Single cell oil production by yeast using low cost substrates

Thesis submitted for the award of

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By

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Declaration

I hereby declare that the work towards this thesis entitled **"Single cell oil production by yeast using low cost substrates"** has been carried out by me under the supervision of Dr. Naseem A. Gaur, Group Leader, Yeast Biofuel Group, at the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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Certificate

This is to certify that the thesis entitled, **"Single cell oil production by yeast using low cost substrates"**, submitted by Kukkala Kiran Kumar for the degree of Doctor of Philosophy to Jawaharlal Nehru University (JNU) is based on the work carried out at International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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Dedicated to

My loving family members, well-wishers and almighty for their relentless support, encouragement and push during this long journey…….

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Contents

ABBREVIATIONS

ACAD	Acyl-CoA dehydrogenase
ACAT	Thiolase or Acetyl-coenzyme A acetyltransferases
ACC	Acetyl-CoA carboxylase
ACL	ATP citrate lyase
AMDIS	Automated Mass Spectral Deconvolution and Identification System
ASTM	American Society for Testing and Materials
ATMT	*Agrobacterium tumefaceins* mediated transformation
BLAST	Basic Local Alignment Search Tool
CFPP	Cold filter plugging point
CN	Cetane number
DGAT	Diacyl glycerol acyl transferase
ECH	Enoyl-CoA hydratase
EN 14214	European 1424
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
GC–MS	Gas chromatography–mass spectroscopy
GPDH	Glycerol-3 phosphate dehydrogenase
HHV	High heating value
HPLC	High-performance liquid chromatography
ICDH	Isocitrate dehydrogenase
ICP-MS	Inductively coupled plasma-induced ion chromatography mass spectroscopy
IL	Ionic liquid
IM	Induction medium
IS156907	Indian standards 56907
IV	Iodine value
KV	Kinematic viscosity
LD	Lipid droplet
ME	Malic enzyme
MFE	Multifunctional enzyme
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NIST	National Institute of Standards and Technology
OS	Oxidative stability
POX	Peroxisomal acyl-CoA oxidase
PUFA	Polyunsaturated fatty acid
RFU	Relative fluorescence unit
SCO	Single cell oil
SCT1	Glycerol -3 phosphate O acyltransferase
SFA	Saturated fatty acid
SSF	Simultaneous enzymatic hydrolysis saccharification and fermentation
SSLP	Simultaneous saccharification and lipid production
TAG	Triacylglycerol
TALDO	Transaldolase
TKT	Transketolase
TLC	Thin-layer chromatography
YE	Yeast extract
YNB	Yeast nitrogen base
YPD	Yeast extract, peptone, dextrose
5-HMF	5-Hydroxyl methyl furfural

LIST OF FIGURES

(Color codes blue and red of the heat map are the scaled normalized expression values and colour codes for pathway were assigned by ClustVis server).

Figure-4.2.3: Reconstructed model of lipid production including glycolysis, TCA cycle, fatty acid synthesis and lipolysis, β -oxidation in *R. pacifica* INDKK is depicted. Also, metabolic reactions, genes that were upregulated and downregulated under nitrogen limitation are highlighted in red and blue, respectively. Abbreviations for the components of the fatty acid synthase system are ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; ER, enoyl reductase; KR, ketoacyl reductase; KS, ketoacyl synthase; MPT, malonyl / palmitoyl transferase; PPT, phosphopantetheine transferase, GPD- Glyceraldehyde 3 phosphate dehydrogenase, PK-pyruvate kinase, PDH -Pyruvate dehydrogenase complex, E-2 component.

Figure-4.3.1: Diagrammatic representation of binary plasmid having ACC1 and its terminator (*R. toruloides* 880) with GAPDH promoter, DGA1 and its terminator (*R. toruloides* 880) with ACL promoter. The double overexpression cassette and nourseothricin resistance cassette were cloned between the left border (LB) and right border (RB) of T-DNA to enable *Agrobacterium tumefaciens* mediated transformation.

Figure-4.3.2: "push-pull" strategy for over expression of Acetyl-CoA carboxylase (ACC1) and diacylglycerol acyltransferase (DGA1)

Figure-4.3.3: (A) Negative control (B) Transformed colonies appeared having plasmid pGI2-880-ACC1-DGA1 on YEM Kan+ plates after 48h of incubation at 28 °C

Figure-4.3.4: (A) Yeast selected on YPD plates (200 µg/mL) NAT (200 µg/mL) and Cefotaxime (600 µg/mL)**:** 1. Transformed colonies 2. Negative control. (B) Transformed and wild type colonies grown on YPD liquid medium: 1. Wild type *R. pacifica* INDKK on YPD medium, 2. Wild type *R. pacifica* INDKK on YPD medium with NAT (100 µg/mL), 3. Transformed *R. pacifica* INDKK on YPD medium with NAT (100 µg/mL) + Cefotaxime (300 µg/mL)**.** (C) Transformed colonies streaked on YPD plates with NAT (200 µg/mL) and Cefotaxime (600 µg/mL) – 1. Generation-1, 2. Generation-2, 3. Generation-3.

LIST OF TABLES

ABSTRACT

Global warming and finite fossil fuels demand for alternative ecofriendly biofuels such as biodiesel. Plant oil-based fuels creates food vs energy concern and microbial lipid-based fuels are being explored these days. Therefore, many unconventional yeasts are being used development of lipid-based bio-refinery.

In this study, a novel oleaginous yeast isolate *R. pacifica* INDKK was identified and selected after screening 57 yeast, as it has shown high lipid producing ability, ability to grow on both C5, C6 sugars and high resistance to inhibitors such as acetic acid, HMF, furfural. It has produced 7.02 g/L of lipids on lignocellulosic waste Pongamia shell hydrolysate (PSH). The maximum lipid production by *R. pacifica* INDKK achieved was 14.65 g/L using media optimization process called response surface methodology (RSM). Omics study plays indispensable role to understand metabolic networks of microbes. A comprehensive whole genome sequencing of *R. pacifica* INDKK was done using hybrid Illumina and Nanopore sequencing, which revealed its of 33.63 Mb genome size. It also has given insights into central carbon, nitrogen and lipid related metabolic pathways. RNAseq analysis, revealed a total of 1228 differentially expressed genes involved in central carbon, nitrogen, energy and lipid metabolic pathways under N limiting conditions. The key genes of lipogenesis, FAS and ACC-1 were found to be upregulated and lipolytic genes of beta oxidation and lipases were downregulated under optimized medium. Also, nitrogen responsive genes such as GATA factors and nitrogen transporters were found to be upregulated. Also, the over expression of ACC-1 and DGA-1 in *R. pacifica INDKK* for lipid production improvement using *Agrobacterium tumefaciens* mediated transformation (ATMT) was attempted. Therefore, insights obtained through this study would aid in the development of *Rhodotorula pacifica* INDKK into a suitable host for feasible SCO production.

CHAPTER-1

Introduction and review of literature

1. INTRODUCTION AND REVIEW OF LITERATURE

The population density is growing exponentially and about to touch ~8.6 billion by 2030 which raise global issues for food and energy [1]. The vegetable oil utilization has also been increasing widely as they are required for various oleochemicals such as polymer processing [2] , drug delivery [3], lubricants [4], cosmetics [5] , biopharmaceuticals [4] and biofuels [6]. Microbes with lipid producing capacity above 20 % of their dry cell weight (DCW) are considered as oleaginous [7,8]. SCOs are produced by several oleaginous microbes including yeast, fungi, microalgae, bacteria but among them yeast serves as most promising unicellular microorganism due to their simple pathway editing, fermentation and high scale SCO production [9–11]. These yeast are also known to utilize various low cost substrates like molasses, acetic acid, industrial wastewaters, municipal sewage sludge, glycerol and lignocellulosic materials [12–14]. This review of literature discusses comprehensive insights about various robust oleaginous yeast strains which can utilize agro-industrial low-cost substrates for lipid production. Furthermore, improvement strategies for enhanced lipid agglomeration such as media engineering and genetic engineering with the aid of omics study in developing new oleaginous strains into industrially competent strains for sustainable SCO bio refinery establishment have been discussed.

1.1 Promising oleaginous yeasts

Several well-known unconventional yeast genera namely, *Rhodosporidium toruloides* [15], *Trichosporon species* [16], *Rhodotorula glutinis* [17], *Yarrowia lipolytica* [18], *Lipomyces starkeyi* [19], *Candida* species [20] have been explored for lipid production using lignocellulosic wastes [21,22]. Exploitation of yeast for production of industrially valuable compounds requires understanding their complex metabolic networks. Hence, in this study, different oleaginous yeast, their lignocellulosic waste conversion ability into lipids and

various strain development strategies that would aid in development of sustainable microbial lipid-based bio refinery.

1.1.1 Mechanism of sugar conversion into lipids by yeast

The conversion process of sugar into lipids by yeast occurs mainly by denovo biosynthesis, which includes three physiological phases: 1) growth phase, 2) oleaginous phase and 3) lipid turnover phase. In growth phase, carbon flux is diverted for making cell mass made of polysaccharides and proteins through glycolysis and the pentose phosphate pathway (PPP) with limited synthesis of polar lipids necessary for the cell membranes construction [23]. During stationary phase, the depletion of one essential nutrient (e.g. nitrogen, magnesium, phosphate or sulphate), induces the accumulation of oil [24]. However, during the final lipid turn over phase, degradation of TAGs occur to meet the energy for cell maintenance [25]. The most prominently studied condition which induces lipid accumulation is nitrogen deficiency, which causes reduction in concentration of adenosine monophosphate (AMP) and inhibition of NAD^+ dependent mitochondrial isocitrate dehydrogenase (NAD^+ - ICDH) [26,27]. This causes krebs cycle deregulation leading to rise in citric acid concentration inside the mitochondria which is then pumped out to cytosol by exchanging malate. Citric acid is then converted to oxaloacetate and acetyl-CoA by ATP-dependent citrate lyase (ACL) in cytosol. High activity of ACL and a null or low activity of isocitrate dehydrogenase (ICDH) in the cytoplasm are the key factors for lipids accumulation [23,28,29]. After complete carbon consumption, most oleaginous microbes begin to utilize storage lipids by lipid turn over or lipid degradation process called beta oxidation which occurs in mitochondria and peroxisomes [30–32]. The lipid yield generated from different carbon sources have different titers and it is estimated from the number of moles of acetyl-CoA produced. Different carbon sources generate different theoretical yields of lipid, *e.g*. glucose gives 1.1 mole acetyl-CoA and xylose gives 1.2 mole acetyl-CoA, which equals to maximum theoretical lipid yield of

0.32 g/g glucose and 0.34 g/g xylose, respectively during SCO production [33]. These values vary based on source of NADPH in fatty acid synthesis. NADPH obtained from malic enzyme (ME) generates the lipid yield of 0.31 g/g of glucose while PPP gives lipid yield of 0.27 g/g of glucose [34]. However, the practical yield of lipid production is ~ 22 % as carbon is diverted towards biomass and secondary metabolites production (Ratledge & Cohen, 2008). One study have shown that *Y. lipolytica* could reach near theoretical lipid yield of 0.29 g/g of glucose [35] and more recently 0.33 g/g of glucose was reported [36].

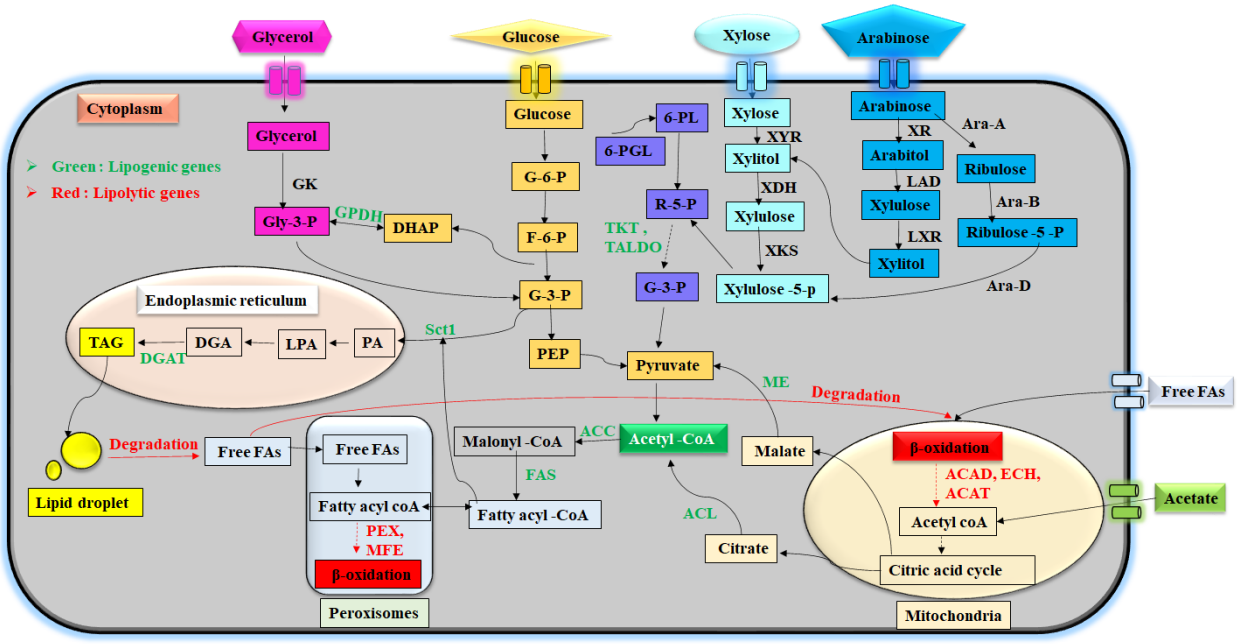

Figure- 1: Schematic representation of lipid production and turn over in yeast

1.1.2 Insights into valorization of agro-industrial wastes into lipids

Lignocellulosic biomass is the largest reservoir of sugars and made of 35–50 % cellulose, 15–25 % hemicellulose and 10-15 % lignin. To establish a sustainable microbial lipid bio-refinery, cheap carbon sources from agro industrial wastes serves as an attractive substrate for production of lipids as they are of low cost, renewable and loaded with fermentable sugars [29,37–39]. From the past decade , lipid production from various agro-industrial wastes by yeast are being explored [37,40]. To obtain fermentable sugars from recalcitrant

lignocellulosic materials special chemical, physical or biological pre-treatment are applied (Table 1.1) which generate several inhibitory compounds such as acetic acid, HMF, furfural, formic acid etc. that hinders microbial fermentation [11,41].

Table-1.1: Comparative analysis of different pre-treatment methods

Pre-treatment	Advantages	Disadvantages	Work condition	References
Ionic liquids (IL)	1. Environmental friendly. 2. ~ 90% fermentable sugars are released after enzymatic treatment	1. Costly and interferes during enzymatic treatment	80–160°C 3–50 % solid load 30 min-8 h 60–80 % w/w	[42–48]
Ammonia fibre expansion (AFEX)	1. Low inhibitor concentration. 2. Removes acetyl groups by deacetylation. 3. Agricultural residues are highly susceptible. 4. ~ 85% Lignin removed. 5. ~ 95% assimilable released after enzymatic hydrolysis.	1. Not suitable for softwoods and hardwoods 2. Costly process 3. Lignin interference with enzymatic process	60–200 °C 10–50 % solid load 5–60 min > 100 % w/w	[12,43,44,49–51]
ARP (Ammonia recycled Percolation) + SAA (Soaking in aqueous Ammonia)	1. High degree of delignification in hardwoods. 2. 99 % solids are recovered. 3. Preserves glucan and xylan.	1. Costly	140–210 °C 10–90 min 5–15 % w/w	[51–53]
CO_2 explosion	1. Delignification in both hard and softwoods and to dissolving cellulose and hemicelluloses. 2. No generation of inhibitory compounds. 3. Low cost and less environmental effects.	2. Lower yield relative to steam and AFEX explosion	31–250 °C 20–60 min 5–15 % w/w	[49–51,54,55]
Hot water	1. Digests hemicelluloses. 2. Do not require catalysts or chemicals. 3. Lignin removed by > 73 %. 4. Up to 95 % assimilable sugars are obtained after enzymatic hydrolysis. 5. Low inhibitors.	1. High water and energy usage to remove inhibitors	121–240 °C 10–20 % solid load 4–60 min	[49,50,56–58]
Oxidative (Wet	Used with alkali (NaOH).	1. Cellulose		

oxidation	2. Generates less inhibitors. 3. Combination with stem exploitation the conversion of cellulose and hemicelluloses is increased 4. 50–70 % of hemicelluloses and lignin are removed 5.Hardwoods and herbaceous biomass are effectively treated 6. ~ 95 % assimilable sugars are obtained	and mannans are not affected. 2. Possibility of non-selective oxidation causing loss of hemicelluloses and cellulose. 3. Large amount of acids and chemical compounds are generated 4. Oxidizing agents, high temperatures and pressure are required. 5. Costly process	25–195 °C 10–20 min 1–2 % w/v	[49,50,59]
Microwave pre-treatment	1. Used with combination of acids and alkaline and steam Exploitation 2. Low cost 3. Short reaction times. 4. Degrades lignin and hemicelluloses. 5. Homogeneous heating of the reaction mixture. 6. Recovery yields of glucose, xylose, and total sugar are ~ 13–27 %, 17–25 %, and 20–21 %, respectively.	1. High inhibitors	150–180 °C 3 min 1–2 % w/v	[47][60][61][12,62]

The pre-treatment methods that produce less inhibitory compounds or detoxification process after pre-treatment are considered to reduce the inhibitor concentration of the hydrolysates [11,63]. Oleaginous yeast that can consume common sugars like glucose, xylose and arabinose with tolerance to inhibitors generated during pre-treatment are preferred [64]. Therefore, necessary strain development, efforts are essential to make yeast assimilate cheap carbon sources and gain tolerance to inhibitors with industrially feasible SCO titers [65]. The final step before fermentation is enzymatic hydrolysis of pre-treated biomass to obtain C6 and C5 sugars from cellulose and hemicellulose fractions respectively [66].The inhibitory

compounds generated during pre-treatment also shows inhibitory effects on enzymatic hydrolysis [67]. One of the major challenges is to increase specificity and activity of enzyme with low-cost process. Non-ionic surfactants such as sorbitol ester and polyethoxylated, Tween-80, Tween-20, dodecylbenzenesulfonic acid, Triton X-100 and PEG 4000 recently have been used which proved to be effective in increasing digestibility of cellulose and decreased nonspecific function of enzymes [50,67–69]. Another alternative called simultaneous enzymatic hydrolysis (saccharification) and fermentation (SSF) have proven to be efficient process due to reduction in processing time, enzyme load, risk of contamination with higher yields than separate stepwise processing [70,71]. SSF for lipid production by *Cryptococcus curvatus,* was demonstrated by using cellulase and cellobioses on corn biomass pre-treated with ionic liquid (IL), resulting in a lipid yield of 112 mg of lipid/g of corn stover [72]. In one study, > 3 g/L lipids were obtained using cellulase on dilute acid pretreated and biodetoxified corn stover by *Trichosporon cutaneum* [73]. In another study, simultaneous process of saccharification and lipid production after dry acid pre-treatment and biodetoxification of corn stover was explored using *Rhodosporidium toruloides* which generated less lipid yield of 0.080 g/g [74]. More recently, 47.9 ± 1.5 mg/g of lipid was obtained on fungal pre-treated palm empty fruit bunches in SHF by *Y. lipolytica* [20].To achieve efficient SSF, parameters such as pH , % sugars, dose of enzymes and temperature to reach high yields at lower costs are needed to be tweaked [75,76]. In this context, the use of thermotolerant oleaginous yeast which can tolerate ~ 40 °C is beneficial as at this temperature the enzymatic hydrolysis of cellulose is more efficient. However, lipid production by yeast found to be low at this high temperature [72,77].

During pre-treatment of lignocellulosic biomass, many inhibitors are generated thus inhibiting the growth and lipid production by microbes which in turn decreases the fermentation efficiency. Hence, detoxification step is necessary to improve fermentation

which is achieved by adding heating vaporization, chemical compounds, enzymatic treatment, microbial treatment method and liquid-liquid or liquid-solid extraction [78].

Table-1.2: Agro-industrial wastes used for lipid production by yeast in various studies

S.no	Yeast	Carbon source	Lipid titer (g/L)	Lipid content (% w/w)	Reference
1	*Rhodotorula. glutinis* ATCC 204091	Wheat straw hydrolysate	3	25	[79]
2	*Cryptococcus curvatus*	Sorghum bagasse hydrolysate	7.93	73.26	[80]
3	*Rhodosporidium kratochvilovae* HIMPA1	Hemp seeds aqueous hydrolysate	8.39	55.56	[81]
4	*Lipomyces starkeyi* CBS 1807	Sweet sorghum stalks hydrolysate	6.4	30	[82]
5	*Rhodotorula glutinis*	Corncob hydrolysate	33.5	47.2	[83]
6	*Cryptococcus curvatus*	Volatile fatty acids from macroalgae fermentation	1.36	61	[84]
7	*Rhodosporidium kratochvilovae* HIMPA1	Cassia fistula fruit pulp hydrolysate	4.86	53.18	[85]
8	*Yarrowia lipolytica*	Food waste derived volatile fatty acids	0.37	18.23	[86]
9	*Rhodotorula toruloides*	Brewers' spent grain	10.41	56.45	[87]
10	*Cryptococcus curvatus*	Vegetable waste hydrolysate	2.11	28.3	[14]
11	*Cryptococcus curvatus*	Waste office paper hydrolysate	1.39-5.75	22-37.8	[88]
12	*Rhodosporidium fluvialis*	Sugarcane top hydrolysate & glycerol	18.2	75%	[89]
13	*Cryptococcus podzolicus* SCTCC30292	Corn stover	5.03	47.6	[90]
14	*Cryptococcus sp.*	Banana peel hydrolysate	1.12	34	[91]
15	*Rhodotorula mucilaginosa*	Sugarcane bagasse hydrolysate	1.99	29.55	[92]
16	*Rhodotorula pacifica* INDKK	Pongamia shell hydrolysate	7.02	55.89	[11]
17	*Rhodotorula taiwanensis* AM2352	Corncob hydrolysate	11.27	60.3	[93]
18	*Trichosporon fermentans*	Sweet sorghum hydrolysate	1.8	11.6	[94]
19	*Cryptococcus curvatus*	Waste office paper hydrolysate	4.9	43.11	[95]
20	*Rhodotorula mucilaginosa* KKUSY14	Durian peel hydrolysate	1.6	16	[96]

The most commonly used detoxification is utilization of $Ca(OH)_2$ where pre-treated lignocellulosic biomass is neutralized which reduces 5-HMF, furfural and phenolic compounds by ~ 20–30% [78,97]. Several researchers have used activated charcoal as detoxifier. Most recently, using activated carbon detoxified rice straw hydrolysate was assimilated by *Trichosporon fermentans* efficient lipid production [98]. Other methods such as resin adsorption, electrodialysis and biological detoxification by bacteria, such as *Bordetella sp.* and *Bacillus* sp., have been considered for efficient strategies to eliminate HMF and furfural [99–102]. Nevertheless, additional step of detoxification causes loss of sugars and raises overall production cost [102]. Apart from lignocellulosic wastes, industrial wastes such as molasses which contain saccharides such as sucrose, fructose, glucose and glycerol [103,104] also being widely used for SCO production.

