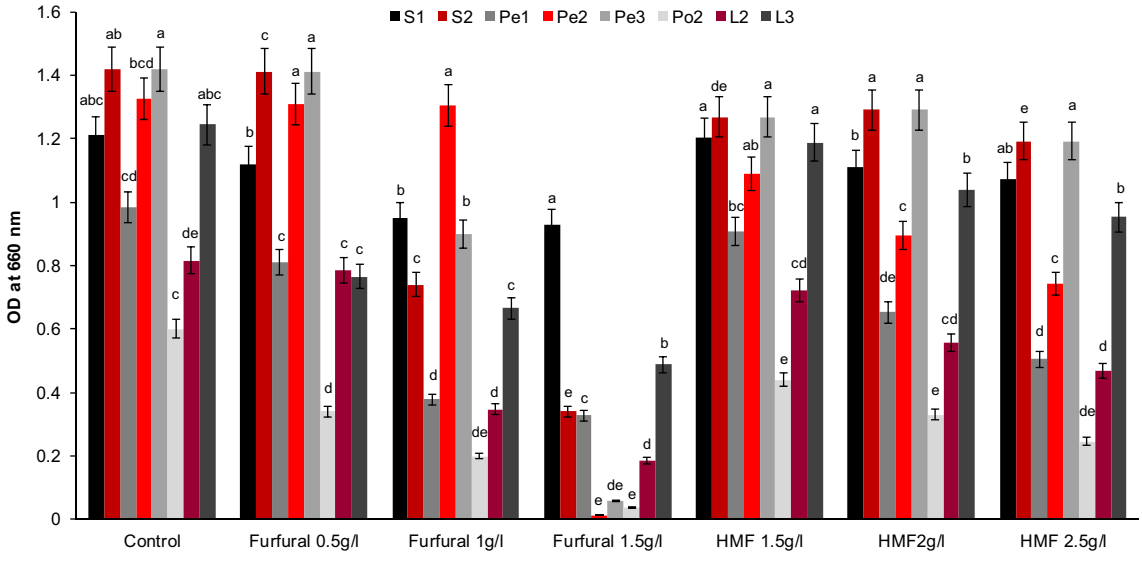

Fig. 2. Tolerance of xylose-utilizing yeast isolates to inhibitors: Furfural and 5-HMF.