1.2 Strategies implemented for improved lipid production

Information regarding optimal media conditions is essential inorder to reach the intrinsic maximum lipid production potential by oleaginous yeast. The omics studies to understand their elusive metabolic networks and strain improvement strategies such as genetic engineering to improve lipid production are being investigated now a days.

1.2.1 Media engineering to improve lipid production

The efficiency of lipid production by yeast depends on optimal cultivation conditions. The main media components such as carbon and nitrogen sources , C/N ratio [105], incubation time, temperature and pH [106,107] at appropriate levels determines sustainable lipid production [108–110]. The major biochemical response of high carbon to low nutrient scenario in oleaginous yeast is that the extra carbon is converted into lipids which further determines fatty acid composition [105,108,111,112]. Another important factor is the initial pH of the medium which plays a key role for lipogenesis as every oleaginous yeast has its own optimal pH for lipid production [113–117]. It has been observed that there is a reduction

in lipid production levels when the pH was < 4.0 and > 8.0 [118] making pH a very critical factor for lipid production [119–121]. The appropriate fermentation time is also critical for efficient fermentation process as futile prolonged fermentation makes the process expensive and unfeasible [31,118]. Conventionally one factor at a time (OFAT) method of medium optimization for lipid improvement is performed where only single component is changed keeping others unchanged at a given time [118]. However, OFAT requires experimental iteration of several components of culture conditions leading to arduous experiments but failing to identify the interactive effects of media components [109]. In order to achieve multi-factorial medium optimization, Box and Wilson proposed statistical methodology called Response Surface Methodology (RSM) [122] has been preferred over OFAT as it reduces experimental time and labor compared relatively with accurate statistically validated data [112,123–126]. In one study , the effect three nitrogen sources (peptone, NH_4SO_4, urea) with molasses on lipid accumulation in yeast were tested using OFAT [127].

1.2.2 Genomics and transcriptomics to understand lipid related metabolic networks

Due to metabolic differences observed from strain to strain, omics studies are indispensable tools in providing information of genetic elements like replicons, promoters, terminators, selection markers, target genes, and transcription factors that aids in understanding the genetic structure of microbes and facilitate in designing tools to modify them genetically. Several genomes of oleaginous yeast were studied which revealed key genes of carotenoids, lipids, carbohydrate metabolism and signal transduction pathways that helps in the production of biofuels, cosmetics and other industrially relevant compounds along with value added products [27,128,137–139,129–136]. In one study, genomics of *R. toruloides* NP11 yeast revealed 20.2 Mb genome with 8,171 genes and transcriptomics to identify highly expressed genes necessary for lipid production under nitrogen limiting conditions [140]. Its mitochondrial genome was also studied [141]. To understand the metabolic network during

high lipid production conditions, transcriptomic and proteome studies were performed in *R. toruloides* NP11 strain and identified regulatory mechanisms of lipid accumulation [140]. Also genomics of *R. toruloides* at the functional level was studied related to lipid accumulation [142]. Transcriptomics changes happening in yeast during the transition from growth phase to lipid production phase is very important to understand. Several researchers have shown that differential gene expression occurs at central carbon metabolism, central nitrogen metabolism, transporters, proteases, vacuole hydrolases, nucleotide metabolism , ribosome biosynthesis, TOR - signaling in relation to lipid metabolism of yeast [140,143–145]. The outcomes of phosphate limitation on lipid metabolism was analyzed by transcriptomic, proteome, and metabolite studies in *R. toruloides* [146,147]. The differential gene expression by transcriptomics in *T. oleaginosus* was studied under lipid accumulating condition [148,149] and the rise in ACL activity to increase the acetyl-CoA and malonyl-CoA flux into lipogenesis was observed. In another study, the transcriptomics study revealed mechanism of glycerol suppression on glucose consumption during co-substrate fermentation in *Y. lipolytica* [150]. Transcriptomic analysis of the physiology and gene expression patterns of *L. starkeyi* on clean sugars and corn stover hydrolysate revealed genes that are involved in detoxification of pre-treatment generated inhibitor compounds [151]. In one study, global transcriptomic analysis was carried out in unicellular yeast *T. cutaneum*, to understand the dimorphic shift induced by pH change and lipid biosynthesis [152]. Also, transcriptomics analysis of *R. toruloides* during growth on glucose, xylose, acetic acid, and lipids showed the involvement of diverse metabolite pathways under different carbon sources [148,153–155]. In another study, relation between TOR - signaling and lipid biogenesis was delineated in oleaginous yeast *Trichosporon oleaginosus* [156]. Recently, the transcriptomic profiling between wild and mutated *Rhodotorula sp.*U13N3 was studied to understand the changes in metabolism which helped in optimizing the lipid fermentation process [153].

1.2.3 Genetic modification to improve lipid titers

Unlike *saccharomyces cerevisiae*, genetic information and tools for modifying the genome of unconventional oleaginous yeast is not well established. But the metabolic engineering aspects of majority of the oleaginous yeast are still at dawn except *Y. lipolytica*. However, over the past decade, many significant efforts have been made to develop effective metabolic engineering strategies, techniques, tools and methodologies for many unconventional oleaginous yeast genera such as *Rhodotorula*, *Rhodosporidium*, *Lipomyces*, *Trichosporon*, and *Candida* with the aid of rigorous bioinformatics and omics data generated. The most explored unconventional oleaginous yeast is *Y. lipolytica* , because of its ability to utilize cheap carbon sources and accumulate high amount of lipids (68 % of DCW)[25,157]. Several metabolic engineering attempts have been made to enhance the lipid content upto 80 % DCW [157,158]. Recently, xylose isomerase pathway genes has been introduced in *Y. lipolytica* which improved lipid titers to 12.01 g/L when grown on hydrolysates of lignocellulosic materials [158]. Another yeast , *Lipomyces starkeyi* belongs to ascomycete has the potential to reach maximum lipid titers of about 85.1 % of its DCW [159], with ability to utilize various cheap carbon materials [27,40,160,161]. But genetic engineering tools to modify it are at still naïve stage and most recently novel electroporation transformation method was developed to integrate the drug-resistance gene markers into its genome [162]. *R. toruloides* is the extensively studied oleaginous yeast because of its intrinsic ability to produce high lipids around 76 % DCW [160,163].

It has several advanced characteristics such as utilization of long range carbon sources [173–175] and lignocellulosic hydrolysates along with the ability to tolerate the effects of wide range of inhibitory compounds [27,173,176].

From the previous metabolic engineering of oleaginous yeast, different regulatory mechanism has been proposed to improve lipid production**.** The important and regulatory enzymes

involved in *denovo* biosynthesis of lipids in yeasts are fatty acyl synthase (FAS), Acetyl-CoA carboxylase (ACC-1) and DGAT. The greater activity of ATP-citrate lyase (ACL), under N-limitation improve acetyl-CoA flux and induces lipogenesis [177].

Table-1.3: Genetic modifications in unconventional yeasts for lipid production improvement

S.no	Organism	Genetic modification	Lipid titer /content/yield	Reference
1	*Y. lipolytica*	Native ACC-1, Diacylglycerol acyl transferase (DGA1) *overexpression*, Glyceraldehyde-3-phosphate dehydrogenase (GPD) (*C. acetobutylicum) and* ME (*M. circinelloides)* expression	0.28 g/g of glucose	[164]
2	*Y. lipolytica*	Overexpression of transaldolase and transketolase	40 % of its DCW	[165]
3	*Y. lipolytica*	Steryl ester hydrolase (TGL-4), DGA-2, GPD-1, phosphoketolase, acetate kinase, xylulose kinase, xylose reductase expression, xylitol dehydrogenase, fatty-acyl coenzyme A oxidase (POX1–*6) deletion*	16.5 g /L	[165]
4	*Y. lipolytica*	Wax ester synthase (*Marinobacter hydrocarbonoclasticus*) expression and Peroxisome biogenesis factor 10 (PEX-10) disruption	1.18 g/L of FAEEs	[129]
5	*Y. lipolytica*	Heterologous expression of wax ester synthase (*M. hydrocarbonoclasticus)* expression in DSM 87986 in a Po1g strain	360.8 mg/L of FAEEs	[166]
6	*R. toruloides*	Overexpression of ACC-1, DGA-1 and SCD	89.4 g/L	[167]
7	*R. toruloides*	Overexpression of ACC-1 and DGA-1	16 g/L	[167]
8	*R. toruloides*	Expression of a second copy of the native genes Diacyl glycerol acyl transferase (DGAT-1) and SCD-1	39.5 g/L	[168]
9	*R. toruloides*	Δ12 desaturase (*Fusarium verticillioides* and *Mortierella alpine)* expression	1.3 g/L linoleic acid	[169]
10	*R. toruloides*	Elongase (ELO-1) overexpression and deletion of Δ12 desaturase (FAD-2)	Oleic acid raised by 23 %	[163]
11	*R. fluviale* DMKU-RK253	DGAT overexpression	1.2 g/L	[170]
12	*T. oleaginosus*	Δ9 elongase (IgASE2), Δ12/ω3 desaturase (Fm1) and linoleic acid isomerase *were expressed* heterologously	9 % Eicosadienoic acid, 16 % eicosatrienoic acid, 2.8 % to 21 % ALA increased	[171]
13	*T. oleaginosus*	Expression of *linolenic acid isomerase* (*P. acne)*	2.6 % rise in linoleic acid	[171]
14	*C. tropicalis*	CtRAP-1 expression	37 % DCW	[172]

Through transcriptomics, lower expression of isocitrate lyase (ICL) and rise in aconitase activity to increase citrate flux during N-limiting scenario was postulated [145]. These studies prove nitrogen limiting condition plays crucial role for increment of lipid production and suitable condition to understand lipid metabolism of oleaginous yeast. ME is known to produce NADPH for fatty acid elongation and in concurrence its overexpression in *R. glutinis* has proved to improve lipid production [178] while in *Y. lipolytica , C. tropicalis* and *L. starkeyi* due to absence of cytosolic ME, PPP supplies NADPH [34,179,180] and cytosolic acetyl-CoA is produced from citrate-pyruvate cycle [181]. The well explored and more genetic tools are developed for *Y. lipolytica* in relative to other unconventional oleaginous yeast. To redirect the central carbon flux into lipogenesis is implemented in *Y. lipolytica* , by increasing activity of in-house genes such as hexokinase, GPD-1(NAD^+-dependent G3P dehydrogenase) , DGA-1 , ACC-1 and 1-acyl-sn-glycerol-3-phosphate acyltransferase (SLC-1) [35,182,183]. Lipid content raised to 90 % DCW in *Y. lipolytica by* increasing activity of adenosine isomerase (AMPD), ACL-1, ACL-2, ME, DGAT genes in combination of PEX-10and multifunctional enzyme (MFE-1) (β-oxidation enzyme) deletion [184]. SCD, DGA-1, ACC-1 overexpression improved lipid production to 55 g/L in *Y. lipolytica* [185] . DGA-1and/or DGA-2 overexpression raised to 39 % DCW of lipids from 33.8 % DCW of lipids in *Y. lipolytica* [35,186]. DGA-1 and DGA-2 overexpression coupled with deletion of TGL-3 achieved 0.21 g/g on glucose lipid yield and also increase in activity of DGA-2 to 236 % lipid content in *Y. lipolytica* [183,187]. GPD (GapC) and malic enzyme (MCE2*)* over expression under the background of DGA-1 and ACC-1 over expression also improved total lipids [188]. The transcriptional regulators MIG-1and MGA-2 deletion or SNF1 mutation improved lipogenesis in *Y. lipolytica* [189–191]. Recently, over expressing PPP genes, transaldolase and transketolase rise in lipid content 40 % DCW [165]. Genetic engineering advancements in the oleaginous yeast *R. toruloides* has been less explored than *Y. lipolytica*

[136]. ACC-1 and DGA-1 upregulation *in R. toruloides* improved lipogenesis by two fold [192] and the improved activity of SCD, DGA-1and ACC-1 raised lipid levels (89.4 g/L) by 1.42 times using high density fermentation [167]. Increasing copy number of DGAT-1and SCD-1in *R. toruloides,* produced highest lipid titer of 39.5 g/L [168]. Recently, oleaginicity of yeast *R. azoricum* was improved by 89 % than wild type by expressing homologous phosphoketolase (Xfpk) and phosphotransacetylase (Pta) [193]. In another study, DGA-1 overexpression in *R. fluviale* DMKU-RK253 led to 2.5-fold increment in lipids [170]. FAD-2 and DGAT-2 (*Vernicia fordii)* was heterologously expressed which improved linoleic acid by 102 % and α - linolenic acid by 174.36 % in *R. glutinis* [194].

T. oleaginosus is known for high lipid accumulation and by expressing phospholipid: diacylglycerol acyltransferase (PDAT-1) from *Rhizopus oryzae* along with pyruvate decarboxylase (PDC-1), acetyl-CoA synthetase (ACS-2) and acetaldehyde dehydrogenase (ALD-6) from *S. cerevisiae* for enhanced lipid production [181]. By heterologous ME (*M. circinelloides*) expression in *R. glutinis* raised lipogenesis by 2.1-fold [195]. Elusive genome information and no genetic tools restricted genetic modification of many capable oleaginous yeasts [196]. Similarly for *T. oleaginous*, genetic engineering tools are yet naïve stage. But phospholipid : diacylglycerol acyltransferase (PDATs) expression in *T. oleaginosus* and bypassing PDH pathway increased lipid titer to theoretically the maximum yield of 0.27 g/g [181]. *C. tropicalis* also been known for its oleaginicity but its genetic manipulation is at dawn. However, recently, it's lipid content has been improved [172,197]. Therefore, based on above mentioned genetic engineering strategies would be helpful to design genetic modifications in newly identified oleaginous yeast to improve lipid production.

1.3 Global SCO lipid and Biodiesel market

The rising demand for lipid applications in biofuels, nutritional and pharmaceutical industries, and commercial range of lipid market value has soared up. The compound annual

growth rate (CAGR) of lipid has reached 9.30 % from period 2021 to 2028 with an estimate market value of USD 19.42 billion by 2028 as reported by Data Bridge Market Research analyses [198]. Also, the demand for environmentally-friendly fuels has raised the biodiesel market value. Majorly biodiesel manufactured from plant oils such as rapeseed, soybean and palm are competitive to food sector. The evaluated market size of biodiesel is to be USD 34.1 billion (2016) and is expected to raise to USD 41.2 billion (2021) at a CAGR of 3.8 %, between 2016 and 2021 [198]. Since last decade, several pilot scale SCO based biodiesel production plants have been established. A SCOs derived biodiesel production pilot scale of 1000 L from *R. toruloides* DEBB 5533 using sugarcane juice [199] and 300 L pilot-scale from *R. glutinis* using starch wastewater [200] were established. These studies have shown that SCO based biodiesel can be produced at higher yield (4172 L/ha) with price (0.76 US$/L) than soya bean-based biodiesel of yield 661 L/ha and price of 0.81 US$/L. SCOs derived biodiesel from cheap raw materials is technically demonstrated at lab scale and pilot scale but at industrial scale has not been accomplished so far.

1.4 Objectives and scope of this study

The demand for SCOs has been increasing owing to their wide industrial application [2–4,6]. Oleaginous yeast factories are being used for production of SCOs. However, the robust oleaginous yeast which can meet the industrial level SCO production yet to be developed. In this context, this thesis work is categorized into three major objectives:

1) Screening and identification of high lipid producing yeast strains.

2) Media optimization to improve lipid production in selected *R. pacifica* INDKK yeast.

3) Improving lipid production by metabolic engineering in *R. pacifica* INDKK.

In the first objective, screening of a pool of yeast for identification of potential oleaginous yeast with high lipid titer was performed. These strains for tested against different inhibitors for evaluating its inhibitor tolerance profile. During the second objective, media optimization

was performed using response surface methodology to establish the optimal media conditions for improving its lipid titer. In the final objective, genomics and transcriptomics was performed in order to understand its metabolism at genomic and transcriptional level. Clues derived from omics study and information from literature, metabolic engineering strategy was developed to further improve lipid titer in *R. pacifica* INDKK.

CHAPTER -2

Screening and identification of high lipid producing yeast strains

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2. Screening and identification of high lipid producing yeast strains

2.1 Materials and methods

2.1.1 Media and other chemicals

PS were collected from International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi campus. All analytical reagents and solvents (chloroform, methanol, n-hexane, diethyl ether, glacial acetic acid, H_2SO_4) were of high-performance liquid chromatography (HPLC) grade. Nile red (9-diethylamino-5-benzo[α] phenoxazinone), Bodipy 493–503 nm, Heptadecanoic acid (internal standard) and FAME external standard (Supelco 37 component FAME mix) for GC–MS analysis were procured from Sigma (USA). Standard for thin-layer chromatography (TLC) (Triolein), sugars (glucose, xylose, arabinose) were procured from Hi-Media laboratories (Mumbai, India). YNB, yeast extract and peptone were purchased from Difco (USA).

2.1.2 Screening and identification of oleaginous yeast strains

Soil sample was collected from the Coringa mangrove forest, Kakinada, Andhra Pradesh (16.83139°N–82.33667°E), India. 10^{-1} to 10^{-6} serial dilutions were made in sterilized (0.9 % w/v) saline and seeded onto YPD agar plates (agar (2 % w/v); glucose (2 % w/v); peptone (2 % w/v); yeast extract (1 % w/v)) with chloramphenicol (35 μg/mL) and neomycin (50 μg/mL). After 72 h incubation at 30 °C and colonies with yeast-like morphology were further streaked on YPD agar plates for pure single cell culture. Yeast strains were also obtained from NCIM (National Collection of Industrial Microorganisms), Pune, and MTCC (Microbial Type Culture Collection and Gene Bank), Chandigarh.

2.1.3 Microwave aided Nile red staining and screening of oleaginous yeast

Yeast isolates were screened for lipid accumulation by using microwave assisted Nile red staining protocol with modifications [201]. Briefly, single colony culture of each strain was grown overnight in YPD medium at 30 ºC and 200 rpm (pre-culture). The pre-culture was centrifuged and washed twice with Milli-Q (MQ) water, re-suspended in 100 mL YNB

medium with glucose (3 %) and $(NH_4)_2SO_4$ (0.5 %) to optical density (OD) of 0.2 at 600 nm and incubated for three days at 30 °C at 200 rpm. Cells corresponding to OD 1 of the above grown cultures were centrifuged (5000 ×*g*, 4 min) and re-suspended in 50 µL of Dimethyl sulphoxide (DMSO) followed by microwave treatment (1250 watts power for 60 sec). Cells were mixed with Nile red solution (10 µg/mL) and again subjected to microwave treatment (1250 watts power, 60sec). Four replicates of each treatment were prepared and relative fluorescence intensity (RFU) was measured at exciting and emission wavelengths of 475 nm and ~ 580 nm, respectively. Relative neutral lipid content was represented as RFU of LD [202].

2.1.4 ITS sequencing and phylogenetic analysis

The 18S rDNA sequence was PCR amplified from the genomic DNA by using ITS universal primers in a PCR machine (Eppendorf, Nexus GSX1, Germany) [203]. The 50 µL PCR reaction (MQ-water, 37.5 µL; Taq DNA polymerase (G-Biosciences, USA), forward primer and reverse primer, 2.5 µL each; deoxyribonucleotide triphosphate (dNTP) mix, 1 µL; PCR Taq buffer, 5 µL; 0.5 µL; and genomic DNA, 1 µL) was used at conditions: 5 min initial denaturation (95 °C), of 30 s denaturation (95 °C, 30 cycles), 30 s annealing (52 °C) and 1.0 min extension (72 °C) and a 5.0 min final extension (72 °C). The Gene JET PCR Purification Kit (Thermo scientific, Lithuania) was used for PCR products cleaned up. The PCR amplified DNA fragments were sequenced (Invitrogen BioServices, India) and used for BLAST (Basic Local Alignment Search Tool) analysis in NCBI (National Centre for Biotechnology Information) database. Sequences were subjected to multiple sequence alignment (ClustalW tool) and MEGA (Molecular evolutionary genetics analysis) X software was used for phylogenetic tree construction using sequences with shared similarity.

2.1.5 Cell growth in presence of different carbon sources and pre-treatment inhibitors

To find the carbon source utilization by *R. pacifica* INDK, the experiment was conducted on individual carbon sources. For this, cells were grown in 100 mL YNB medium supplemented with 2 % carbon source individually (sucrose, cellobiose, glucose, mannose, galactose, rhamnose, arabinose, xylose and glycerol) and cultured at 200 rpm, 30 ℃ for three days. The inhibitor tolerance profile was tested by growing cells in YNB with 2 % glucose supplemented with varying concentrations of 5-hydroxy methyl furfural (5-HMF) (0.5 to 3 g/L), furfural (0.5 to 3 g/L) and acetic acid (0.2 to 0.7 g/L). YNB without inhibitors was used as control medium for cell growth.

2.1.6 Biomass and lipid production on PSH

PS were washed thoroughly with water, dried in oven (60 ℃ for 48 h) and crushed in grinder. ~ 20 g of dried powder was subjected to dilute acid (2 % v/v) as well as alkaline (2 % v/v) treatments in autoclave for 90 min at 121℃. The liquid portion of acid treatment was detoxified by activated charcoal (15 % w/v) at 30 ℃ for 3 h. Solid fraction of both acid and alkali treatments were neutralized and subjected to enzymatic saccharification using 20 FPU of cellulases/g (Sigma, U SA) of biomass at 50 ℃, 150 rpm for 72 h [204]. The hydrolysate was filtered and used as cultivation medium after supplementation of micro-nutrients such as $MgSO_4$ (1.5 g/L), NH_4SO_4 (0.5 g/L), KH_2PO_4 (1.5 g/L). In parallel, YNB supplemented with 46.5 g/L of sugars (glucose 28 g/L, xylose 18.18 g/L, and arabinose 0.3 g/L) was used as control for cell growth and lipid accumulation. Lipid production experiment was executed in Erlenmeyer flasks (250 mL) containing 100 mL medium by adding overnight grown cells of OD 0.2 and incubating at 30 ℃, 200 rpm for 196 h (pH 6.8). Harvesting of cells were done using centrifuge, washed with MQ water, lyophilized (Labconco, USA) and DCW (g/L) was determined. Lipids were extracted from lyophilized cells by using modified Bligh and Dyer method [205], lipid titer (g/L) was determined gravimetrically. Biomass productivity (g/L/h),

lipid productivity (g/L/h) and lipid contents (%) were calculated as described previously [206].

2.1.7 Lipid analysis

Lipid analysis was performed by confocal microscopy (Nikon, India) after staining the cells with Bodipy dye (0.5 µg/mL DMSO) [85]. Cell sizes and LD sizes were measured by using Nikon software. TAG analysis was performed on TLC plates (Merck, India) along with standard triolein for comparison as reported earlier [207]. The TAG estimations were performed by using Image-J software.

2.1.8 Analytical methods

Sugar (glucose, xylose, arabinose) and inhibitor (5-HMF, furfural and acetic acid) concentrations in PSH were analyzed by using HPLC (Agilent 1260 Series) equipped with Aminex HPX-87H column (Bio-Rad, USA) and refractive index (RI) detector. The mobile phase H_2SO_4 (4 mM) was used at a flow rate of 0.3 mL/min at column temperature of 40 °C [208]. Trace elements were analyzed by Inductively Coupled Plasma Induced Ion Chromatography- Mass Spectroscopy (ICP-MS) analysis (Agilent 7900) using argon as carrier gas and sample flow rate was 2.0 mL/min with approximately 2.5 min total analysis time per sample. The samples were acidified with nitric acid to pH below 2.0 and filtered through a membrane filter of 0.45-µm pore diameter [206]. The protein concentration was estimated by Bradford method [209]. Holocellulose, cellulose and xylan content were determined by using standard Association of Official Analytical Chemists (AOAC) methods of analysis [210]. The dried PS powder was also subjected to Energy dispersive X-ray (EDX) elemental analysis.

2.1.9 Transesterification and GC-MS analysis

Transesterification was performed by previously described method [211] with some modifications. Briefly, lyophilized yeast cells and 6 % methanolic-H_2SO_4 in 1:20 ratio were

taken in teflon-sealed tube and heated at 80 °C for 1 h. FAMEs were extracted into hexane phase and analyzed by GC-MS (7890A series) equipped with Omegawax (30m × 0.25mm ID, 0.25µm thickness) and Agilent 7000 QQQ MS [212]. Identification and quantification of FAMEs were performed by NIST (National Institute of Standards and Technology) mass spectral database, AMDIS (Automated Mass Spectral Deconvolution and Identification System) and mass hunter software. Physical properties of biodiesel were computed by using previously reported experimental equations [213] and collated with rape seed oil methyl ester, jatropha oil methyl ester and to EN 14214, ASTM D6751 and Indian standards IS156907 [214].

2.2 Results and discussion

2.2.1 Screening and molecular identification of the selected yeast isolate

In this study, a pool of potential oleaginous yeast isolates (57) including strains procured from collection centres in India (NCIM and MTCC) as well as by screening samples from various sites of biomass degradation were used (Table 2.1).

Table-2.1: List of yeast strains screened in this study by microwave aided nile red spectrofluorimetry

S.no	Strain	RFU*	S.no	Strain	RFU*
1	*Saccharomyces cerevisiae*-NG1, *(*Sugar distillery waste, Bijnor, Uttar Pradesh, India) (Lab isolate)	284.12	30	*Saccharomyces cerevisiae* (NCIM-3219)	174.43
2	*Kluyveromyces marxianus* (Mother dairy, New delhi, India)(Lab isolate*)*	85.17	31	*Yarrrowia lipolytica* (NCIM- 3590)	365.23
3	*Pichia kudriavzevii*-NG19 *(*Brewery boiler, Bulandhshahar, Uttar Pradesh, India) (Lab isolate)	32.8	32	*Yarrrowia lipolytica* (NCIM-3472)	121.54
4	*Wikerhamomyces anomalus-NG25(*Sugar distillery waste, Bijnor, Uttar Pradesh, India) (Lab isolate)	60.48	33	*Torulospora delbrueckii* (MTCC-2893)	202.7
5	*Ogataea thermophile*-NG10 (Sugarcane distillery waste, Bulandshahar, Uttar pradesh, India) (Lab isolate)	27.35	34	*Torulospora delbruecki* (MTCC-3415)	41.89

6	*Candida tropicalis -NG 26*, (Sugarcane bagasse distillery waste, Bijnor, Uttar Pradesh, India) (Lab isolate)	68.69	35	*Scheffersomyces spartinae* (MTCC-9717)	31.23
7	*NG-45* (Sugarcane bagasse distillery waste, Bijnor, Uttar Pradesh, India) (lab isolate)	64.23	36	*Rhodosporidium paludigenum* (MTCC-2889)	112.28
8	*Candida lusitaniae* (NCIM -3484)	31.63	37	*Pichia Manshurica saito* (MTCC-4799)	122.54
9	*Kluyveromyces lactics* (NCIM-3551)	83.19	38	*Pichia Manshurica saito* (MTCC-4051)	63.48
10	*Candida shehatae* (NCIM-3500)	103.29	39	*Rhodosporidium paludigenum* (MTCC-2890)	61.89
11	*Pichia stipites* (NCIM-3507)	53.21	40	*Rhodosporidum kratochvilovae* (MTCC-190)	248.16
12	*Schwanniomyces occidentalis* (NCIM-3424)	102.19	41	*Rhodosporidum kratochvilovae* (MTCC-247)	957.23
13	*Trichosporan pullulans* (NCIM-3151)	226.43	42	*Starmerella bombicola* (MTCC-1910)	31.76
14	*Rhodosporidium toruloides* (NCIM-3547)	136.76	43	*Lipomyces starkeyi* (MTCC-1400)	236.96
15	*Rhodosporidium toruloides (NCIM-3641)*	1144.3	44	*Yarrowia lipolytica* (MTCC-9517)	47.03
16	*Rhodosporidium dibovatum* (NCIM-3657)	276.01	45	*Cryptococcus curvatus* (MTCC-2698)	145.98
17	*Rhodosporidium dibovatum* (NCIM-3658)	307.22	46	*Cryptococcus albidus* (MTCC-2661)	208.81
18	*Rhodotorula glutinis* (NCIM-3168)	160.03	47	*Blastobotrys adeninovorans* (MTCC-2517)	263.12
19	*Rhodotorula glutinis* (NCIM-3169)	310.54	48	Coringa soil isolate (KS-1)	148.63
20	*Rhodotorula minuta* (NCIM-3359)	83.75	49	Coringa soil isolate (KS-2) (Lab isolate)	82.75
21	*Rhodotorula minuta* (NCIM-3427)	58.92	50	Coringa soil isolate (KS-3) (Lab isolate)	126.58
22	*Pichia anomala* (NCIM-3341)	205.65	51	Coringa soil isolate (KS-4) (R. pacifica INDKK)	1316.1
23	*Hansenula beijernikii* (NCIM-3343)	76.8	52	Coringa soil isolate (KS-5)	40.90
24	*Rhodotorula rubra* (NCIM-3260)	721.02	53	Coringa soil isolate (KS-6)	45.37
25	*Hansenula californica* (NCIM-3438)	106.69	*54*	Coringa soil isolate (KS-7)	96.12

26	*Cryptococcus laurentii* (NCIM-3373)	38.69	*55*	Coringa soil isolate (KS-8)	24.06
27	*Candida tropicali* (NCIM-3121)	136.52	*56*	Coringa soil isolate (KS-9)	135.3
28	*Candida magnolia* (NCIM-3646)	216.6	*57*	Coringa soil isolate (KS-10)	115.41
29	*Candida guilliermondii* (NCIM-3124)	78.66			

⁎ *RFU:* Relative fluorescence intensity (RFU) values estimated in this study by microwave aided nile red spectrofluorimetry

Next, molecular identification of new yeast isolate was carried out by PCR amplification of the ITS region (using genomic DNA template) followed by phylogenetic relationship analysis. Top hits of the BLAST analysis showed 99 % identity with yeast belonging to *Rhodotorula* species such as *Rhodotorula* sp. SY-96 (Acc. No. AB026006.2 of 1194 bp), *Rhodotorula* sp. LH23 (Acc. No. HQ832796.1 of 643bp), *Rhodotorula pacifica* (Acc. No. AB193175.1 of 1194bp) and *Rhodotorula pacifica* AUMC 10761 (Acc.no. KY495729.1 of 614 bp), indicating that selected isolate might belongs to *Rhodotorula* species. Phylogenetic relationship with oleaginous yeasts was established by MEGA X software [215]. The evolutionary background of the taxa was determined by the Maximum likelihood method [208]. The bootstrap consensus of tree was obtained from 1000 replicates and units of branch length are represented by number of nucleotide substitutions per site.

As shown in Figure 2.1, 4 clades were formed; Clade-1 belongs to *R. toruloides* strains. Clade-includes *Yarrowia lipolytica.* Our lab isolate (appendix) was grouped with *R. pacifica* strains in clade-3. Indicating evolutionary closeness to *R. pacifica.*

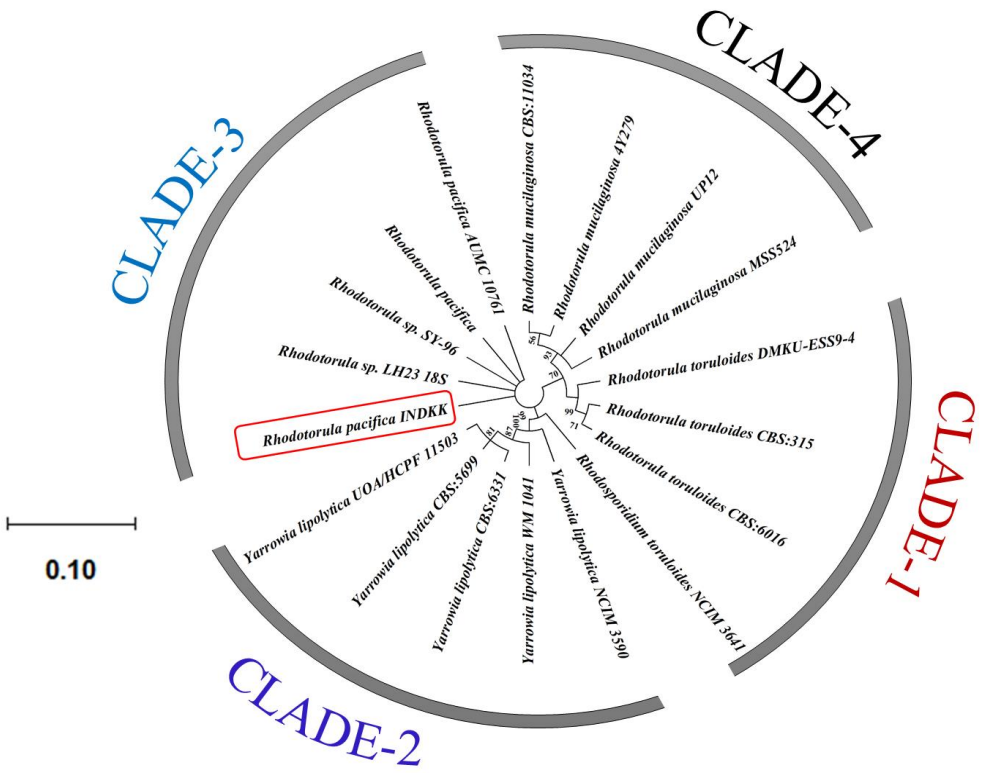


Figure-2.1: Phylogenetic tree of *Rhodotorula pacifica* INDKK (Acc. No. MN560184) showing evolutionary history constructed by MEGA X software using the maximum likelihood method. Bootstrap values greater than 50 % are represented as numbers above the branches.

Clade-4 comprised of strains belonging to *R. mucilaginosa*. The ITS sequence was submitted in NCBI as *R. pacifica* INDKK with GenBank Accession No. MN560184.The evolutionary hierarchy of *Rhodotorula pacifica* INDKK was Eukaryota > Fungi > Dikarya > Basidiomycota > Pucciniomycotina > Microbotryomycetes > Sporidiobolales > Sporidiobolaceae >*Rhodotorula pacifica* INDKK.

2.2.2 Selection of high lipid accumulating yeast isolates

Yeast isolates accumulating more than 20 % TAG are considered as good candidates for microbial lipid based fuel production [216]. For screening high lipid accumulating strain, cells were grown in nitrogen (N) limiting yeast nitrogen base without amino acids and ammonium sulphate (YNB) medium [217]. Initially, conventional Nile red staining based spectrofluorimetry method [218] was tested with known high and low TAG accumulating

yeast strains, *R. toruloides* (NCIM-3641) and *Saccharomyces cerevisiae* respectively. Surprisingly, no emission was found at ~ 580 nm (corresponding to neutral lipids) and the emission at ~ 620 nm, (corresponding to polar lipids) was not able to distinguish between oleaginous and non-oleaginous yeast [219]. Hence, we optimized microwave aided Nile red staining for yeast generating emission peak at ~580 nm and clearly differentiating the Relative fluorescence unit (RFU) values of *R. toruloides* (NCIM-3641) and *S. cerevisiae* (Figure 2.2).The 6 strains, *Rhodotorula pacifica* INDKK (lab isolate), *Rhodosporidium toruloides* (NCIM-3641), *Rhodosporidium kratochvilovae* (MTCC-248), *Rhodotorula rubra* (NCIM-3260), *Rhodotorula glutinis* (NCIM-3168), *Rhodosporidium dibovatum* (NCIM-3658) showed higher or comparable RFU value (Figure 2.3a) to *Yarrowia lipolytica* (NCIM-3590), a previously reported high lipid accumulating yeast isolate [36].

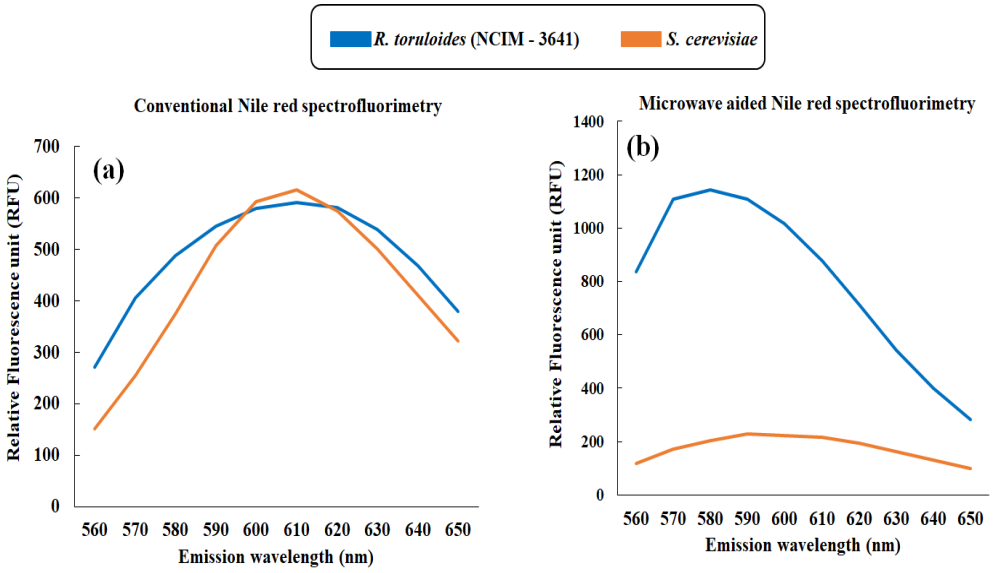


Figure-2.2: (a)Conventional Nile red spectrofluorimetry showing emission peaks at ~ 620nm for both *R. toruloides* (NCIM -3641) (high lipid) strain and *Saccharomyces cerevisiae* (low lipid) strain, (b) Microwave aided Nile red spectrofluorimetry showing emission peaks at ~580 nm for both *R. toruloides* (NCIM -3641) (high lipid) strain and *Saccharomyces cerevisiae* (low lipid) strain.

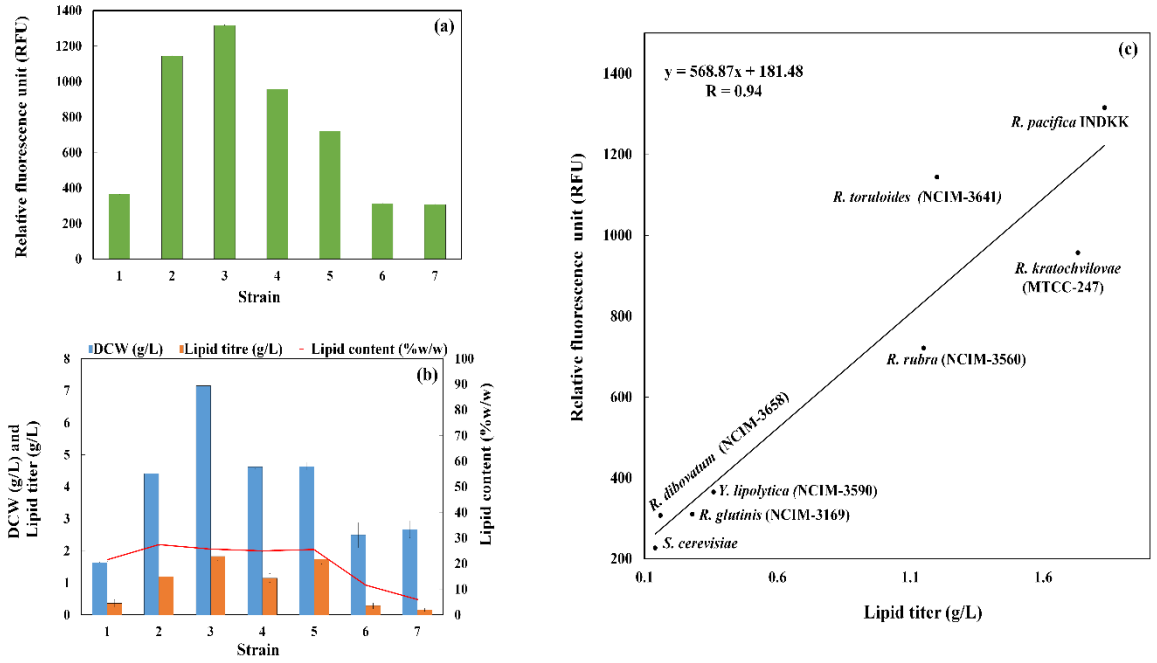


Figure-2.3: a) RFU values of (1) *Y. lipolytica as control*, (2) *R. toruloides* (NCIM-3641), (3) *R. pacifica* INDKK, (4) *R. kratochvilovae* (MTCC-248), (5) *R. rubra* (NCIM-3260), (6) *R. glutinis* (NCIM-3168), (7) *R. dibovatum* (NCIM-3658) from Nile red spectrofluorimetry. b) Comparison of DCW (g/L), lipid titer (g/L) and lipid content (% w/w) of six selected strains with (1) *Y. lipolytica as control*, (2) *R. toruloides* (NCIM-3641), (3) *R. pacifica* INDKK, (4) *R. kratochvilovae* (MTCC-248), (5) *R. rubra* (NCIM-3260), (6) *R. glutinis* (NCIM-3168), (7) *R. dibovatum* (NCIM-3658) after 72 h (one-way ANOVA test, $P < 0.05$). c) Correlation plot showing liner correlation between lipid titers (g/L) from gravimetric analysis and RFU values of *S. cerevisiae*, *Y. lipolytica* (NCIM-3590), *R. toruloides* (NCIM-3641), *R. pacifica* INDKK, *R. kratochvilovae* (MTCC-248), *R. rubra* (NCIM-3260), *R. glutinis* (NCIM-3168) and *R. dibovatum* (NCIM-3658).

The high lipid accumulation in these isolates was further confirmed by lipid droplet (LD) size measurement using confocal microscopy (Figure 2.4). DCW (g/L), lipid titers (g/L) and lipid content (%) of these isolates were also determined gravimetrically and *R. pacifica* INDKK

showed maximum lipid titer (1.8 g/L) followed by *R. kratochvilovae* (1.6 g/L), *R. toruloides* (1.4 g/L) and *R. rubra* (1.2 g/L) at 72 h as shown in Figure 2.3b.

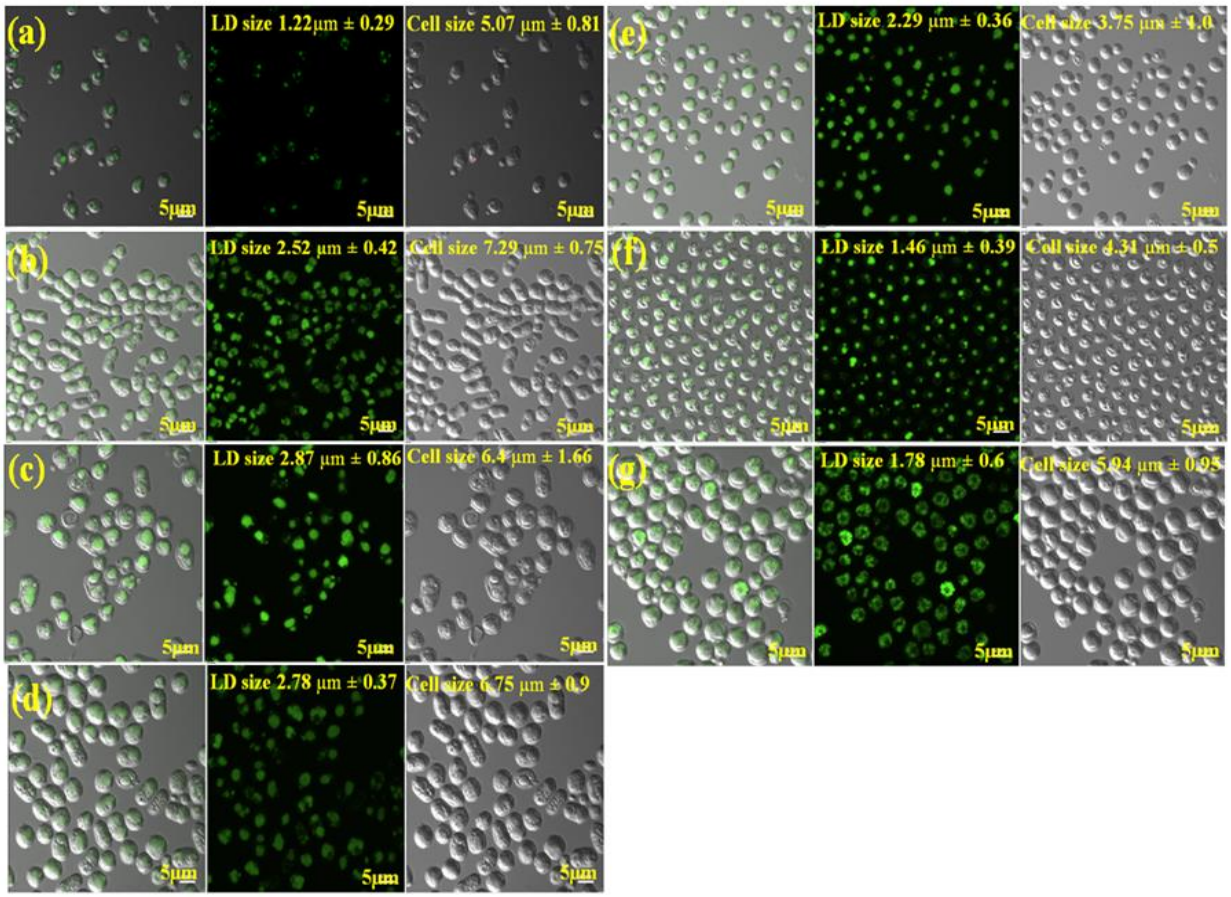
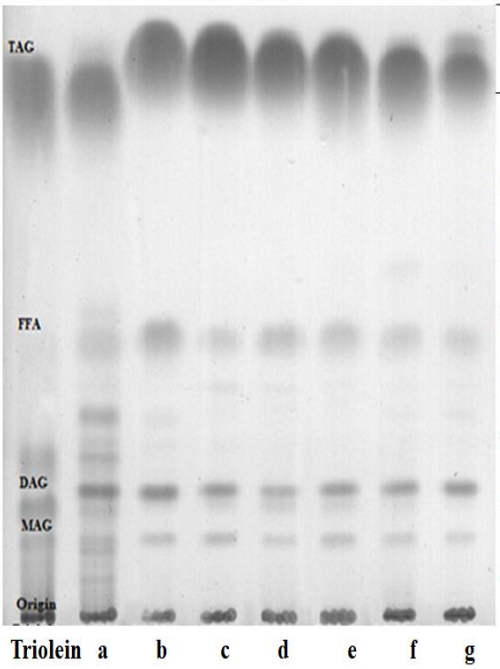


Figure-2.4: Confocal microscopy of (a) (Control) *Y. lipolytica* (NCIM-3590), (b) *R. toruloides* (NCIM-3641) (c) *R. pacifica* INDKK (d) *R. kratochvilovae* (MTCC-248) (e) *R. rubra* (NCIM-3260) (f) *R. glutinis* (NCIM-3168) (g) *R. dibovatum* (NCIM-3658) showing cell size and lipid droplet size.