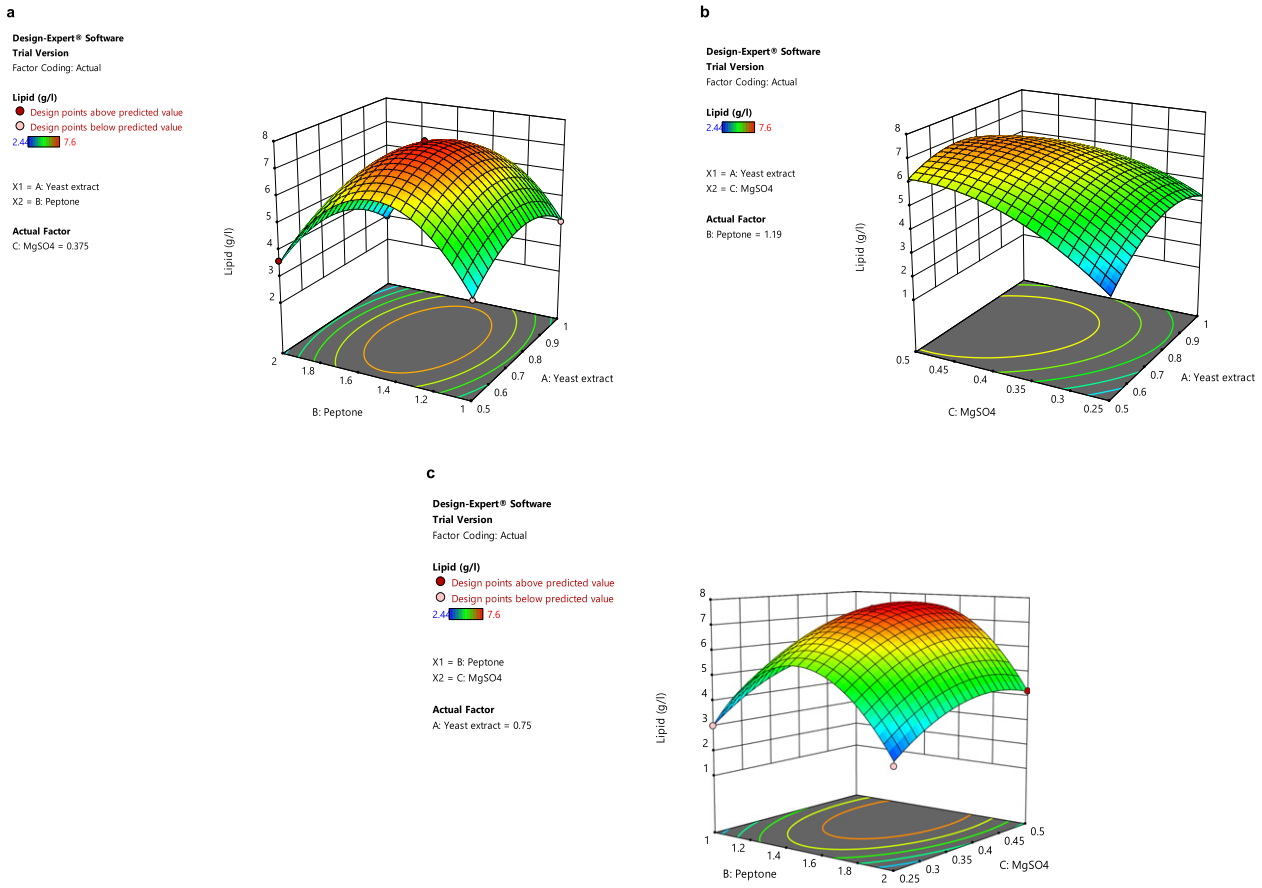

Fig. 3. Response surface plot of lipid production as a function of (a) yeast extract and peptone at a fixed concentration of $MgSO_4$ (0.375 g L^{-1}), (b) yeast extract and $MgSO_4$ at a fixed concentration of peptone (1.19 g L^{-1}), (c) peptone and $MgSO_4$ at a fixed concentration of yeast extract (0.75 g L^{-1}).

$$\text{Lipid yield}\left(\text{g L}^{-1}\right) = -45.90583 + 34.93 * A + 36.055 * B + 66.36 * C - 1.96 * A * B - 17.92 * A * C - 2.56 * B * C - 16.9733 * A^2 - 11.4433 * B^2 - 55.4133 * C^2$$

where lipid yield is the response; A, B and C were independent variables (A, Yeast; extract; B, Peptone; and C, $MgSO_4$).

The above equation mentions the role of each independent variable and the effect of their interactions on the lipid yield.

The analysis of variance (ANOVA) results of the experimental model are represented in Table S5. High F value of the model (149.51) confirmed that the model is significant. Smaller p value (<0.0001) indicates the chance that F value occurs by noise was only 0.01%. Lack of fit test was insignificant (p value = 0.1877 > 0.005) explaining the adequacy of the model. Interactions between all variables are significant (p value <0.05) except the interaction between peptone and yeast extract (p value 0.13). The coefficient of determination (R^2) was 0.9963, which indicates that 99% variability in the response (lipid yield) is due to the independent variables. A sound agreement was found between predicted coefficient of determination (0.9474) and adjusted coefficient of determination (0.9963). The adequate precision of 32.98 indicates low signal-to-noise ratio. The precision and reliability of the model were explained by low coefficient of variance of about (C.V % = 3.66).

The contour plot analysis presented in Fig. 3 indicates the lipid yield (response) as a function of interaction between two variables with one variable kept as constant. It can be seen that lipid production is enhanced with an increase in the concentration of both yeast extract and peptone up to a point, and any further increase in the concentration of two variables had a negative effect on lipid yield (Fig. 3a). Response surface plot of lipid production as a function of yeast extract and $MgSO_4$ shows that lipid concentration improves exponentially with subsequent increase in the concentration of $MgSO_4$, while higher concentration of yeast extract decreases lipid yield (Fig. 3b). It was also seen from the interaction between peptone and $MgSO_4$ that lipid concentration increases with the concentration of yeast extract up to a point and any further supplementation has negative effect in lipid yield, while it increases exponentially with increase in $MgSO_4$ concentration (Fig. 3c). The optimum conditions proposed by the model for the predicted lipid yield of about 7.32 g L^{-1}were (A) yeast extract 1.0 g L^{-1}, (B) peptone 1.5 g L^{-1}, and (C) MgSO4 0.5 g L^{-1}. The validation of the results from optimized conditions (Table 4) illustrated an increase in the lipid yield from nondetoxified paddy straw hydrolysate by 41.59%. Optimization also resulted in a comparatively similar lipid yield from *T. mycotoxinivorans* S2 with synthetic carbon source (9.78 g L^{-1}) and nondetoxified paddy straw hydrolysate (7.32 g L^{-1}) as substrates (Fig. 4 and Table S6).