Gravimetric lipid analysis of the selected strains showed good correlation with microwave aided Nile red staining (Correlation Coefficient R = 0.94), suggesting the reliability of microwave aided Nile red spectrofluorimetry for different yeast species and genera (Figure 2.3c). Densitometric analysis of TLC revealed major % triacylglycerols (TAG) but low % of free fatty acids (FFA), diacylglycerols (DAG), monoacylglycerols (MAG) in all lipid extracts cultivated in YNB medium.



Densitometric analysis

Yeast	TAG (%)	FFA (%)	DAG (%)	MAG (%)
(a) *Y. lipolytica* (NCIM-3590)	46.38	14.42	32.14	7.057
(b) *R. toruloides* (NCIM-3641)	58.97	16.20	12.47	12.34
(c) *R. pacifica* INDKK	67.92	9.67	10.29	12.11
(d) *R. kratochvilovae* (MTCC-248)	69.01	15.89	6.49	8.60
(e) *R. rubra* (NCIM-3260)	60.03	12.54	12.08	15.33
(f) *R. glutinis* (NCIM-3168)	56.00	14.36	16.46	13.17
(g) *R. dibovatum* (NCIM-3658)	58.90	11.68	19.36	10.06

Figure-2.5: TLC analysis Control (Triloein), (a) *Y. lipolytica* (NCIM-3590), (b) *R. toruloides* (NCIM-3641) *(c) R. pacifica* INDKK (d) *R. kratochvilovae* (MTCC-248) *(e) R. rubra* (NCIM-3260) *(f) R. glutinis* (NCIM-3168) *(g) R. dibovatum* (NCIM-3658).

Moreover, quantitative analysis of lipid was performed by TLC. The chromatogram showed highest lipid accumulation by *R. pacifica* INDKK with TAG (67.92 %), MAG (12.11 %), DAG (10.29 %) and FFA (9.67 %). Notably, TAG content in *R. pacifica* INDKK was 1.46-fold higher than *Yarrowia lipolytica* (NCIM-3590) (Figure 2.5). We further performed kinetic studies with four isolates showing > 20 % w/w of lipid content in gravimetric analysis (*R. pacifica* INDKK, *R. toruloides, R. kratochvilovae, R. rubra)*. Kinetics of growth and lipid accumulation among these isolates revealed lowest DCW (6.88 ± 0.22 g/L), lipid titer (3.47 ± 0.05 g/L), lipid productivity (0.024 ± 0.05 g/L/h) and slow glucose utilization rate (0.17 g/L/h) by *R. toruloides* at 120 h (Figure-2.6a). However,

R. pacifica INDKK showed maximum DCW (12.8 ± 0.66 g/L), lipid titers (6.8 ± 0.4 g/L) and lipid productivity (0.056 ± 0.4 g/L/h) with glucose consumption rate of 0.25 g/L/h (Figure2.6b). Though glucose consumption rate (0.025 g/L/h) of *R. kratochvilovae* is similar

to *R. pacifica* INDKK but it showed lower lipid titer (6.32 g/L) **(**Figure 2.6c). *R. rubra* also showed low lipid titer (5.35 g/L) and poor sugar consumption rate (0.02 g/L/h) as compared to *R. pacifica INDKK* (Figure-2.6d). Together, among all the tested isolates *R. pacifica* INDKK showed maximum potential for lipid accumulation and was selected for further analysis in this study.

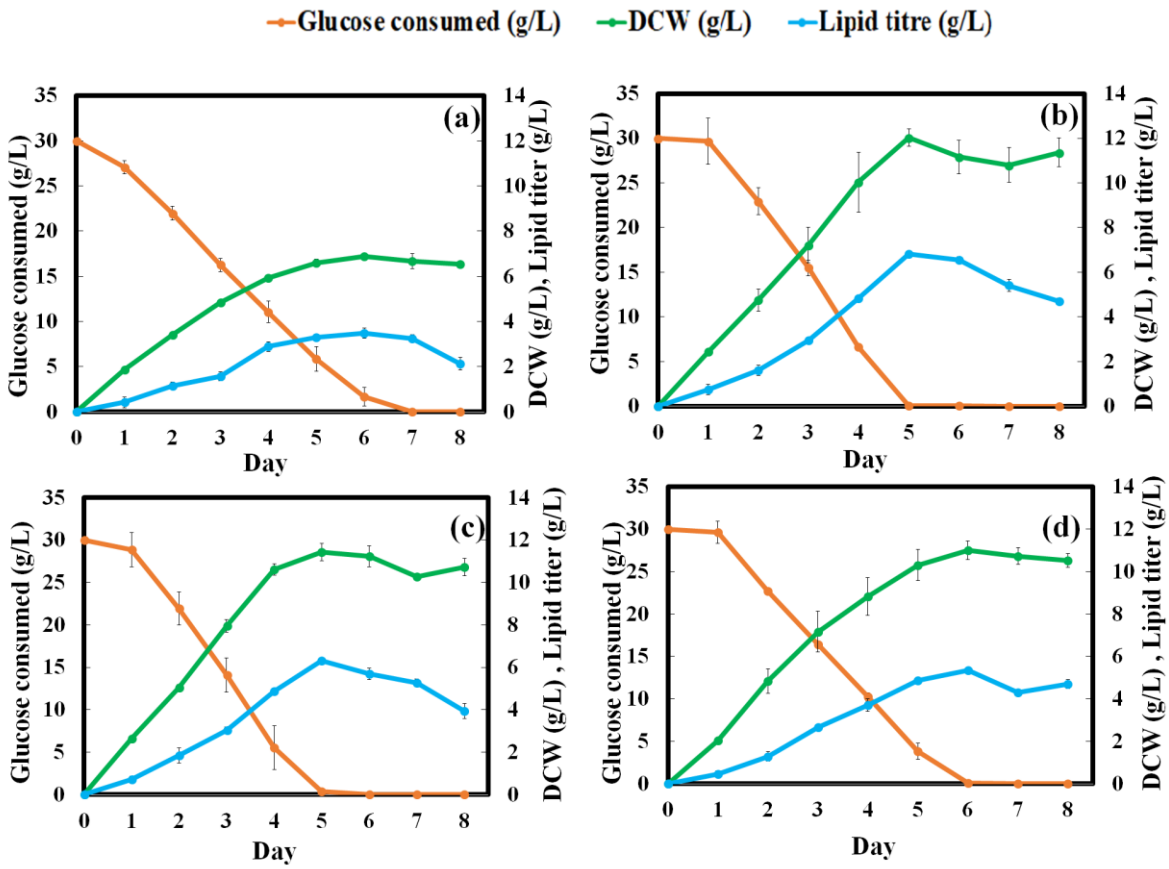


Figure-2.6: Time-course study of DCW, lipid titer and glucose consumption by selected oleaginous yeasts in YNB medium. a. *R. toruloides* (NCIM-3641); b. lab isolate *R. pacifica* INDKK; c. *R. kratochvilovae* (MTCC-247); d. *R. rubra* (NCIM-3560) (one-way ANOVA test, $P < 0.05$)

2.2.3 *R. pacifica* INDKK assimilated wide range of sugars and displayed inhibitor tolerant phenotypes

Hydrolysates of lignocellulosic biomass contain mixture of C5 and C6 sugars and toxic inhibitors generated during pre-treatment such as furfural, acetic acid and 5-HMF [220]. These inhibitors reduce cell growth as well as lipid yield and productivity [221]. Therefore, yeast isolates capable of assimilating wide range of sugars (C5 and C6) along with enhanced

tolerance to pre-treatment inhibitors are very important for economical microbial lipid production [222].

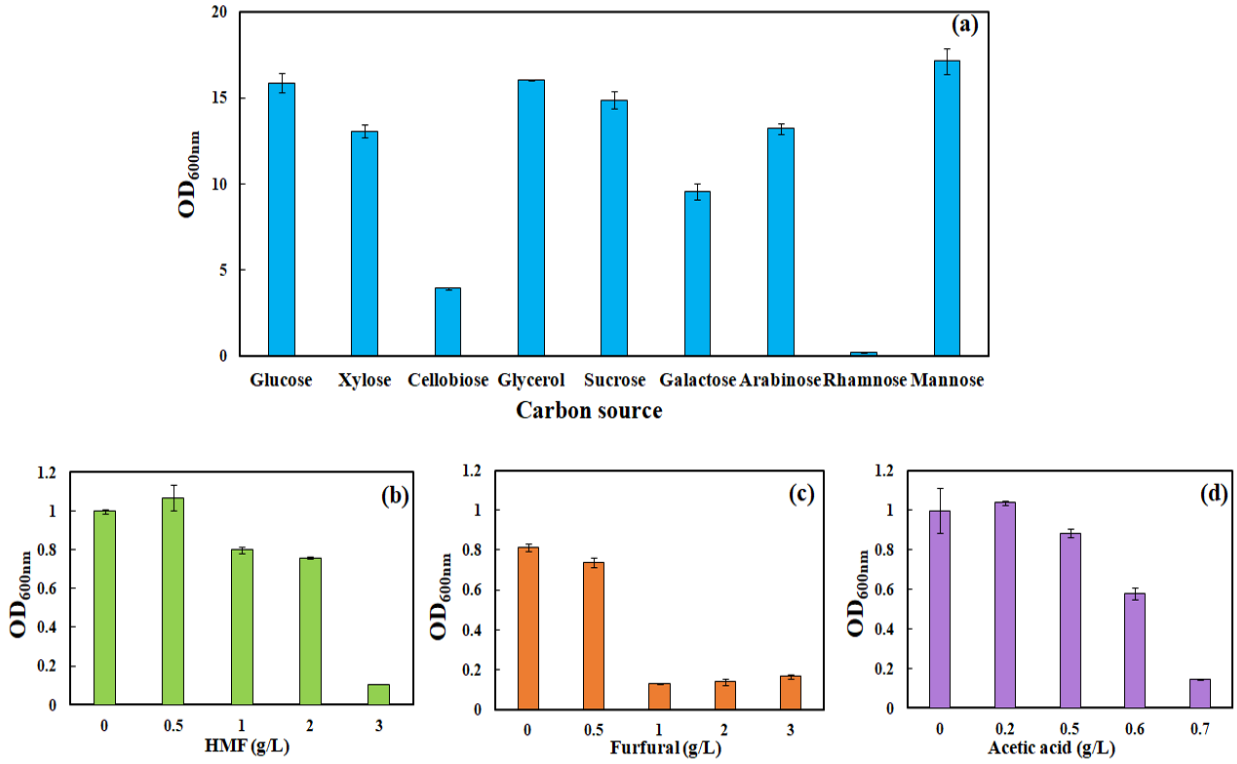


Figure-2.7: a) Growth study of *R. pacifica* INDKK in different C6, C5 and C3 carbon sources and tolerance study in pre-treatment-generated inhibitors; b) 5-HMF; c) furfural and d) acetic acid

2.2.4 Microbial lipid production using PS

Pongamia tree bears non-edible fruits whose shells after oil extraction from the seeds are generally discarded or burned [223].Compositional analysis showed that PS contains 56.8 % w/w holocellulose, 12 % w/w cellulose and 8 % w/w of xylan (Table 2.2). However, PS has not been considered as source of carbon and nitrogen for microbial cell growth thus far. In this regard, we explored hydrolysate of *Pongamia pinnata* shells for growth and lipid production by our newly isolated yeast isolate *R. pacifica INDKK*.PS were subjected to acid as well as alkali pre-treatments (appendix). Liquid fraction of acid treatment showed higher sugar concentration (37.38 g/L) while alkali treated liquid fraction obtained negligible amount of fermentable sugars (0.7 g/L total sugars). Therefore, liquid fraction of acid

treatment was detoxified by activated charcoal, which reduced acetic acid concentration from 5.61 ± 0.035 g/L to 0.11 ± 0.005 g/L, completely removed furfural and 5-HMF with slight reduction in sugar concentration (14.63 %). The Acid treated and detoxified liquid fraction of PSH (AD-PSH) contains 31.91 ± 0.042 g/L of total sugars (0.45 g/L glucose, 29.01 g/L xylose and 2.45 g/L arabinose). Time course study revealed that *R. pacifica* INDKK consumed all the sugars after 120 h of growth and produced 10.63 ± 0.004 g/L of DCW with 4.48 ± 0.02 g/L of lipid titer and 0.037 ± 0.001 g/L/h of lipid productivity (Fig.5. Panel-1 5a). The solid fraction of both acid treated and alkali treated PS were subjected to enzymatic hydrolysis (as described in "methods"). The enzymatic saccharification of solid fraction from acid treated PS yielded very less fermentable sugars (7.63 g/L) along with inhibitors such as acetic acid (0.3 g/L) and furfural (0.19 g/L) while enzymatic saccharification of solid fraction of alkali treated PS resulted in high amount of total sugars (46.47 g/L), wherein glucose was the most abundant (28.05 ± 0.01 g/L) followed by xylose (18.13 ± 0.04 g/L) and arabinose (0.29 ± 0.01 g/L). As expected very low amount of inhibitors (5-HMF 0.013 ± 0.005 g/L, furfural 0 g/L and acetic acid 0.046 ± 0.01 g/L) was detected. Interestingly, trace elements such as Mn (0.37 ± 0.05 mg/L), K (1.53 ± 0.005 g/L), Ca (107 ± 0.87 mg/L), Fe (0.67 ± 0.009 mg/L), Na (1.08 ± 0.15 g/L) and Mg (1.53 ± 0.003 g/L) were also found in alkaline treated-saccharified PSH (AS-PSH), which could be helpful in cell growth and lipid accumulation. There was a drastic reduction (17-fold) in calcium ions which might be due to increase in calcium influx into the cell to cope up with physiological stress conditions [224,225]. Time course analysis of *R. pacifica* INDKK in AS-PSH showed that all the sugars (C6 and C5) were consumed after 120 h of growth and maximum 12.6 ± 0.5 g/L of DCW, 7.02 ± 0.7 g/L of lipid titer, 0.104 ± 0.004 g/L/h of biomass productivity and 0.058 ± 0.006 g/L/h of lipid productivity were achieved (Fig.5. Panel-1 5b).

Table-2.2 The Pongamia shell hydrolysate (PSH) nutrient composition before and after consumption by *Rhodotorula pacifica* INDKK.

Component	**PS (Dry)**	**Before cultivation**	**After cultivation**
Cellulose (%)	12	-	-
Holocellulose (%)	56.8	-	-
Xylan (%)	8	-	-
Glucose (g/L)	-	28.05 ± 0.01	-
Xylose (g/L)	-	18.13 ± 0.04	0.05 ± 0.08
Arabinose (g/L)	-	0.29 ± 0.01	-
Total proteins (g/L)	-	0.33 ± 0.02	0.23 ± 0.005
Total Nitrogen (g/L)	1.90	0.053 ± 0.01	0.037 ± 0.02
Calcium (mg/L)	-	107 ± 0.87	6.3 ± 2.28
Sodium (g/L)	-	1.08 ± 0.15	0.84 ± 0.28
Magnesium (g/L)	-	1.53 ±0.003	0.22 ± 0.06
Phosphorous (g/L)	2.05	1.51 ± 0.001	0.36 ± 0.12
Potassium (g/L)	6.32	1.53 ± 0.005	0.38 ± 0.12
Manganese (mg/L)	-	0.37 ± 0.05	0.24 ± 0.07
Iron (mg/L)	-	0.067 ± 0.009	0.036 ± 0.021
Sulphur (g/L)	0.276	-	-
5-HMF (g/L)	-	0.013 ± 0.005	0.01 ± 0.01
Acetic acid (g/L)	-	0.046 ± 0.01	-

-not detected

Remarkably, the DCW and lipid productivities were 1.02-fold and 1.12-fold higher in AS-PSH as compared to YNB (0.101 ± 0.005 g/L/h of biomass productivity and 0.052 ± 0.003 g/L/h of lipid productivity) (Fig.5. Panel-1 5c). Moreover, after 120 h of growth 30.0% reduction in protein content and 94.11 %, 22.22 %, 85.62 %, 73.71 %, 75.16 % and 35.13 % utilization of trace elements corresponding to Ca, Na, Mg, P, K and Mn were also observed.

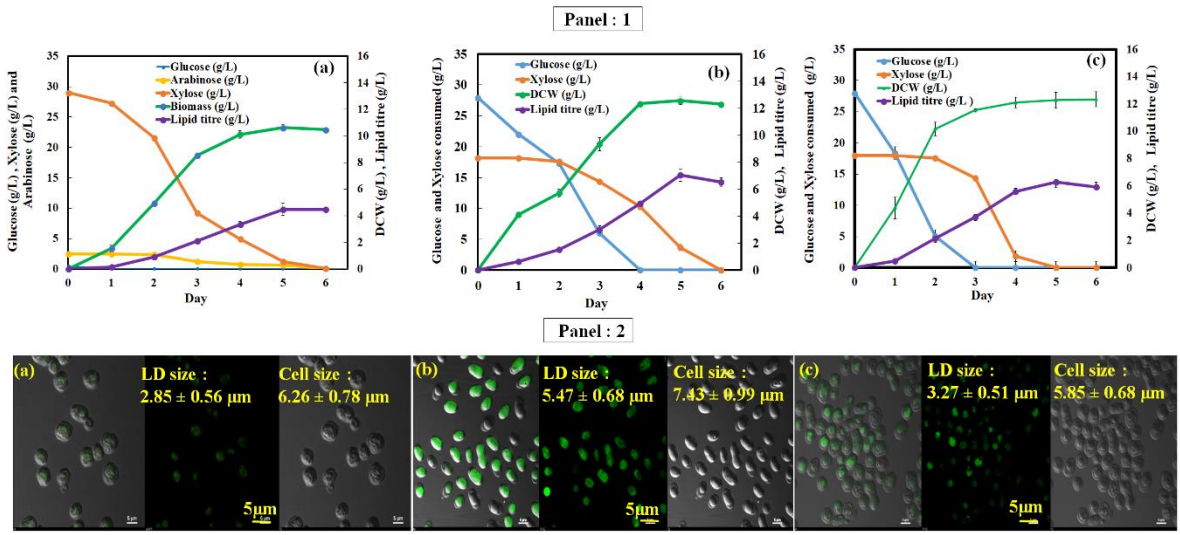


Figure-2.8: Panel:1 Time-course study of DCW, lipid titer and sugar consumption by *R. pacifica* INDKK in (**a)** AD-PSH (**b)** AS-PSH and (**c)** Control (YNB) (one-way ANOVA test, $P < 0.05$). Panel: 2 Confocal microscopy of *R. pacifica* INDKK cells cultivated in (**a)** AD-PSH, (**b)** AS-PSH, and (**c)** control (YNB) after 120 h showing average LD size and cell size at 100x

The lipid accumulation in PSH batch cultivated cells was also confirmed by confocal microscopy. The average cell size (7.43 ± 0.99 µm) and average LD size (5.47±0.68 µm) was 1.67-fold and 1.27-fold higher in AS-PSH cultivated cells as compared to YNB medium, respectively (Fig.5. Panel-2)**.** However, cell size and LD size was 1.18-fold and 1.56-fold higher in AS-PSH grown cells as compared to AD-PSH (6.26 ± 0.78 µm and 3.49 ± 0.56 µm), respectively. Together, batch cultivation in AS-PSH significantly showed more biomass and lipid productivity as compared to AD-PSH (P value < 0.05).

2.2.5 FAME and biodiesel properties analysis

The fatty acid profile of lipids extracted from *R. pacifica* INDKK cultivated in AS-PSH and YNB were analyzed by Gas Chromatography-Mass Spectroscopy (GC-MS) after transesterification (as described in "Methods"). The 91.77 % of FAME yield was achieved after transesterification with stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3) and palmitic (16:0) fatty acids, which is desirable for biodiesel production [226].

The lipids extracted from AS-PSH grown *R. pacifica* INDKK showed higher amount of C18:1 (52.58 %) followed by C16:0 (28.85 %), C18:2 (12.45 %), C18:3 (1.37 %), C14:0 (0.89 %), C22:0 (0.46 %) and C15:0 (0.1 %) fatty acids as depicted in Table 2.3. The fatty acid profile of *R. pacifica* INDKK grown in AD-PSH also showed higher C18:1 (61.98 %) followed by C18:2 (16.86 %), C16:0 (14.45 %), C18:0 (6.13 %) and C18:3 (1.37 %). Interestingly, C18:2, C18:3 and C22:0 fatty acids were not detected in YNB grown *R. pacifica* INDKK. Moreover, saturated fatty acid (SFA, 30.19 %) and monounsaturated fatty acid (MUFA, 52.58 %) were higher in AS-PSH grown cells as compared to YNB (SFA 10.95 % and MUFA 43.61 %). The CN in AS-PSH and YNB were 57.29 and 86.86, respectively. The CN of *R. pacifica* INDKK grown in AS-PSH is higher as compared to AD-PSH (CN 53.92). The result showed that IV of AS-PSH (73.58 g I_2/100 g) and YNB (39.02 g I_2/100 g) grown cells were in accordance with the standards of EN 14214. Compared to rape seed oil and jatropha oil, *R. pacifica* INDKK oil in AS-PSH grown cells contains more MUFA, SFA and less polyunsaturated fatty acid (PUFA), conferring greater CN. Other estimated biodiesel properties such as kinematic viscosity (KV, 3.7 mm^2/s) and density (0.84 g/cm^3) were comparable to rape seed oil, jatropha oil and standard values specified by EN 14214, ASTM D6751 and IS 15607 (Table 2.3). The above speculated biodiesel property values obtained from AS-PSH grown *R. pacifica* INDKK satisfies the specifications precise by EN-14214, ASTM-D6751and IS-15607 standards suggesting it as an ideal biodiesel feedstock.

Table-2.3: Comparative FAME profile and biodiesel properties of *R. pacifica* INDKK cultivated in PSH and YNB

Fatty acid/ Biodiesel properties	**Rape seed oil methyl ester** [217]	**Jatropha oil methyl ester** [217]	**AD - PSH**	**AS-PSH**	**Control (YNB)**	**EN 14214**	**ASTM D6751**	**IS 15607**
C14:0 (%)	ND	ND	ND	0.89	0.55	-	-	-
C16:0 (%)	11.9	14.9	14.45	28.85	10.4	-	-	-
C16:1 (%)	ND	1	ND	ND	ND	-	-	-
C18:0 (%)	4.1	6.1	6.13	ND	ND	-	-	-

C18:1 (%)	20.8	40.4	61.98	52.58	43.61	-	-	-
C18:2 (%)	53.8	36.2	16.86	12.45	ND	-	-	-
C18:3 (%)	9.3	0.3	ND	1.37	ND	-	-	-
C20:0 (%)	ND	ND	ND	ND	ND	-	-	-
C22:0 (%)	ND	ND	ND	0.46	ND	-	-	-
C24:0 (%)	ND	ND	ND	ND	ND	-	-	-
Iodine value (IV) (g of I_2/100 g)	107.76	98.02	86.29	73.58	39.02	120 (max.)	-	-
Cetane number (CN)	54.35	55.23	53.92	56.72	86.86	47	51	51
High heating value (HHV) (MJ/kg)	40.78	40.55	39.42	38.14	21.56	-	-	-
Density (g/cm^3)	0.80	0.88	0.87	0.84	0.47	-	0.86-0.90	0.86 – 0.89
Kinematic viscosity (KV) (mm^2/s)	4.4	4.48	3.90	3.7	2.13	1.9-6	3.5-5	2.5-6
Cold Filter Plugging Point (CFPP)	ND	ND	-6.1	-5.97	-13.2	-	-	-

– Not mentioned , ND: not detected

Table-2.4 Biodiesel production from *R. pacifica* INDKK using PS as substrate

Pongamia shell powder used (%)	***R. pacifica* INDKK**			
	DCW produced (g /L)	**Lipids produced (g/L)**	**Biodiesel produced (g/L)**	**Transesterification Efficiency (%)**
20	12.8	6.8	6.24	91.77

It was estimated that 6.8 g microbial lipid was obtained from 200 g of dry PS (20 % w/v), from which 6.24 g of biodiesel was produced (Table 2.4) in this study.

2.3 Conclusion

In this study, 57 yeast isolates were screened for lipid accumulation by microwave aided Nile red spectrofluorimetry. Among them, novel oleaginous yeast isolate *R. pacifica* INDKK (Gen Bank accession No: MN560184) showed highest lipid accumulation, ability to grow on diverse carbon sources and also displayed pre-treatment inhibitor tolerant growth phenotypes. *R. pacifica* INDKK showed maximum DCW (12.8 ± 0.66 g/L), lipid titer (6.8 ± 0.4 g/L) and

lipid productivity (0.056 ± 0.4 g/L/h) with glucose consumption rate of 0.25 g/L/h in YNB, among all the tested strains. *R. pacifica* INDKK can utilize wide range of C5 and C6 sugars and showed pre-treatment inhibitor tolerant phenotypes. *R. pacifica* INDKK produced more DCW (12.56 ± 0.5 g/L), lipid titer (7.02 ± 0.4 g/L) and lipid productivity (0.058 ± 0.006 g/L) in AS-PSH as compared to AD-PSH (DCW, 10.63 ± 0.37 g/L; lipid titer, 4.48 ± 0.78 g/L; lipid productivity, 0.037 ± 0.006 g/L/h) and YNB (DCW, 12.26 ± 0.68 g/L; lipid titer, 6.65 ± 0.35 g/L; lipid productivity, 0.055 ± 0.003 g/L/h) grown cells. C18:1 (52.58 %), C16:0 (28.85 %) and C18:2 (12.45 %) were major fatty acids detected in AS-PSH grown cells, attributing better quality of the fuel. The present study demonstrates lipogenic potential of *R. pacifica* INDKK for the production of biodiesel using PS as feasible feedstock.

CHAPTER -3

Media optimization to improve lipid production in selected *R. pacifica* INDKK oleaginous yeast

3. MEDIA OPTIMIZATION TO IMPROVE LIPID PRODUCTION IN SELECTED *R. PACIFICA* INDKK OLEAGINOUS YEAST

3.1 MATERIALS AND METHODS

Rhodotorula pacifica INDKK was isolated from mangrove forest soil as mentioned in chapter 1. It has shown potential to produce lipids and could utilize diverse carbon sources along with tolerance to inhibitors. All the solvents and the reagents used in this study were of HPLC grade. Bodipy (493–503 nm), heptadecanoic acid and supelco 37 component FAME mix were acquired from Sigma (USA).

3.1.1 Cultivation of *R. pacifica* INDKK for enhanced lipid production

R. pacifica INDKK was incubated for growth and lipid production in YNB media supplemented with glucose as carbon source. For the RSM study, glucose (4 to 7 % *w/v*), ammonium sulphate (0.14, 0.21 and 0.28 % w/v), initial pH of medium (4, 5, 6) and temperature (30 °C) was selected. All the flasks (100 mL) containing 30 mL of media were inoculated with yeast inoculum of OD_{600nm} of 0.2. The lipid titer (g/L) and DCW (g/L) were measured as described previously in chapter 2.

3.1.1.1 Experimental design for optimization of lipid production

The classical approach of OFTA is commonly used for media optimization to enhance lipogenesis [227]. Response surface methodology (RSM) is also being widely used for optimization of media conditions [228]. In OFTA the range of input values for each media component are identified While RSM performs multifactorial optimization. Hence, in this study, the combinatorial approach of OFTA and RSM was used to optimize the lipid production.

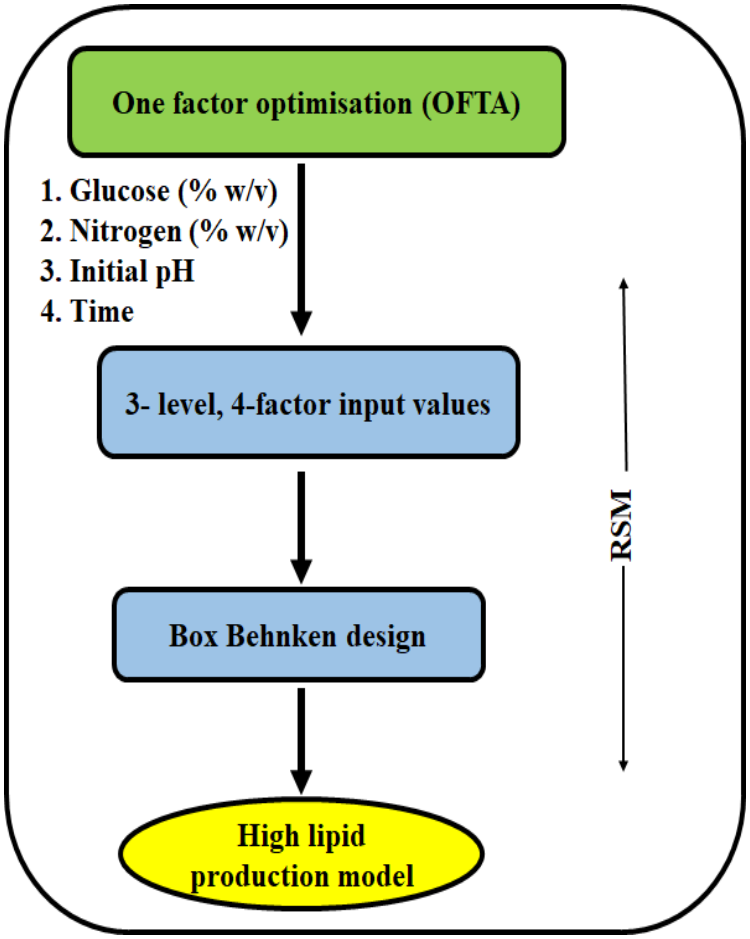


Figure-3.1: Media optimization process flow chart for improvement of lipid production improvement

3.1.1.2 Methodology of one factor at a time (OFTA) media optimization process

During initial screening of yeast isolates for lipid production ability on basal medium (YNB medium with glucose (3 % w/v) and ammonium sulphate (0.5 % w/v), *R. pacifica* INDKK could produce 6.78 ± 0.46 g/L lipid titer in 120 h. To further optimize medium conditions of *R. pacifica* INDKK for improved lipid production OFTA approach was applied and cultivated in YNB media supplemented with glucose in 250 mL flasks. Various concentrations of glucose (4 to 7 % w/v), 0.5 % w/v nitrogen source (ammonium sulphate, yeast extract, peptone and urea), different initial pH levels (2, 4, 5, 6, 7, 8, 10) and (% w/v) nitrogen (0.28, 0.21, 0.14, 0.07) at 30 °C for 120 h were studied using OFAT to ascertain the optimum medium components and pH for enhanced lipid production.

3.1.1.3 Experimental design using Response Surface Methodology

Using the obtained range of input values for each media component from OFTA, a three-level-four-factor, quadratic model was constructed and the effects of glucose (% w/v), ammonium sulphate (% w/v), initial pH and cultivation time on the lipid production of *R. pacifica* INDKK was constructed by Box Behnken design (BBD) using Design expert (Version 12.0, Stat-Ease Inc., Minneapolis, USA) software [229]**.**

Table-3.1: Selected range of RSM input values for 4 factors at 3 level derived from OFTA

S.no	Factor	-1	0	+1
1	Glucose (% w/v)	5	6	7
2	Ammonium sulphate (% w/v)	0.14	0.21	0.28
3	Initial pH	4	5	6
4	Time (h)	144	168	192

In this study, the independent variables at 3 - different levels, i.e low (−1), medium (0) and high (+1) were selected (Table- 3.1) and experimental design has total 29 trials (Figure 3.1) conducted in triplicates. The lipid titer measured from these experiments was considered as response factor. The 2^{nd} order model was used to correlate the response variables to the respective independent variables. The enumeration and analysis of second order polynomial coefficients was performed with the help of 'Design Expert' software.

The general representation of second-degree polynomial equation (1).

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_i X_i^2 \ldots\ldots\ldots (1)$$

where, Y is the predicted response; β_0 the intercept, β_i the linear coefficient, β_{ij} the quadratic coefficient, β_{ii} is the linear-by-linear interaction between X_i and X_j regression coefficients and X_i, X_j are input variables that influence the response variable Y. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included

Table-3.2: RSM Experimental design and runs

Run	Glucose (% w/v)	Ammonium sulphate (% w/v)	Initial pH	Cultivation time (h)	Actual Lipid titer (g/L)	Predicted lipid titer (g/L)
1	6	0.28	4	168	11.12	10.84
2	6	0.21	5	168	13.56	13.25
3	7	0.21	5	192	10.96	10.59
4	7	0.14	5	168	8.89	9.03
5	6	0.21	4	144	9.58	9.68
6	7	0.21	4	168	7.86	8.02
7	6	0.14	5	192	14.67	14.68
8	6	0.14	4	168	11.45	11.43
9	5	0.21	6	168	10.45	10.33
10	6	0.21	6	192	13.10	13.32
11	5	0.14	5	168	11.31	11.22
12	6	0.21	4	192	12.67	12.75
13	6	0.21	5	168	12.83	13.25
14	6	0.28	5	144	11.21	11.23
15	7	0.28	5	168	8.69	9.10
16	5	0.28	5	168	8.87	9.05
17	6	0.21	5	168	13.41	13.25
18	7	0.21	6	168	8.17	8.25
19	5	0.21	5	192	11.10	11.16
20	6	0.14	5	144	11.87	11.91
21	5	0.21	4	168	8.13	8.08
22	6	0.28	6	168	11.96	11.62
23	7	0.21	5	144	8.12	7.70
24	6	0.21	5	168	13.36	13.25
25	5	0.21	5	144	9.25	9.26

26	6	0.28	5	192	13.25	13.25
27	6	0.14	6	168	13.22	13.14
28	6	0.21	6	144	11.35	11.59
29	6	0.21	5	168	13.10	13.25

Fisher's *F*-test (overall model significance), its associated probability *p* (*F*), correlation coefficient *R*, determination coefficient R^2 which measure the goodness of fit for regression model. For each variable, response surface curves representing quadratic models were generated.

3.1.2 Analytical methods

Glucose concentration was measured as mentioned in section 2.1.7.

3.1.3 Fatty acid analysis by GC-MS

GCMS analysis were performed as mentioned in section 2.1.8.

3.1.4 Cell size and lipid droplet size analysis

Bodipy stained yeast were observed under confocal microscopy (Nikon, India) and the cell size and lipid droplet size were measured using Image-J software.

3.2 RESULTS AND DISCUSSION

3.2.1 OFAT media optimization to improve lipid production

The efficiency of lipid production by yeast depends on optimal cultivation conditions. The major media components like carbon and nitrogen sources , C/N ratio [105], incubation time, and pH [106,107] at optimal levels determines sustainable lipid production [108–110]. Therefore, in order to find out the optimal range for glucose (% w/v), C/N ratio or nitrogen source (% w/v), and initial pH, the OFAT method was performed by varying one component at a time and keeping others constant.

3.2.1.1 Glucose (% w/v)

Glucose is most preferred carbon source for yeast though they can utilize different sugars. But appropriate concentration of carbon source is essential to prevent incomplete and inefficient fermentation. While low carbon concentration leads to low lipid production below its ability and high carbon concentration levels decreases both growth and fermentation due to osmolarity stress [230]. So, optimal carbon concentration is critical for efficient lipid production. Consequently, 4 to 7 (% w/v) glucose concentrations were tested at selected 30 °C (Figure 3.2.A). Among all 6 (% w/v) glucose was found to be optimal for both DCW (18.18 g/L) and lipid production (11.64 g/L), followed by glucose (5 % w/v, lipid titer – 11.23 g/L) and glucose (4 % w/v, lipid titer - 8.89 g/L). But interestingly, at 7 (% w/v) glucose (w/v) both DCW (14.25 g/L) and lipid titer (8.21 g/L) were relatively low perhaps due to increased osmolarity stress on yeast [230].

3.2.1.2 Nitrogen source

Nitrogen source is another key player which determines the biomass and lipid production in yeast [231,232]. They also constitute the most important component of body building units such as amino acids and nucleic acids. Therefore, different nitrogen sources like ammonium sulphate, peptone, yeast extract and urea at 0.5 (% w/v) concentration were used to find the appropriate nitrogen source that induce high DCW and lipid production at selected 30 °C and 6 % glucose which were similarly used by recent studies [94,233] (Figure 3.2.B). Among them, ammonium sulphate was found to induce higher lipid titer (11.41 g/L) similar to other previously studies where lipid tire of 6.05 g/L was obtained by oleaginous red yeast *Rhodotorula glutinis* [234]. In another study, 52.38 lipid content was obtained by *Meyerozyma guilliermondii* BI281A with ammonium sulphate as nitrogen supplement [235]. According to previous studies, different nitrogen sources support different oleaginous yeasts uniquely. In one study, *R. glutinis* cultivated with peptone media has increased lipid production while supplementation of yeast extract improved DCW production and

ammonium sulphate supported less lipid accumulation relatively [236]. In this study, peptone and yeast extract induced high DCW production of 22.1 g/L and 20.34 g/L respectively might be due to high complex organic nitrogen components which supports DCW production [236] but with less lipid titers (~ 8.2 g/L). Surprisingly, in presence of urea, relatively less DCW (12.83 g/L) and reduced lipid titer (3.89 g/L) were produced similar to previous studies [127,237].

3.2.1.3 Initial pH

Every yeast has its own optimal pH for efficient fermentation as it influences cellular energy process critically [107]. Earlier reports have already shown that pH of the medium effects the lipid accumulation [118,228,238–241]. Therefore, initial pH range of 2, 4, 5, 6, 7, 8 and 10 were tested at previously selected 30 °C, glucose (6 % w/v) and ammonium sulphate (0.5 % w/v) (Figure 3.2.C). The previously reported optimal pH for high lipid production for most yeast lies between 4 to 6 [240,242,243] and in this study, the optimal range was found to be 4 to 6, with 5 as most optimal with high DCW (18.9 g/L) and lipid titer (11.77 g/L). Interestingly, the extreme pH values such as 2, 8 and 10 were found to have negative effect on both DCW and lipid production.

3.2.1.4 Ammonium sulphate concentration (% w/v)

The well-studied condition to understand the lipid production is nitrogen starvation [244–246]. It is a known phenomenon that high concentration of nitrogen source induces high DCW production with less lipid titer while very low concentration hinders growth of microorganisms. So, the subtle optimal nitrogen concentration becomes critical for both high DCW and lipid production. Therefore, different ammonium sulphate concentration (% w/v) range of 0.28, 0.14, 0.093 and 0.07 that corresponds to C/N values of 25, 50, 75 and 100 respectively were tested (Figure 3.2.D). The 0.21 (% w/v) and 0.14 (% w/v) ammonium sulphate concentrations were found to induce both DCW and lipid titers while 0.21 (% w/v)

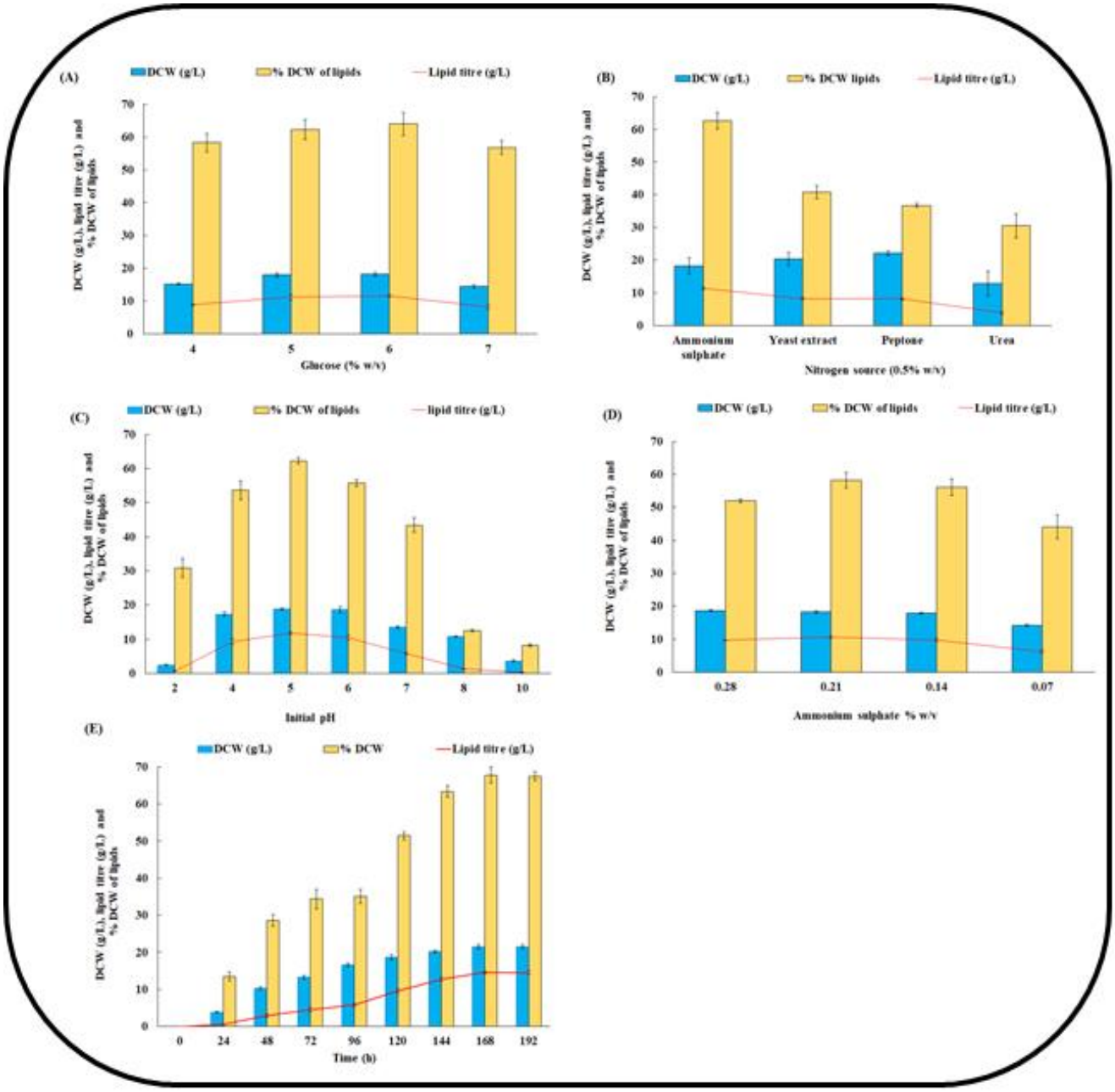


Figure-3.2: OFTA optimization to understand the effects of (A) Glucose (% w/v) (B) Nitrogen source (C) Initial pH (D) Ammonium sulphate (% w/v) (E) Time on DCW and lipid production by *R. pacifica* INDKK

was inducing more lipid titer (10.62 g/L). In corroboration to above statement, in one study, a range of C/N ratios (60, 80, 100, and 120) for *Rhodotorula toruloides* were used and found regardless of the carbon source, high C/N ratios induce lipid yields but at the cost of decreased growth [105,247]. At 0.07 (% w/v) ammonium sulphate, both DCW and lipid

titers tend to decrease which is supported by previous study, where they observed C/N 70 to 120 for *Rhodotorula glutinis* did not enhance lipid production further [248]. OFTA study, revealed a temperature of 30 °C, glucose (6 % w/v), ammonium sulphate (0.21 % w/v) or C/N ratio (33.33) was determined as optimal fermentation conditions for high lipid production of 13.87 g/L lipid titer in 168 h (Figure 3.2.E) for *R. pacifica* INDKK using OFTA.