Table 4
Validation results of optimized variables for higher lipid production from paddy straw hydrolysate with *T. mycotoxinivorans* S2.

Std. Order	A	B	C	Lipid yield (g L^{-1})
7	-1	0	1	7.32 ± 0.70
8	1	0	1	5.15 ± 0.31
15	0	0	0	6.58 ± 0.60

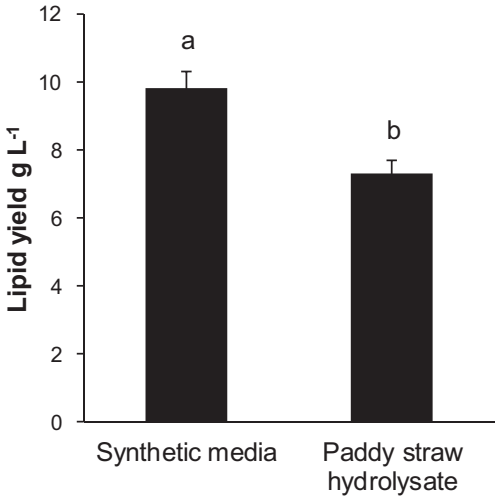

Fig. 4. Comparison of lipid production from *T. mycotoxinivorans* S2 between complex synthetic media and paddy straw hydrolysate.

3.7. FAME analysis of SCO produced by T. mycotoxinivorans S2

The fatty acid profile of lipid from *T. mycotoxinivorans* S2 analyzed by GC is listed in the Table 5. The major fatty acids were shown as oleic acid (30.84%), palmitic acid (18.28%) and stearic acid (17.64%) which indicates the potential of SCO obtained from the dual sugar-utilizing yeast *T. mycotoxinivorans* S2 as a potential feedstock for biodiesel production.

4. Discussion

Valorization of lignocellulosic biomass for the production of biofuel and other feedstock chemicals is being seen as the most promising way of reducing environmental pollution and dependence on fossil fuels. Bioethanol production from lignocellulosic biomass such as paddy straw has been investigated and reviewed by many researchers. One of the major obstacles in bioethanol production is the unavailability of co-fermenting yeast that can utilize both xylose and glucose; otherwise, residual xylose has to be utilized in separate fermentation with a different organism that incurs additional cost. Though few co-metabolizing yeasts are available, their use at the commercial scale is yet to be tested. Production of SCO by various yeasts on synthetic medium, molasses, or starchy material has been reported, but the strains were capable of utilizing glucose only [13,14]. Moreover, only few attempts have been made to utilize biomass hydrolysate for production of lipids using yeasts.

Use of low cost and abundant substrates can make SCO production economically feasible and sustainable. Among the low cost substrates, lignocellulosic biomass serve as the most promising, abundant, inexpensive and biorenewable resource for the production of biofuels and other value added products. A case study on life cycle assessment by Cherubini and Ulgiati [15] showed that the use of agricultural residues for biofuel production caused 50% reduction in greenhouse gas emission.

Table 5
Fatty acid profile of single cell oil from *T. mycotoxinivorans* S2.

Component	Relative percentage (%)
Lauric acid ($C_{12:0}$)	5.36
Palmitic acid ($C_{16:0}$)	18.28
Palmitoleic acid ($C_{16:1}$)	7.60
Stearic acid ($C_{18:0}$)	17.64
Oleic acid ($C_{18:1}$)	30.84
Linoleic acid ($C_{18:2}$)	2.36

Among different microbes, yeasts are generally preferred because of their ability to grow at low pH thereby reducing the risk of contamination and easier downstream processing due to larger cell size [4]. The present study focused mainly on the isolation of dual sugar (xylose and glucose)-utilizing oleaginous yeasts, as both are the predominant reducing sugars found in the lignocellulosic hydrolysate. Among the 11 yeast isolates, eight belonging to genera *Candida*, *Meyerozyma*, and *Trichosporon* were found to utilize both glucose and xylose. Several other researchers have reported these oleaginous genera isolated from soil, fruits, and guts of insects are capable of utilizing different sugars [16,17,18]. Our study is the only report where *Meyerozyma* and *Trichosporon* are utilized for the production of SCO.