3.2.2 Optimization of fermentation parameters using RSM

RSM has gained attention for its ability to perform multi factorial media optimization and adopted by several researchers to maximize lipid production by oleaginous yeast [120,163,249–254]. Therefore, in this study, RSM was used to find the interactive effects among multifactors of fermentation and obtained statistically significant high lipid producing model. The OFTA results have suggested that the temperature of 30 °C, glucose (6 % w/v), ammonium sulphate (0.21 % w/v), or C/N ratio of 33.33 effectively influence the lipid production in *R. pacifica* INDKK. Hence, keeping 30 °C temperature constant and considering glucose concentration, ammonium sulphate concentration, initial pH and with additional cultivation time were considered for 3-level 4-factor input values (Table: 3.2). The BBD model was constructed and corresponding experimental data to understand the influence of above four factors at different concentrations on the lipid accumulation by *R. pacifica* INDKK (Table-3.2). The regression equation obtained by analysis of variance (ANOVA) performs multiple regression analysis representing response level as a function of 4 independent variables. The quadratic model interms of coded factors representing lipid titer of *R. pacifica* INDKK is given below:

$$\text{Lipid titer} = 13.252 + -0.534583 * A + -0.525833 * B + 0.620417 * C + 1.1975 * D + 0.56 * AB + -0.50375 * AC + 0.2475 * AD + -0.2325 * BC + -0.19 * BD + -0.335 * CD + -3.37017 * A^2 + -0.280792 * B^2 + -1.21267 * C^2 + -0.203292 * D^2$$

Where *A*, *B*, *C* and *D* were glucose (% w/v), ammonium sulphate (% w/v), initial pH and cultivation time (h) respectively.

Table-3.3: Anova analysis for constructed BBD model

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model	110.70	14	7.91	88.30	< 0.0001	significant
A-Glucose	3.43	1	3.43	38.30	< 0.0001	
B-Ammonium sulphate	3.32	1	3.32	37.05	< 0.0001	
C-Initial Ph	4.62	1	4.62	51.58	< 0.0001	
D-Cultivation time	17.21	1	17.21	192.17	< 0.0001	
AB	1.25	1	1.25	14.01	0.0022	
AC	1.02	1	1.02	11.34	0.0046	
AD	0.2450	1	0.2450	2.74	0.1203	
BC	0.2162	1	0.2162	2.41	0.1425	
BD	0.1444	1	0.1444	1.61	0.2248	
CD	0.4489	1	0.4489	5.01	0.0419	
A^2	73.67	1	73.67	822.74	< 0.0001	
B^2	0.5114	1	0.5114	5.71	0.0315	
C^2	9.54	1	9.54	106.52	< 0.0001	
D^2	0.2681	1	0.2681	2.99	0.1056	
Residual	1.25	14	0.0895			
Lack of Fit	0.9210	10	0.0921	1.11	0.5021	not significant
Pure Error	0.3327	4	0.0832			
Cor Total	111.95	28				

R^2 = 0.9888; Adj. R^2 = 0.9776

A significant model (*F*-value (88.30) & *P*-value ($P < 0.0001$)) was obtained (Table 3.3). The obtained R^2 value (0.9888) suggests good correlation between the empirical and predicted values indicating reliability of model to estimate lipid titer. The adjusted R^2 (0.9776) denotes the total variation of 97.76 % for the lipid titer was associated to the independent variables.

The P-value of 0.502 for lack-of-fit suggested it wasn't significant and also 50.21 % chance that F-value of lack of fit was due to noise and the equation was sufficient enough to predict lipid titer under different conditions. Significant corresponding coefficient was indicated by the larger F-value and smaller P-value of the model. The model terms, A, B, C, D, AB, AC, CD, A^2, B^2, C^2 were found as significant model terms, demonstrating the positive interactive influence on lipid accumulation by all factors. The link between response variable and experimental levels of each variable was represented by response surface curves as shown in (Figure 3.4).

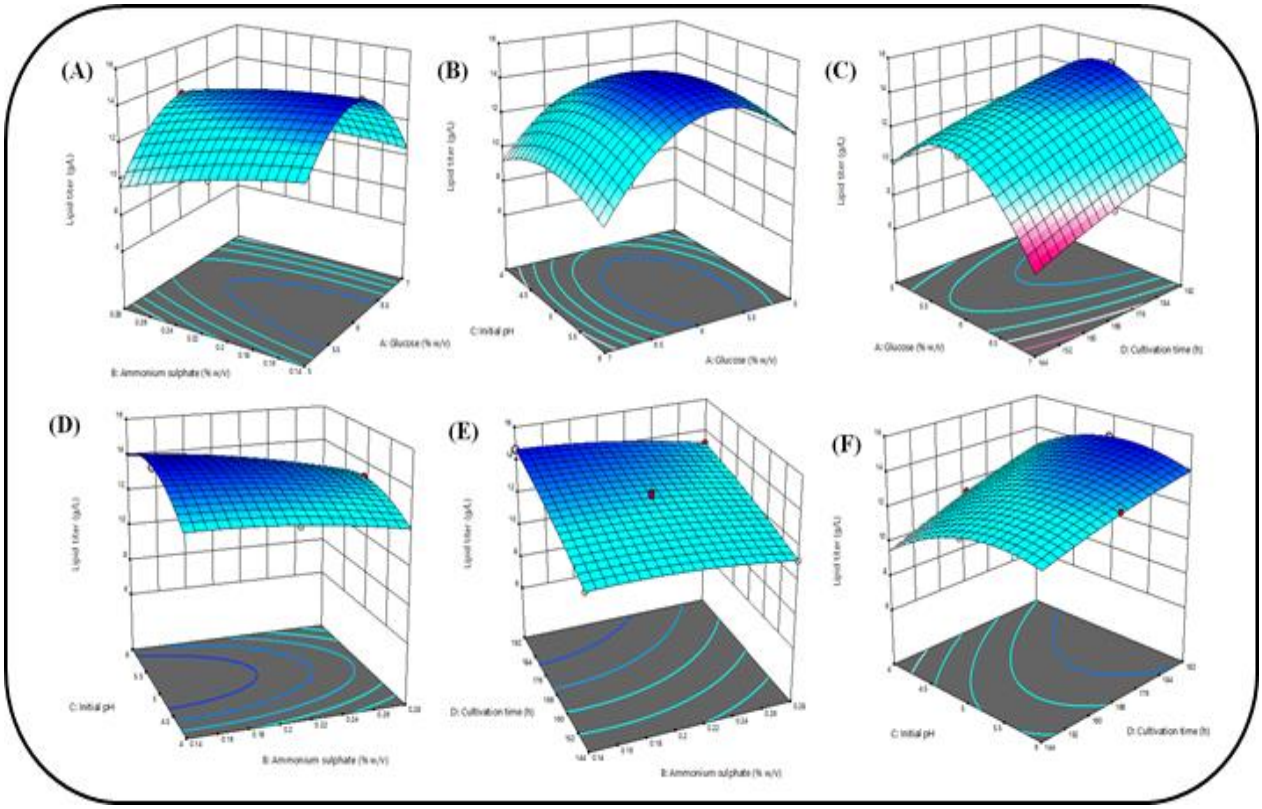


Figure-3.4: The response surface plots showing possible interactions between each pair of variables for the model.

Each pair of variables in many possible ways and among them the interaction between glucose (A) - initial pH (C), ammonium sulphate (B) - initial pH (C) and glucose (A) – cultivation time (D) have significant interactive effects on lipid production. While other factor interactions were not significant, indicating their less influence on lipid production.

3.2.3 Kinetic study of RSM validation experiment

The model built by the 'Design-expert' software has suggested glucose 5.88 (% w/v), ammonium sulphate 0.14 (% w/v), C/N ratio 49, incubation time of 190 h and initial pH 5.4 as the optimal values for *R. pacifica* INDKK for maximum lipid production.

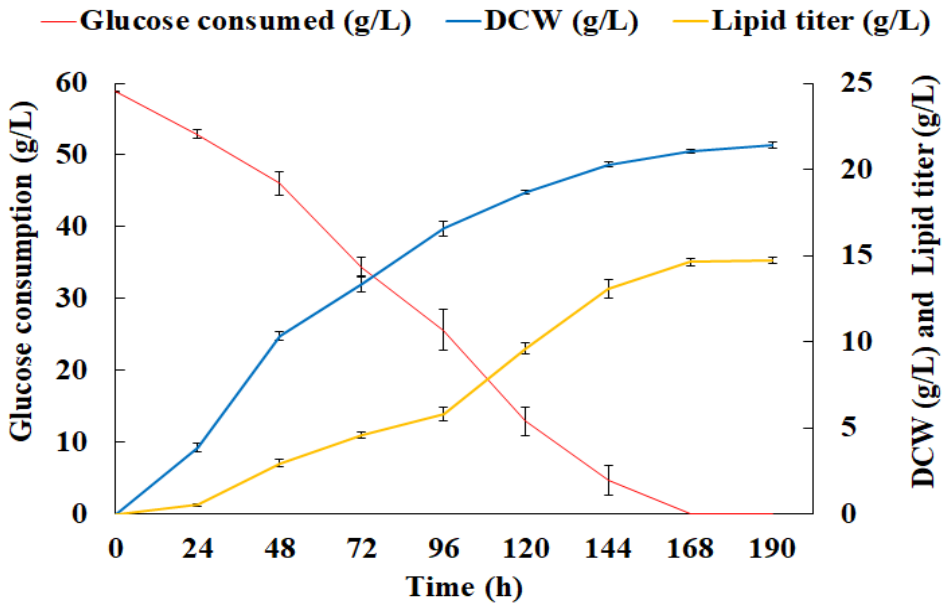


Figure-3.5: Kinetic study of DCW and lipid production by *R. pacifica* INDKK under RSM optimized conditions.

Table-3.5: Recently reported media optimization using RSM for lipid production improvement in oleaginous yeast

S.no	Organism	RSM model	Carbon source	Mode	Lipid titer (g/L)	Reference
1	*Rhodosporidiobolus fluvialis* DMKU-RK253	Box–Behnken	Crude glycerol	Fed batch	27.81 ± 1.86	[252]
2	*Candida viswanathii* Y-E4	Box–Behnken	Bio-diesel-derived crude glycerol	Batch	13.6	[255]
3	*Rhodosporidiobolus fluvialis* DMKU-SP314	Box–Behnken	Sugarcane top hydrolysate	Batch	15.85	[255]
4	*Cutaneotrichosporon oleaginosus*	Box–Behnken	Lactose	Fed batch	9 ± 0.34	[256]
5	*Rhodotorula graminis* TISTR 5124	Box–Behnken	Glucose	Batch	17.4	[257]

6	*Meyerozyma. guilliermondii* Bl281A	Central Composite	Glucose	Batch	1.31	[258]
7	*R. pacifica* INDKK	Unoptimized	Glucose	Batch	6.78 ± 0.46	This study
8	*R. pacifica* INDKK	Box–Behnken	Glucose	Batch	14.65 ± 0.14	This study

Kinetic study of DCW and lipid production was carried out to validate the constructed RSM model at above mentioned optimal values and found lipid titer of 14.65 ± 0.14 (g/L), lipid content (% w/w) of 68.66 ± 0.45, lipid productivity of 0.077 ± 0.0015 (g/L/h), lipid yield of ~ 0.25 (g of lipid /g of glucose) which were in agreement with proposed model validation result suggesting the significance of RSM model (Figure 3.4).

3.2.4 Assessment of biodiesel properties

The fatty acid profile from *R. pacifica* INDKK cultured in unoptimized and optimized media were analyzed by GC–MS (as described in "Methods"). In unoptimized medium, palmitic acid (16 :0), oleic (18:1) and stearic (18:0) fatty acids were only produced while in optimized medium, a different lipid profile consisting of palmitic acid (16:0), stearic (18:0), oleic (18:1) and polyunsaturated fatty acids (PUFA) like linoleic (18:2) and eicosadienoic acid (EDA) (C20:2) were produced. The C16:0, C18:0, C18:1 fatty acids were increased by 6.36, 3.56, 4.14-fold relative to unoptimized media. Interestingly, unlike unoptimized media, PUFA such as linoleic acid (18:2) and EDA (C20:2) of 6.3 % and 3.56 %, respectively were produced on optimized media which are known to provide health benefits like reducing LDL cholesterol, non-HDL cholesterol and with neutralizing effect on blood pressure [259–261].

The FAME profile of both optimized and unoptimized media majorly shows palmitic acid (16:0), stearic (18:0) and oleic (18:1) fatty acids suitable for biodiesel production [226,262] as depicted in Table-3.5. The CN of oil from *R. pacifica* INDKK grown in optimized media was higher (CN 62.63) than unoptimized (CN 61.94). High MUFA, SFA and less PUFA of

R. pacifica INDKK oil in optimized or unoptimized media grown cells has given higher CN as compared to plant oils (Table-3.5). The result showed that IV of optimized (47.6 g I_2/100 g) and unoptimized (48.5 g I_2/100 g) media grown cells met the standards of EN 14214. The kinematic viscosity (KV) and density of FAMEs from both optimized and unoptimized media standard values specified by EN 14214, ASTM D6751 and IS 15607 (Table 3.5). The estimated biodiesel properties of FAMES from *R. pacifica* INDKK grown in both optimized and unoptimized media incongruence with standard values mentioned by EN-14214, ASTM-D6751 and IS-15607, implying it as potential feedstock for biodiesel production. The PUFAs obtained from optimized media, suggests it as promising source of health benefitting dietary fatty acids.

Table-3.5: Comparative fatty acid profile and estimated biodiesel properties of *R. pacifica* INDKK grown in optimized and unoptimized media

Fatty acid/biodiesel properties	**Rape seed oil methyl ester** [217]	**Jatropha oil methyl ester** [217]	**Optimized N limited-media**	**Unoptimized N sufficient - media**	**EN 14214**	**ASTM D6751**	**IS 15607**
C10:0 (%)	–	–	1.76	–	–	–	–
C16:0 (%)	11.9	14.9	42.07	36.65	–	–	–
C16:1 (%)	–	1	–	–	–	–	–
C18:0 (%)	4.1	6.1	6.04	9.42	–	–	–
C18:1 (%)	20.8	40.4	40.24	53.94	–	–	–
C18:2 (%)	53.8	36.2	6.3	–	–	–	–
C18:3 (%)	9.3	0.3	–	–	–	–	–
C20:2 (%)	–	–	3.56	–	–	–	–
C22:0 (%)	–	–	–	-	–	–	–
Iodine value (IV) (g of I_2/100 g)	107.76	98.02	47.597	48.505	120 (max.)	–	–
Cetane number (CN)	54.35	55.23	62.631	61.943	47	51	51
High heating value (HHV) (MJ/kg)	40.78	40.55	37.954	39.497	–	–	–
Density (g/cm^3)	0.80	0.88	0.84	0.87	–	0.86–0.90	0.86–0.89

Kinematic viscosity (KV) (mm^2/s)	4.4	4.48	3.703	4.058	1.9–6	3.5–5	2.5–6
Cold filter plugging point (CFPP)	–	–	6.228	9.835	–	–	–

3.2.5 Cell size and lipid droplet size analysis

The differences in the lipid droplet size and the cell size under optimized and unoptimized media were investigated using confocal microscopy. Interestingly, the difference in cell size was not observed under unoptimized condition (9.47 ± 1.64 µm) and optimized condition (9.61 ± 1.92 µm). But the lipid droplet size of *R. pacifica* INDKK measured in optimized medium (7.84 ± 1.31 µm) is 1.61-fold higher than unoptimized medium (4.87 ± 0.86 µm), confirming the augmentation of lipid production after media optimization.

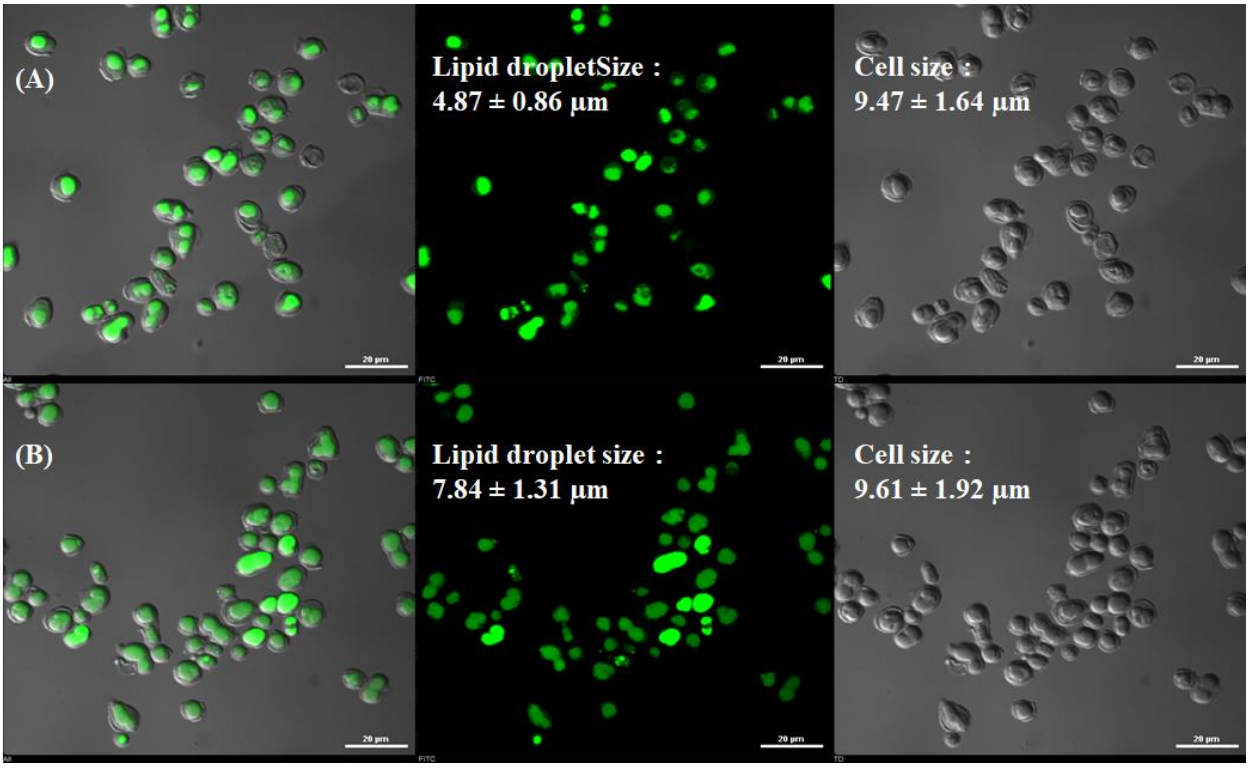


Figure-3.6: Measurement of cell size and lipid droplet size using confocal microscopy under (A) Unoptimized medium N sufficient media (B) Optimized medium N limited media

3.3 CONCLUSION

The DCW (21.39 g/L) and lipid titer (14.62 g/L) of *R. pacifica* INDKK in RSM optimized medium was achieved which are 1.73, 2.16-fold improved values compared to unoptimized media. Also, the presence of major fatty acids such as C16, C18, C18:1 and PUFAs (C18:2, C20:2) were detected in optimized media. These results indicate that media optimization led to improved lipid titer levels in *R. pacifica* INDKK with enhanced biodiesel properties.

CHAPTER-4

Improving lipid production by metabolic engineering in *R. pacifica* INDKK

4. Improving lipid production by metabolic engineering in *R. pacifica* INDKK

In previous chapter 3, lipid production was improved by media optimization in novel oleaginous yeast *R. pacifica* INDKK. In this chapter, genomics and transcriptomics study was conducted to unravel its metabolic networks related to lipid metabolism. Based on the clues derived from omics data and literature survey, overexpression of ACC-1 and DGA-1 genes were attempted to improve *R. pacifica* INDKK lipid production.

4.1 HYBRID DENOVO GENOME SEQUECNING OF THE OLEAGINOUS YEAST *R. PACIFICA* INDKK

4.1.1 MATERIALS AND METHODS

4.1.1.1 Culturing, DNA extraction and quality control

R. pacifica INDKK was cultured at 30 °C in YPD media, 200 rpm until it attained mid-log stage. Cells harvested by centrifugation at 8000 rpm, were washed twice with 1X PBS. Further cells were snap frozen immediately and stored at -80 °C. Genomic DNA from frozen *R. pacifica* INDKK was extracted using enzymatic lysis with standard CTAB method [263,264]. Briefly, 1X PBS washed cell pellet was resuspended in 300 µL sorbitol buffer (1M sorbitol, 14mM beta-mercaptoethanol (β-ME), 100mM EDTA, 200 U of zymolyase T20 was added just before incubation at 30 ºC for 30 minutes. Equal volume of pre-warmed CTAB buffer (2 % CTAB, 1.4 M NaCl, 0.1 M TrisHCl, 2mM β-ME, 1 % polyvinyl pyrrolidine (PVP) and 0.02 M EDTA) was added and further incubated at 65 °C for 90 min. Debris was removed by centrifugation and supernatant was subjected to phenol: chloroform: isoamyl alcohol extraction and DNA was precipitated by standard alcohol precipitation method. DNA pellet was re-suspended in 10mM Tris-Cl (pH-8.0). Residual RNA was removed by RNase (20mg/ml) treatment (at 65°C for 10minutes). The quantity of the genomic DNA and its quality was determined using Nanodrop2000 (Thermo Scientific, USA), Qubit (Thermo

Scientific, USA), respectively and the integrity was deduced from agarose gel electrophoresis.

4.1.1.2 Culturing, RNA Extraction and quality control

A preculture of *R. pacifica* INDKK in YPD medium at 30 ºC and 200 rpm was grown overnight. The preculture was centrifuged, washed with MQ, resuspended in YPD media to OD of 0.2 at 600 nm and cultured to reach log phase (12 h). Further, the obtained cells were harvested following centrifugation at 8000 rpm at 4 ºC, washed twice with 1X PBS followed by immediate snap freeze and stored at - 80 °C. Extraction of total RNA was performed using Qiagen RNeasy Mini Kit in combination with DNase treatment. The concentration of RNA and it's purity were evaluated using Nanodrop spectrophotometer and Qubit flurometer. The integrity of RNA sample was analyzed by Agilent Bioanalyzer chip.

4.1.1.3 Sanger sequencing for genomic DNA purity check

18SrDNA was PCR amplified using 30-50 ng of the genomic DNA as a template with 18SrDNA primers (appendix) and Takara ExTaq in a 25 µL reaction mix.

4.1.1.4 DNA Illumina library preparation

Library construction was carried out at Genotypic Technology using the QIASeq FX DNA Library Preparation protocol (Cat#180475) according to manufacturer's instructions (Figure 4.1.1).

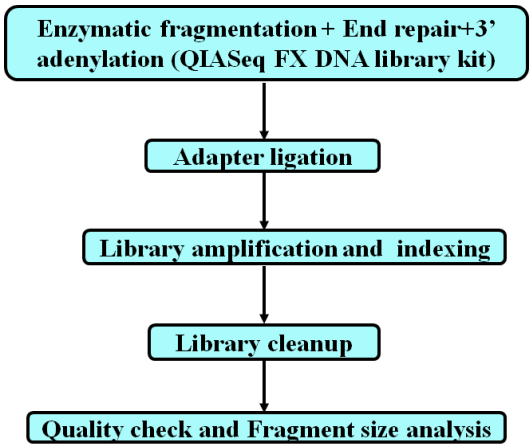


Figure-4.1.1: Workflow for QIASeq FX DNA library preparation protocol

Briefly, 100 ng of DNA was enzymatically fragmented, end-repaired, A-tailed in one-tube reaction using the FX Enzyme Mix available in the QIASeq FX DNA kit. The end-repaired and adenylated fragments were subjected to adapter ligation, whereby index-incorporated Illumina adapter was ligated, to generate sequencing library. This library was subjected to 6 cycles of Indexing-PCR (98˚C for 20 sec; initial denaturation, cycling (98˚C for 20sec, 60˚C for 30sec, 72˚C for 30 sec) and final extension at 72˚C for 1 min) to enrich the adapter-tagged fragments. Finally, the amplified library was purified using JetSeq Magnetic Beads (Bio, # 68031). Further, the sequencing library was quantified by using Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and its size distribution of the fragment was further analyzed by Agilent TapeStation.

4.1.1.5 Nanopore DNA library preparation

A total of 600ng of purified gDNA from the obtained sample was the end-repaired (NEBnext ultra II end repair kit, New England Biolabs, MA, USA) and accordingly cleaned up using 1x AMPure beads (Beckmann Coulter, USA).

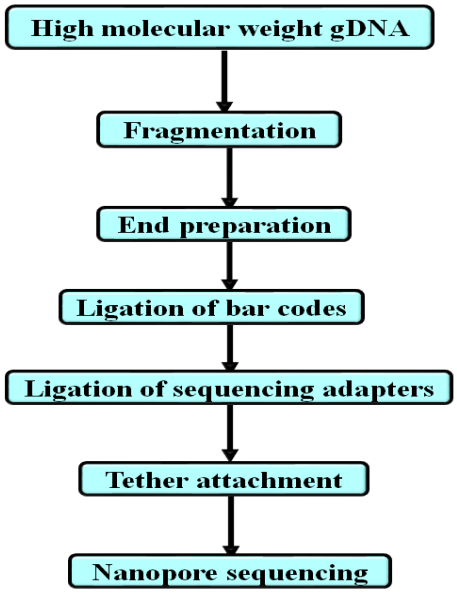


Figure-4.1.2: Overview of Nanopore library preparation

Barcode ligation was carried out using NEB blunt / TA ligase (New England Biolabs, MA, USA) using EXP-NBD114 (ONT) and then cleaned with 1xAmPure beads. Barcode ligated DNA sample was adapter ligated for 15 min using NEBNext Quick Ligation Module (New England Biolabs, MA, USA). Following barcode ligation, the library was then cleaned up with 0.6X AmPure beads (Beckmann Coulter, USA) and finally sequencing library was further eluted in 15 µL elution buffer and was used for downstream sequencing (Figure 4.1.2).

4.1.1.6 RNA library preparation

RNA sequencing libraries (Illumina-compatible) were made with NEBNext® (Ultra™ 2 directional RNA library preparation kit, New England BioLabs, MA, USA) at Genotypic Technology Pvt. Ltd., Bangalore, India. Total mRNA was isolated from 100 ng of total RNA fragmented and primed to cDNA synthesis. The ds cDNA was purified using JetSeq magnetic beads (Bio, # 68031) was further end-repaired, adenylated and ligated to Illumina adapters as mentioned in NEB kit protocol. At temperature 37 ˚C for 15mins the second strand removal was done using the USER enzyme. The schematic showing the steps involved in preparation of library was carried out using NEBNext® Ultra™ II Directional RNA Library Prep Kit (Part #E7760L) was shown in figure 4.1.3. The cDNA (adapter ligated) obtained was then purified using JetSeq beads and was further placed for indexing (12 cycles, 98˚C for 30 sec, cycling (98˚C for 10sec, 65˚C for 75sec and 65˚C for 5min) and the obtained adapter-linked fragments were then further enriched. PCR products (sequencing library) were purified with JetSeq beads for ensuring quality control. Following purification, the library was then quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and the distribution of size of the fragments was investigated using Agilent 2200 TapeStation (Agilent, #5067-5585 (reagents) #5067-5585(tapes).

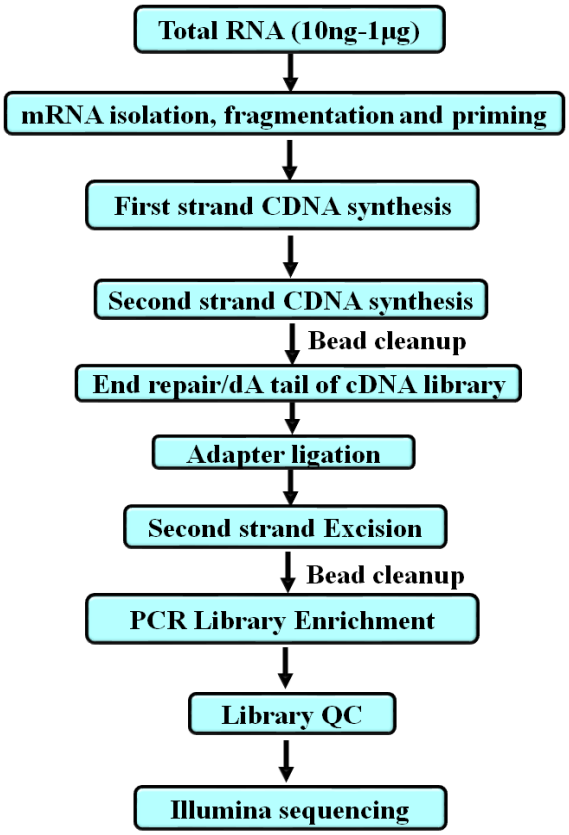


Figure-4.1.3: Work flow for RNA library preparation using NEBNext® Ultra™ II Directional kit.

4.1.1.7 DNA Illumina sequencing

The prepared DNA libraries were paired-end sequenced on Illumina HiSeq X Ten sequencer (Illumina, San Diego, USA) for 150 cycles.

4.1.1.8 RNA Illumina sequencing

Prepared CDNA library was queued to sequencing on Illumina Platform with 150 PE sequencing chemistry generating total of 40 million reads per sample (20 million Paired end reads per sample).

4.1.1.9 Data analysis

The raw reads generated from both Nanopore and Illumina platforms were processed using Nanofilt v2.6.0 and FastQC v0.11.9 respectively and the obtained good quality reads were kept for further analysis. Denovo hybrid assembly was carried out by MaSuRCA v4.0.3 hybrid assembler using Illumina paired end and nanopore data. The assembly resulted in ~

33.6 Mbp size genome. The quality of generated assembly was further evaluated using QUAST v.5.02 and BUSCO v4.1.4. The repeats in assembly were modelled by RepeatModeler v2.0.1 and masked by RepeatMasker v4.1.1. The complete methodology can be seen in the below Figure 4.1.4. The assembled genome was annotated using MAKER v3.01.03 by iteratively running it for 5 rounds using the denovo assembled RNA seq data, employing SNAP, GeneMark and AUGUSTUS abinitio gene predictors and all available *Rhodotorula* proteins from uniport as evidence. Functional annotation was done using InterProScan v5.48-83.0 and BLAST v2.9.0+ against UniProt databases. The completeness of genome annotation was assessed using BUSCO v4.1.4. Further GenomeScope v.1.0.0 was used to estimate the genome size and ploidy. The predicted proteins were searched against KEGG database for pathway analysis.

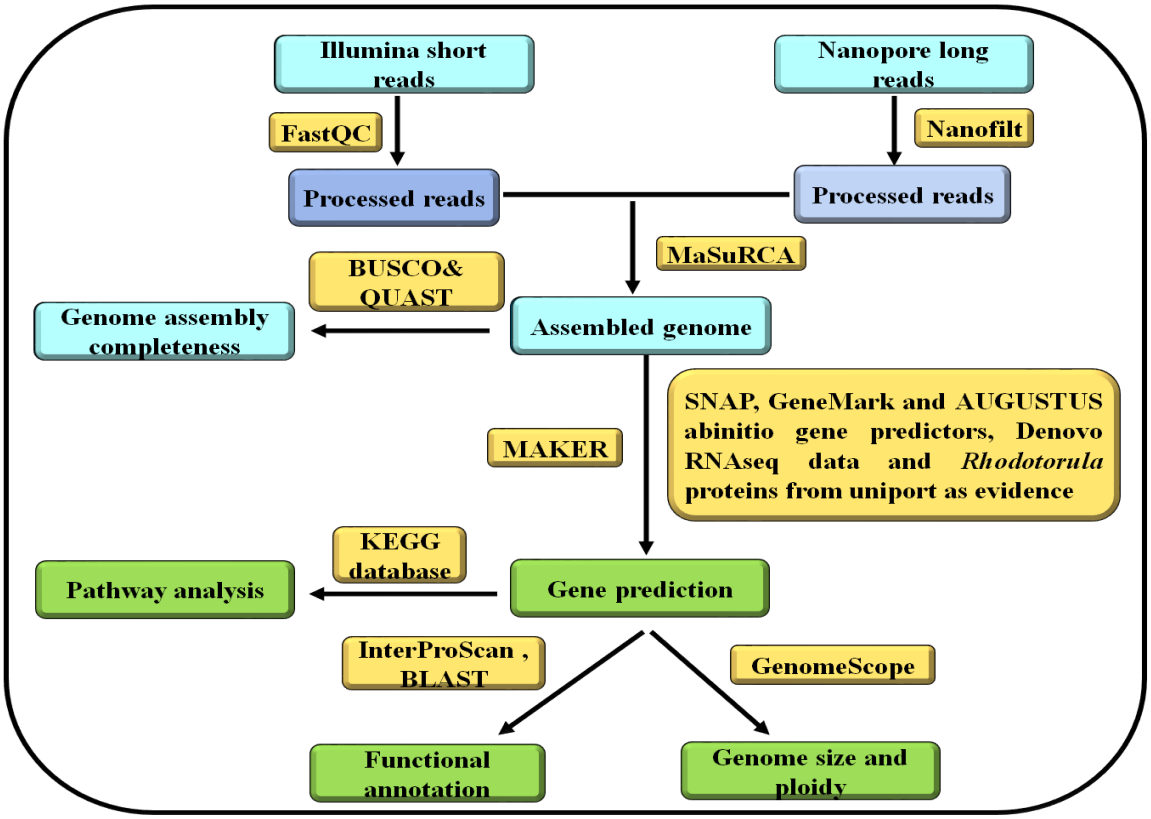


Figure-4.1.4: Methodology for denovo analysis of hybrid genome assembly and annotation of *R. pacifica* INDKK

4.1.2 RESULTS AND DISCUSSION

4.1.2.1 DNA extraction, Illumina library

DNA extracted has optimal yield and concentration suitable for Illumina and Nanopore for library preparation with 260/280 ratio of 2.03 (Table 4.1.1). The Illumina DNA library showed average fragment size of 406 bp with optimal concentration for desired sequencing on Illumina and also concentration and yield for Nanopore was also optimal for sequencing on GridionX5.

Table-4.1.1: Estimated DNA concentration and purity

Sno	Concentration (ng/µL)	260/280	260/230	Volume (µL)	Yield (ng)
1	32.5	2.03	1.27	38	1235

Table-4.1.2: DNA concentration estimated by Qubit

Sno	Qubit concentration (ng/µL)	Volume loaded (µL)	Yield (ng)
1	412	20	820

4.1.2.2 Data de-multiplexing and quality check

The data demultiplexing was performed using Bcl2fastq (v2.20) and the sequence FastQ files obtained using unique dual barcode sequences was assessed by FastQC (v0.11.8). The low-quality bases were filtered off during read pre-processing, adapter sequences were removed and bases above Q30 were selected for Illumina sequencing. Raw reads obtained from nanopore sequencing were base-called (‘fastq5’) and de-multiplexed by Guppy v2.3.4. The pre-processing of data retained more than 21 million paired end reads for the sample (Table 4.1.3).

Table-4.1.3: Processed read Statistics for Illumina sequencing

Sno	Raw reads	Processed reads	% Reads retained
1	22371447	21899078	97.88

Similarly, the nanopore reads were pre-processed using Nanofilt and the read statistics for nanopore data is shown in Table 4.1.4.

Table-4.1.4: Raw and processed Read statistics for Nanopore sequencing

Parameter	Raw reads	Processed reads
Reads generated	293418	291354
Maximum Read Length	102664	102543
Minimum Read Length	87	1
Average Read Length	4358.1	4280.2
Median Read Length	8227.5	5785.5
Total Reads Length	1278734670	1247067369
Reads >= 100 bp	293410	290540
Reads >= 200 bp	289838	264088
Reads >= 500 bp	214255	200866
Reads >= 1 Kbp	169580	164181
Reads >= 10 Kbp	41391	40720
N50 value	11048	11128

4.1.2.3 RNA extraction and quality control

Total RNA from each type of frozen cell pellet sample was extracted using Qiagen RNeasy mini kit in combination with DNase treatment [265]. Extracted RNA concentration was determined using Nanodrop spectrophotometer and Qubit flurometer. RNA is relatively unstable and can rapidly digested by RNase enzymes and degraded RNA can potentially

hinder the downstream process. Therefore, the integrity of RNA molecules is crucial which reflects the gene expression of a cell. The RNA integrity was checked by running an aliquot of the samples on Agilent Bioanalyzer chip. As shown in Table – 4.1.5 and Table – 4.1.6, the calculated values for RNA yield and integrity values are optimal along with RIN value closer to 10 for all samples which were ideal for library preparation [266].

Table -4.1.5 Estimated RNA yields using Nanodrop

S.no	Sample name	ng/µL	260/280	260/230	Volume (µL)	Yield (ng)
1	YPD Medium-1	82.1	2.35	0.14	22	1806.2
2	YPD medium-2	56.7	2.47	0.2	22	1247.4
3	YPD medium-3	18	2.63	0.03	22	396

Table-4.1.6: RNA yields from Qubit QC and RIN values from Agilent Bioanalyzer chip

Qubit QC					Sample Quality Control		
S.no	Sample Name	Qubit concentration (ng/ µL)	Volume (µL)	Yield (ng)	Qubit yield	TAPE RNA integrity	TAPE # RIN
1	YPD Medium-1	50.6	22	1113.2	Optimal	Optimal	9.7
2	YPD medium-2	27.2	22	598.4	Optimal	Admissible	9.5
3	YPD medium-3	8.92	22	196.2	Optimal	Optimal	9.9

4.1.2.4 RNAseq library preparation and RNA Illumina sequencing

Library was prepared using the extracted RNA which was optimal in terms of yield and RIN value. The library constructed was shown in the Table – 4.1.7, has a mean fragment size of ~ 403 bp and with sufficient concentration for sequencing. Thus, the library suitable for Illumina sequencing was made and queued for sequencing on Illumina platform with 150 PE sequencing chemistry, which obtained 20 million paired end reads per sample (Table – 4.1.8)

Table-4.1.7: Description of constructed RNA library

S.no.	Sample ID	Qubit Conc. (ng/µL)	Volume (µL)	Yield (ng)	Barcode ID	Index Sequence
1	YPD Medium-1	3.48	10	34.8	NEB31	CACGAT
2	YPD medium-2	3.42	10	34.2	NEB34	CATGGC
3	YPD medium-3	5.1	10	51	NEB37	CGGAAT

Table-4.1.8: Statistics of RNA reads obtained

S.no	Sample name	Reads obtained - million reads
1	YPD Medium-1	18.799339
2	YPD medium-2	15.914571
3	YPD medium-3	22.059381

4.1.2.5 Hybrid genome sequencing, assembly and annotation

The denovo whole genome sequencing of lab isolate *Rhodotorula pacifica* INDKK was performed followed by transcriptomics-based assembly as described in materials and methods. To attain gap free assembled genome, short reads of 140 bp were obtained by Hiseq Illumina sequencing with 21.8 million reads. Followed by long read sequencing by Nanopore (MinION) sequencer where the average read length (4560 bp) was acquired and further proceeded for hybrid assembly as described in methods.

Table-4.1.9: Genome assembly statistics

Parameter	Number
No of contigs (>= 0 bp)	117
No of contigs (>= 1000 bp)	117
No of contigs (> = 5000 bp)	116
No of contigs (>= 10000 bp)	116
No of contigs (>= 25000 bp)	57
No of contigs (>= 50000 bp)	49
Total length (>= 0bp)	33632480
Total length (>= 1000bp)	33632480
Total length (>= 5000bp)	33627892
Total length (>= 10000bp)	33627892
Total length (>= 5000bp)	32630969
Total length (>= 10000bp)	32357659
Total no of contigs	117
Largest contig	2764732
Total length	33632480
GC %	59.49
N50 value	1123312

The processed Illumina and Nanopore reads were used for the hybrid fungal genome assembly using MaSuRCA v3.3.7 [267]. Both de Bruijn graph and Overlap Layout-

Consensus (OLC) approach was used to assemble short reads and long reads. The assembled fungal genome resulted in ~ 33.6 Mbp genome size.

Table-4.1.10: Previously reported sequenced genomes of *Rhodotorula* strains, assembly statistics and sequencing platforms used

Strain	Sequencing platform	Coverage	Genome size (Mb)	GC content (%)	No of contigs	No of coding sequences	Reference
R. toruloides MTCC 457	Illumina	121 X	20.09	62	689	NA	[268]
R. toruloides NP11	Illumina	96 X	20.2	NA	17,814	8171	[140]
R. toruloides CECT1137	Illumina, Roche 454 FLX	NA	20.45	61.9	NA	8206	[269]
R. toruloides ATC 204091	Illumina, Roche 454 FLX, Sanger	NA	20.48	61.9	186	3359	[270]
R. toruloides IFO0559	Illumina	170 X	20.28	NA	NA	8100	[271]
R. toruloides IFO0880	Illumina	139 X	20.36	NA	NA	7920	[271]
R. toruloides ATCC 10788	Illumina	241 X	20.75	62.01	NA	7730	[272]
R. toruloides ATCC 10657	Illumina	233 X	21.49	61.81	NA	7800	[272]
R. toruloides CGMCC 2.1609	Illumina, Roche 454 FLX	13 X	33.4	61.9	868	9820	[273]
R. toruloides VN1	Illumina	125 X	20.02	61.8	424	8021	[274]
R. toruloides CBS 14	Illumina, Oxford Nanopore	1000 X	20.53	61.83	23	9464	[275]
R. mucilaginosa JGTA-S1	Illumina, Oxford Nanopore	262X	20.11	NA	341	5,922	[276]
R. pacifica INDKK	Illumina, Oxford Nanopore	360X	33.63	59.64	117	12,270	This study

-NA, not available

The basic assembly statistics are shown in Table 4.1.9. Sequencing depth of 360 X was achieved by hybrid technology which generated 117 contigs with a maximum contig length of 2764732 bp and N50 of 1123312 bp (Table: 4.1.9). This number of contigs obtained were

117 contigs which were lower than previous studies so far as shown in Table 4.1.10. The denovo genome assembly of *R. pacifica* INDKK resulted in 33.63 Mbp genome size which is similar to *Rhodotorula toruloides* strain CGMCC 2.1609 genome size of 33.4 Mbp as shown in (Table 4.1.10). The total GC content calculated was 59.63 similar to previously reported average GC content of 61.9 % (Table: 4.1.10).

The overall genome assembly statistics are tabulated in Table: 4.1.9. The genome annotation was carried out with SNAP, Augustus, Genemark from the assembled using Maker tool. Using GenMark-ET and AUGUSTUS tools, genomic and RNA-Seq data were used for automatically predicting gene models in novel genome. A complete set of 12,270 functional genes were identified which are higher than previously reported *Rhodotorula* genomes (Table: 4.1.10). DNA sequences were submitted to BlastKOALA to find unique KEGG ortholog (KO) identifier for the sequences and identify their orthologous gene groups [277]. The obtained transcripts were submitted to KEGG database to predict proteins and their KO ids. The KO ids obtained were submitted to KEGG server to predict and classify the metabolic pathways they involve. The gene ontology (GO analysis) was performed using Eggnog mapper and the final annotated final genome comprised of identified genes from major metabolic pathways such as central carbon, central nitrogen, lipid, cofactors, energy metabolism, transport, cell cyle, terpenoid and secondary metabolism.

Table-4.1.11: Total number of annotated genes and transcripts

No of annotated genes or transcripts	
Total annotated genes	12270
Total annotated transcripts	15135

The annotated genome was checked for completeness using BUSCO on the predicted proteins and fungi as lineage which showed 92.6 % complete single copy orthologues genes,

single-copy genes (36.7 %), duplicated (55.9 %), fragmented (1.2 %) and 6 % were missing genes [278]. While the transcriptome data has shown 91.9 % of the genes as complete with single-copy genes (35.8 %), duplicated (56.1 %), fragmented (0.8 %) and 7.3 % were missing. Therefore, genome sequencing of *R. pacifica* INDKK, with more contiguity, accuracy and completeness was obtained.