The study showed xylose, dextrose, and galactose utilization by all yeast isolates, whereas sugar alcohols (sorbitol, mannitol, and adonitol) and sugars (melezitose, inulin) were found to be utilized by all isolates except *Trichosporon mycotoxinivorans* S2. *Candida tropicalis* S1, Pe1, Po2 and L2 were found to utilize inulin which is not commonly assimilated by *Candida tropicalis*. However inulin assimilation by some atypical *Candida* isolates were reported earlier [19,20]. Lactose utilization by *Trichosporon mycotoxinivorans* S2 paves the way for the utilization of whey as a substrate for lipid production [21]. Cellobiose commonly encountered in lignocellulosic hydrolysate was found to be utilized by *Trichosporon mycotoxinivorans* S2, *Candida dubliniensis* Pe2, *Meyerozyma caribbica* Pe3, and *Candida tropicalis* L3. *Trichosporon mycotoxinivorans* and *Meyerozyma caribbica* have already been reported to grow in lignocellulosic hydrolysate [17].

Two isolates, namely *C. tropicalis* S1 and Pe2, were found to accumulate lipid with glucose as the carbon source, whereas *M. caribbica* Pe3 was found to accumulate lipid with xylose as substrate. Previous reports also indicated higher lipid production by *Candida tropicalis* with glucose as the carbon source [22].The yeast isolate *T. mycotoxinivorans* S2 was found to be capable of lipid accumulation with both glucose and xylose as carbon source suggesting that *T. mycotoxinivorans* S2 can be used for lipid production from lignocellulosic hydrolysate. Molnar et al. [23] first time reported the isolation of co-fermenting *T. mycotoxinivorans* S2 from the hind gut of the lower termite *Mastotermes darwiniensis* and biological detoxification of various food mycotoxins.

Lignin degradation compounds (furfural, hydroxymethyl furfural, syringaldehyde, acetic acid, 4-hydroxybenzaldehyde, vanillin, formic acid, and levulinic acid) produced during pretreatment and saccharification affect cell viability and lipid production. Furfural and HMF concentration is generally reported to be 0.25 g L^{-1} and 0.15 g L^{-1} in paddy straw hydrolysate, respectively [24]. Generally, detoxification of hydrolysate is necessary to reduce the load of inhibitors but it increases the cost of production and also results in loss of sugars therefore, the selected yeast strains were also evaluated for growth inhibition in the presence of furfural and hydroxyl methyl furfural.

Among different yeasts, *T. mycotoxinivorans* S2 was found to be relatively tolerant to furfural at the concentration of 1.0 g L^{-1} with 47% growth reduction, and highly tolerant to 5-HMF at its higher concentration (2.5 g L^{-1}), with only 15% growth reduction. Poontawee et al. [25] investigated yeast *Rhodosporidium fluvial* for its tolerance to lignocelluloses degradation products namely acetic acid, formic acid, furfural and 5-HMF and reported the negative effect of some of these compounds on lipid yield, but yeast strain showed high tolerance to acetic acid,5-HMF and vanillin in synthetic media. Similarly, in an earlier study, oleaginous yeasts such as *Trichosporon cutaneum, Trichosporon fermentas, Rhodosporidium toruloides, Lipomyces starkeyi* have been reported to be tolerant against low level of lignocellulosic degradation compounds [6]. In general, strains of

Candida exhibited better inhibitor tolerance than *Saccharomyces* and *Pichia* because of their inherent capacity for detoxification/degradation of these inhibitors [26,27].

Production of undesirable co-metabolite like organic acids, esters, alcohols and sugar alcohol is reported in most of fermentations, and their concentration depends on the strain, production medium and environmental condition. In the present study, the different genera of yeasts were also found to co-produce ethanol, lactic/malic/citric and succinic acid. These results indicated the diversion of metabolic machinery for the production of co-metabolites. The production of succinic acid also indicates the possibility to develop a process for the coproduction of succinic acid and lipid by *T. mycotoxinivorans* S2 and *M. caribbica* Pe3. Qian et al. [28] also demonstrated coproduction of SCO intracellularly and gluconic acid extracellularly using oleaginous *Cryptococcus podzolicus*. In future, these yeast strains may be explored for the coproduction of one or more metabolite through bio-based refineries.

Alkali pretreatment used in the present study for pretreating paddy straw is known to increase lignin solubilization and decrease crystallinity of cellulose effectively in agricultural biomass [29,30,31]. The concentrated paddy straw hydrolysate inoculated with selected yeast *T. mycotoxinivorans* S2 resulted in lipid yield of 5.17 g L^{-1} with productivity of 0.03 g L^{-1} h^{-1}. Results indicated that lipid production was low in the case of paddy straw hydrolysate than synthetic complex media with 50% reduction in lipid productivity. This can be attributed to the presence of lignin degradation compounds and less amount of sugars present in paddy straw hydrolysate [32,33,34,35,36].

Statistical optimization was perfprmed for higher lipid production from oleaginous yeasts using response surface methodology with Box–Behnken design. The validation of the optimized parameter was undertaken and lipid yield increased by 41.59% to 7.35 g L^{-1}as compared with lipid yield of 5.17 g L^{-1} under the unoptimized condition. The lipid yield is comparable to the yields of various oleaginous yeasts from the previous reports (Table 6) proving the superiority of *T. mycotoxinivorans* S2 strain for higher productivity of SCO from nondetoxified paddy straw hydrolysate. Another research group, Ruan and co-workers [37] recorded low lipid yield (3.02–4.4 g L^{-1}) from different lignocellulosic biomass hydrolysates (corn stover,switch grass, miscanthus and giant reed), without detoxification within 4–6 d.

In the present study, the fatty acid profile was determined after trans-esterification of fatty acids to FAME and subsequent analysis by GC. FAME analysis indicated the major fatty acids of *T. mycotoxinivorans* S2 to be palmitic acid (C16:0) 18.28%, oleic acid (C18:1) 30.84% and stearic acid (C18:0). Similarly, in the past, Knothe [38] reported the presence of palmitic, stearic, oleic, linoleic and linolenic acids in SCO. The presence of these fatty acids in oil can serve as a potential triglyceride feedstock for biodiesel [39]. Our results therefore indicate that the SCO from dual sugar-utilizing *T. mycotoxinivorans* S2 may serve as a potential triglyceride feedstock for biodiesel.

Table 6
Lipid yield from different oleaginous yeasts by using lignocellulosic hydrolysates.

Yeast	Substrate used	Lipid yield (g L^{-1})	References
Trichosporon mycotoxinivorans S2	Paddy straw	7.32	Present study
Cryptococcus curvatus	Wheat straw	5.80	[40]
Rhodosporidium kratochvilovae	*Cassia fistula*	4.86	[41]
Rhodotorula glutinis	Wheat straw	1.40	[42]
Trichosporon fermentans	Paddy straw	7.70	[43]
Candida tropicalis	Palm empty fruit bunch	2.73	[44]

5. Conclusions

In the present study, a promising oleaginous yeast *T. mycotoxinivorans* S2 capable of utilizing both glucose (C6) and xylose (C5) sugars was isolated and characterized. The ability of the strain to achieve high productivity even in the presence of inhibitors makes it a potential candidate for commercial exploitation. This yeast strain exhibited almost similar lipid yield from synthetic medium (9.78 g L^{-1}) and nondetoxified rice straw hydrolysate (7.32 g L^{-1}) under optimized conditions within 6 d of fermentation. The major fatty acids were palmitic, oleic, and stearic acids, which are similar to lipid composition of the currently used biodiesel. In future, metabolic engineering strategies may help to improve the lipid yield to develop a commercially viable process of SCO production from lignocellulosic biomass.

Conflict of interest

The authors declare that they have no conflict of interest.

Financial support

The first author (SS) would like to acknowledge the Indian Council of Agricultural Research, New Delhi, for providing fellowship for the MSc. Program.

Acknowledgment

The first author (SS) thanks Dr. Vinay Kalia for providing facilities of biomass lyophilization, Dr. Sujata Vasudev for gas chromatography, and Dr. Radha Prasanna for her critical inputs in English editing. All the authors are also grateful to the ICAR-Indian Agricultural Research Institute for providing necessary facilities.

This study was also supported by UGC, India (F.15-1/2017/PDFWM-2017-18-DEL-3915(SA-II UG).

Supplementary material

https://doi.org/10.1016/j.ejbt.2020.01.009

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