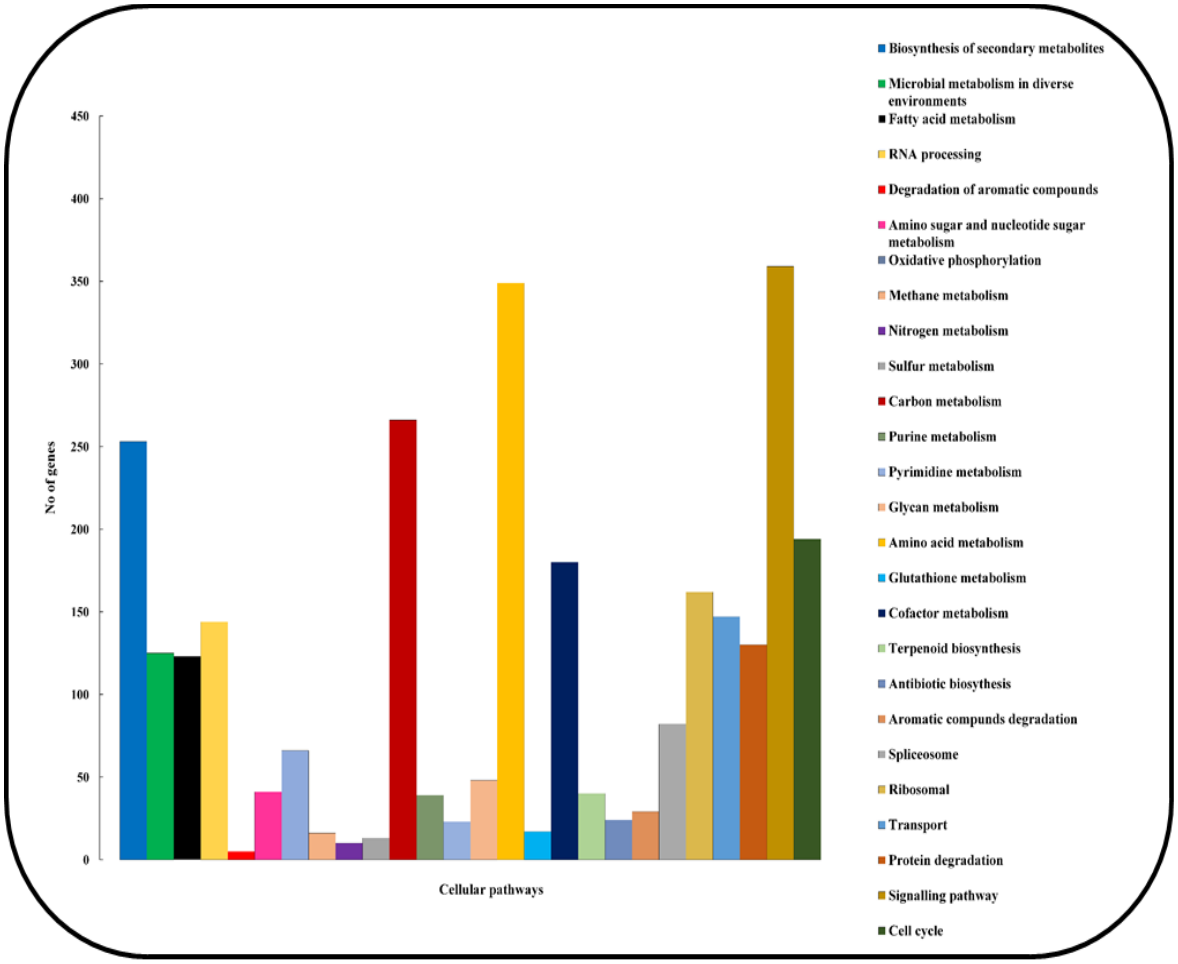


Figure-4.1.5: Cellular pathway genes identified in *R. pacifica* INDKK determined using KEGG database

4.1.2.6 Gene prediction and pathway analysis

The metabolic pathways were reconstructed using KEGG database [279]. Inorder to identify orthologues, sequences were submitted to BLAST KOALA [280] and their KEGG ortholog (KO) were identified.

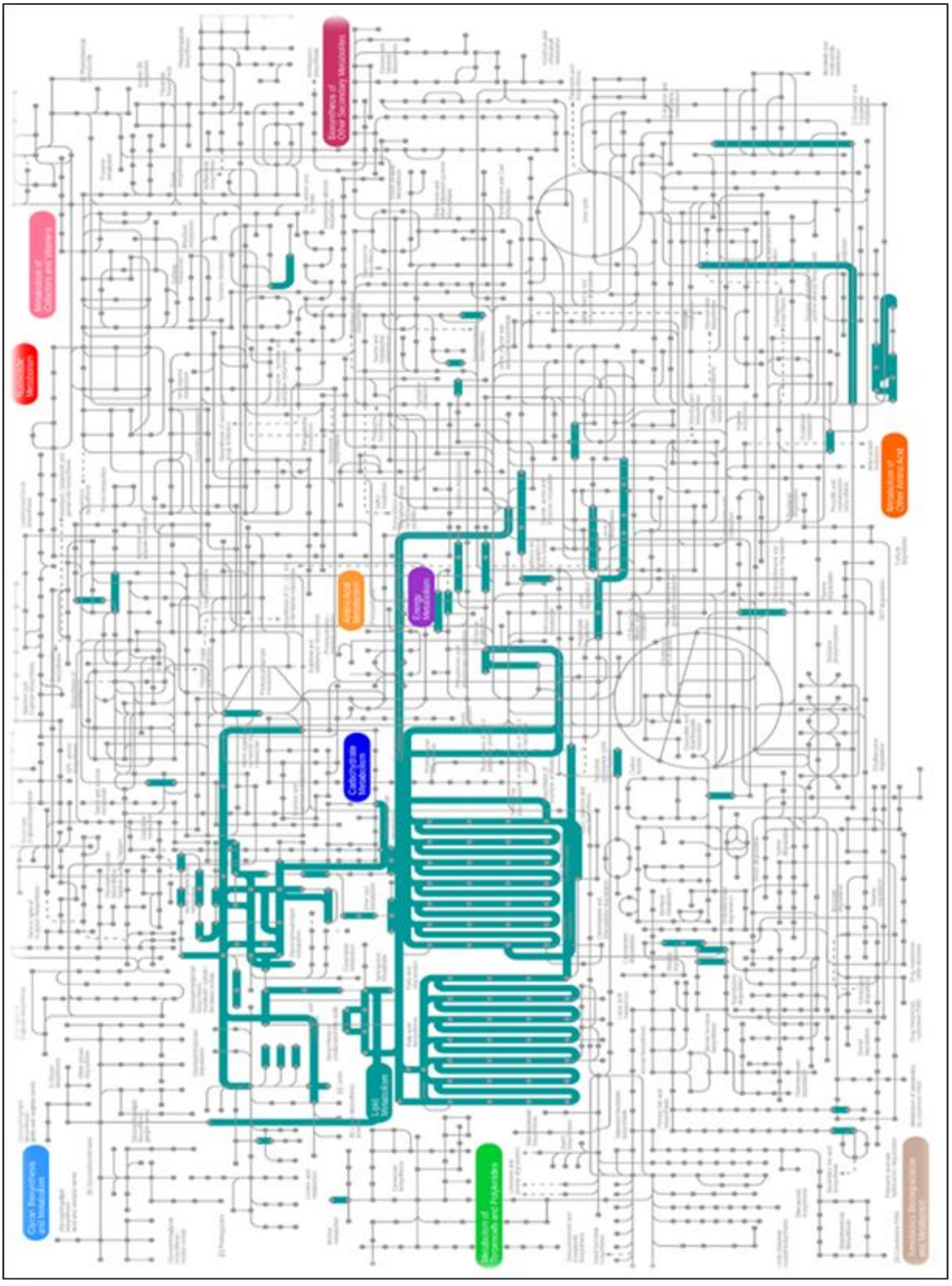


Figure-4.1.6: Lipid metabolism genes (Persian colour) of *R. pacifica* INDKK constructed on interactive pathway explorer using KO ids of identified lipid genes. While other cellular pathway genes are represented in grey color.

The description of pathway was generated from KEGG database with the obtained KEGG ID with respect to fungal organism. Complete set of annotated genes and their related pathways of *R. pacifica* INDKK such as carbon metabolism, nitrogen metabolism, cell cycle, signaling pathway, oxidative phosphorylation, protein degradation, transport, ribosomal, spliceosomal, aromatic compound degradation, antibiotic biosynthesis, terpenoid biosynthesis, cofactor metabolism, nucleotide metabolism, fatty acid (FA) metabolism, secondary metabolite biosynthesis, RNA processing, glutathione metabolism was identified. Using Ipath3, lipid metabolic genes such as FA activation and export, FA desaturation, galactolipid, phospholipid, sulpholipid and membrane lipid biosynthesis, TAG degradation of *Rhodotorula pacifica* INDKK were reconstructed with their KO ids as shown in Figure 4.1.6.

4.1.2.7 Domain identification of FAS

FAS is an important enzyme key enzyme which regulates the de novo biosynthesis of fatty acids Also, it is central to metabolism for biofuel production. Classification of protein domains is widely done by Pfam database [279]. For better understanding of fatty acid synthase of *R. pacifica* INDKK, its structural domains are important. Therefore, its domains were predicted and matched with existing non oleaginous yeast such as *S. cerevisiae* and oleaginous yeast (*R. toruloides* NP11, *Y. lipolytica*,) to understand the differences in their domain architecture.

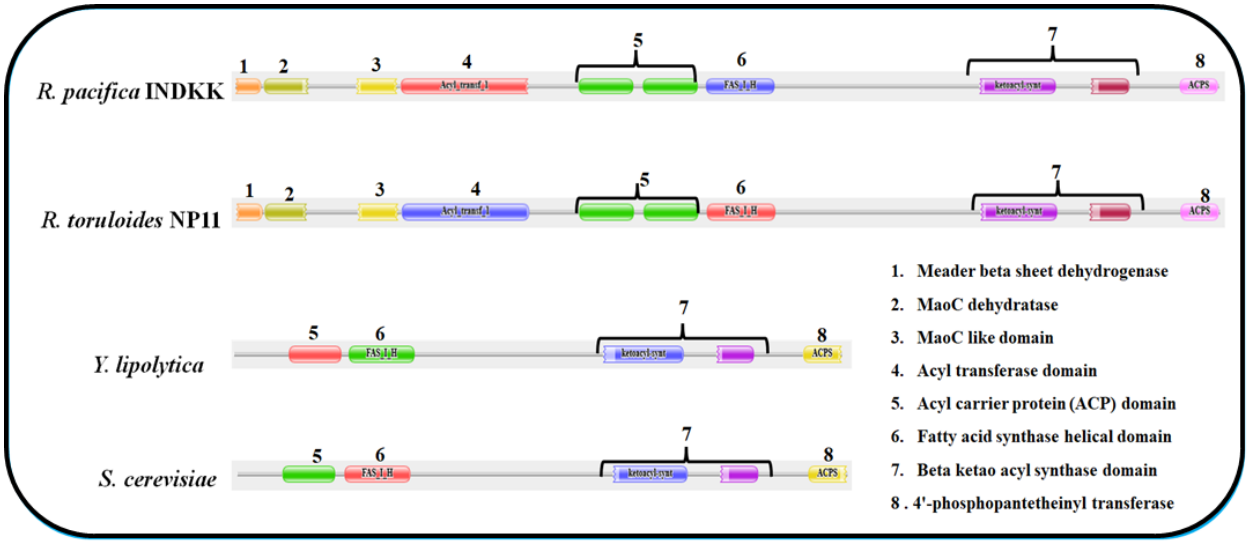


Figure-4.1.7: Fatty acid synthase (FAS) domains of *R. pacifica INDKK* identified by Pfam.

Pfam has identified MaoC dehydratase domain, Acyl transferase domain, Fatty acid synthase type I helical domain, two ACP domains, Beta-ketoacyl synthase, 4'-phosphopantetheinyl transferase domains similar to *Rhodosporidium toruloides* NP11 but significantly different from *Saccharomyces cerevisiae* and *Yarrowia lipolytica* where they possess only one ACP domain without MaoC dehydratase, Acyl transferase domain. The presence of extra domains catalyzes robust fatty acid synthesis than non-oleaginous yeast such as *S. cerevisiae* [140][281]. This, critically explains the high oleaginicity of *R. pacifica* INDKK.

4.1.3 Conclusion

The genome sequencing of oleaginous yeast *R. pacifica* INDKK was completed using hybrid Illumina and Nanopore sequencing platforms. The genome size of 33.63 Mb with 117 scaffolds was estimated. All vital genes and pathways were identified by genome reconstruction. This study also helped in extracting complete fatty acid metabolism related genes and lipid pathway reconstruction. The functional domains of FAS genes involved in lipid biosynthesis were delineated in relation with other yeast genera. Hence, the genomics study and gene mining process provide insights into lipid biosynthesis with their related genes and other central pathways in *R. pacifica* INDKK which should aid in advancement of

metabolic engineering of yeast for lipid yield improvements and production of lipid based renewable biofuels.

4.2 Differential gene expression (DGE) study of *R. pacifica* INDKK in unoptimized (N-sufficient (N+)) medium and optimized (N-limited (N^-)) media

4.2.1 MATERIALS AND METHODS

4.2.1.1 Culture conditions, RNA extraction and quality control

Yeast was cultured overnight in YPD medium at 30 ºC, centrifuged and washed twice with MQ, re-suspended in 100 mL of nitrogen sufficient (N^+) YNB medium [glucose (3 % w/v) and $(NH_4)_2SO_4$ (0.5 % w/v)], nitrogen limited (N^-) YNB medium [glucose (5.88 % w/v) and $(NH_4)_2SO_4$ (0.14 % w/v)] to 0.2 OD_{600nm} and grown at 30°C for 72 h at 200 rpm. From each condition, the cells were centrifuged at 8000 rpm, washed with 1X PBS followed by immediate snap freeze and stored at -80 °C. Total RNA extraction was performed using Qiagen RNeasy Mini Kit in combination with DNase treatment. The RNA concentration, purity of samples and the integrity of RNA sample was checked as described in section 4.1.2.3.

4.2.1.2 RNAseq library preparation and Illumina sequencing

The preparation of RNA sequencing libraries was carried out as discussed in section 4.1.2.4. The libraries were paired-end sequenced on Illumina HiSeq X Ten sequencer (Illumina, San Diego, USA) for 150 cycles following manufacturer's instructions.4.2.1.3 Data analysis

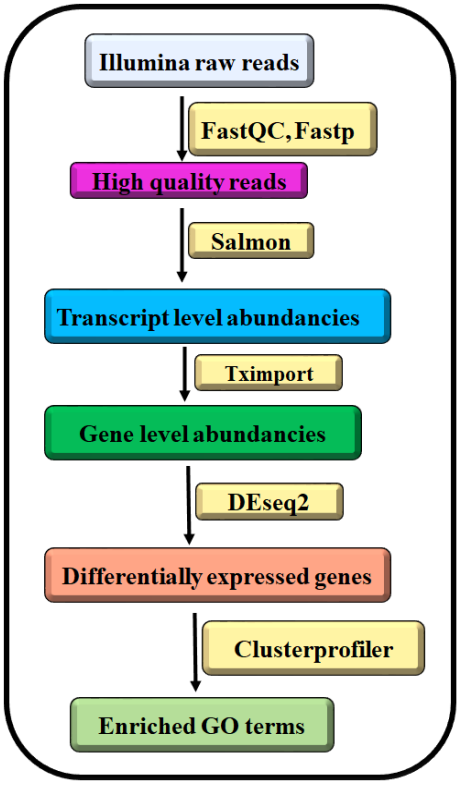


Figure-4.2.1: Flow chart for differential gene expression analysis

The raw reads generated from Illumina Highseq were processed using FastQC v0.11.9, the trimming of reads was done by Fastp and the good quality reads were retained. Salmon (v1.3.0) was used for mapping to total transcripts and collapsing of transcript level count to gene level counts using Tximport (v1.20.0) in R package (v4.1.1). Differential gene expression was performed using DESeq2 (v1.32.0) using R package (v4.1.1). Gene ontology (GO) enrichment analysis was done using Clusterprofiler (v4.0.5).

4.2.2 Results and Discussion

4.2.2.1 RNA extraction and quality control

Total RNA from each type of frozen cell pellet sample was extracted using Qiagen RNeasy mini kit (references) in combination with DNase treatment. Extracted RNA concentration was determined by Nanodrop Spectrophotometer and Qubit Flurometer. The RNA Integrity was checked by running an aliquot of the samples running on Agilent Bioanalyzer chip. As shown in Table – 4.2.1 and Table – 4.2.2, the calculated values for RNA yield and integrity

values are optimal along with RIN value closer to 10 for all samples which were ideal for library preparation.

Table-4.2.1: RNA yields of extracted RNA from N^- and N^+ samples

S.no	Sample Name	ng/ µL	260/280	260/230	Volume (µL)	Yield (ng)
1	N+ sample-1	26.4	2.36	0.06	22	580.8
2	N+ sample-2	55.4	2.51	0.11	22	1218.8
3	N+ sample-3	21.4	3.47	0.05	22	470.8
4	N^- sample-1	36.5	2.96	0.13	22	803
5	N^- sample-2	8.4	4.68	0.01	22	184.8
6	N^- sample-3	22.2	3.62	0.06	22	488.4

Table-4.2.2: RNA yields obtained from Qubit QC and RIN values from Agilent Bioanalyzer chip

Qubit QC					Sample quality control		
S.no	**Sample Name**	**Qubit conc. (ng/ µL)**	**Volume (µL)**	**Yield (ng)**	**Qubit yield**	**TAPE RNA Integrity**	**TAPE # RIN**
1	N+ sample-1	19.3	22	424.6	Optimal	Optimal	9.9
2	N+ sample-2	32.2	22	708.4	Optimal	Optimal	10
3	N+ sample-3	10.5	22	231	Optimal	Optimal	9.6
4	N^- sample-1	20.2	22	444.4	Optimal	Optimal	10
5	N^- sample-2	6.96	22	153.12	Optimal	Optimal	9.5
6	N^- sample-3	12.8	22	281.6	Optimal	Optimal	10

4.2.2.2 cDNA Library preparation and RNA Illumina sequencing

Library was prepared using the extracted RNA which was optimal in terms of yield and RIN value. The library constructed was shown in the Table – 4.2.3, which has mean fragment size of ~ 403 bp with concentration optimal for sequencing. Thus, the library which was suitable for Illumina sequencing was made.

Prepared library was queued to sequencing on Illumina Platform with 150 PE sequencing chemistry generating total of 40 million reads per sample (20 million paired end reads per sample). The obtained raw reads were trimmed using Fastp tool and the quality was checked using FastQC and where the quality was found to be optimal for differential gene expression analysis.

Table -4.2.3: Description of constructed RNA library

S. no	Sample name	Qubit concentration (ng/ µL)	Volume (µL)	Yield (ng)	Barcode ID	Index Sequence
1	N+ sample-1	10.9	10	109	NEB32	CACTCA
2	N+ sample-2	6.12	10	61.2	NEB35	CATTTT
3	N+ sample-3	5.14	10	51.4	NEB38	CTAGCT
4	N^- sample-1	7.12	10	71.2	NEB33	CAGGCG
5	N^- sample-2	3.9	10	39	NEB36	CCAACA
6	N^- sample-3	3.28	10	32.8	NEB39	CTATAC

Table-4.2.4: Statistics of obtained reads during Illumina sequencing

S.no	Sample name	Reads obtained - million reads
1	N+ sample-1	21.801166
2	N+ sample-2	20.628959
3	N+ sample-3	17.120719
4	N^- sample-1	18.702725
5	N^- sample-2	17.527032
6	N^- sample-3	18.423432

4.2.2.3 RNAseq analysis under N-limited (N^-) and N-sufficient (N+) media

Microbial derived long-chain fatty acids have prominent application as drop in biofuels and industrial valuable chemicals. Therefore, natural oleaginous yeast which can produce neutral lipids should be explored for enhancement of lipid production. The high titres of lipid production by the *Rhodotorula* species has been well reported [103,282–284]. Nitrogen limiting condition is a well-studied phenomenon to understand lipid production in for oleaginous yeast [245,285–287] which implied it as a key regulator for lipid overproduction [24,245,246,286]. Under N limiting (N^-) conditions, the activity of mitochondrial NAD^+-dependent isocitrate dehydrogenase (IDH) tend to get impaired under nitrogen limitation condition as the cellular AMP level is lowered [288]. Thus, increasing citrate in mitochondria and pumped into cytosol to form acetyl-CoA for fatty acid synthesis. The cytosolic $NADP^+$-dependent malic enzyme (ME) also known to supplement nicotinamide adenine dinucleotide phosphate (NADPH) for *de novo* lipid synthesis [289,290]. Previously, several transcriptomic

analysis have been studied under N^+ and N^- conditions to understand nitrogen triggered lipid production in oleaginous yeast [140,151,245]. These studies have revealed critical transcriptional changes undergoing in lipid metabolism linked with major central carbon, central nitrogen and other pathways. Therefore, to delineate the influence of nitrogen limitation on the process of lipid accumulation in *R. pacifica* INDKK, to obtain insights into nitrogen metabolism and lipid production. The differential gene expression was studied in nitrogen sufficient (N^+) YNB medium having glucose (3 % w/v) and $(NH_4)_2SO_4$ (0.5 % w/v) and nitrogen limiting (N^-) YNB medium with glucose (5.88 % w/v) and $(NH_4)_2SO_4$ (0.14 % w/v). A total of 40 million reads per each sample was obtained from both N^+ and N^- medium on Illumina Platform with 150 pairend (PE) sequencing. Of these, 74.87 % reads from the N^+ medium and 73.66 % reads from the N^- medium were mapped to annotated transcripts and the genome. There was total 1228 genes were found to be significantly differentially expressed ($P < 0.05$) that were expressed in N- and N+ media of which 221 and 482 genes upregulated and downregulated, respectively. The differential gene expression values between N^- limiting and N^+ sufficient were obtained as log2 fold change (log2FC) with false discovery rate (FDR < 0.05). The differential expression of several genes related to important metabolic pathways such as central carbon metabolism, central nitrogen metabolism, oxidative phosphorylation, lipid metabolism, transporters, protein degradation that delineates lipogenesis of yeast were identified and depicted in heatmap generated by ClustVis server as shown in Figure 4.2.2.

Fatty acid metabolism genes such as FAS, phospholipid-transporting ATPase (SPAC24Bc), hydroxymethylglutaryl-CoA synthase (hcs1), acyl-CoA desaturase (OLE1), carnitine O-acetyltransferase (CAT2) were upregulated (P-value < 0.05). The Acetyl-CoA carboxylase (cut6) was found to be overexpressed (0.47-fold , P-value = 0.001) under N^- medium which is the main enzyme to catalyze irreversible carboxylation of acetyl-CoA to produce malonyl-

CoA for fatty acid biosynthesis [167,291]. Fatty acid synthase enzyme with two tandem ACP domains conserved among oleaginous red yeast was found in *R. pacifica* INDKK. The expression levels of FAS was (0.42-fold , P-value = 0.01) higher in N^- medium as shown by other previous studies [140,292]. Long chain fatty acid producing genes such as long-chain-fatty-acid-CoA ligase, FAA1 (0.36-fold, P-value = 0.0004), Delta (12) fatty acid desaturase enzyme involved in creating double bonds in saturated fatty acids were upregulated (0.45-fold, P-value = 0.006). While lipolytic genes like lipid droplet phospholipase, LPL-1, (0.44-fold, P-value = 0.01), esterase (CG5412) (0.39-fold, P-value = 0.04), lipase B (0.52-fold, P-value = 0.00006), beta oxidation enzymes such as peroxisomal acetyl-coenzyme A thioesterase (ACOT12) (0.27-fold, P-value = 0.03), mitochondrial Acyl-CoA dehydrogenase (ACAD10) (0.22-fold, P-value = 0.03) were down regulated relative to N^+ medium which explains how N^- condition augments lipid production [293,294]. Previously, omic studies have already shown, the role of GATA binding zincfinger transcription factors as regulators of lipogenesis in response to nitrogen limitation. The regulation of NCR genes and nitrogen regulation under N limitation was observed in yeast [140]. In *R. toruloides,* a total of 11 GATA transcription factors were identified which are present in upstream of upregulated N metabolism genes.

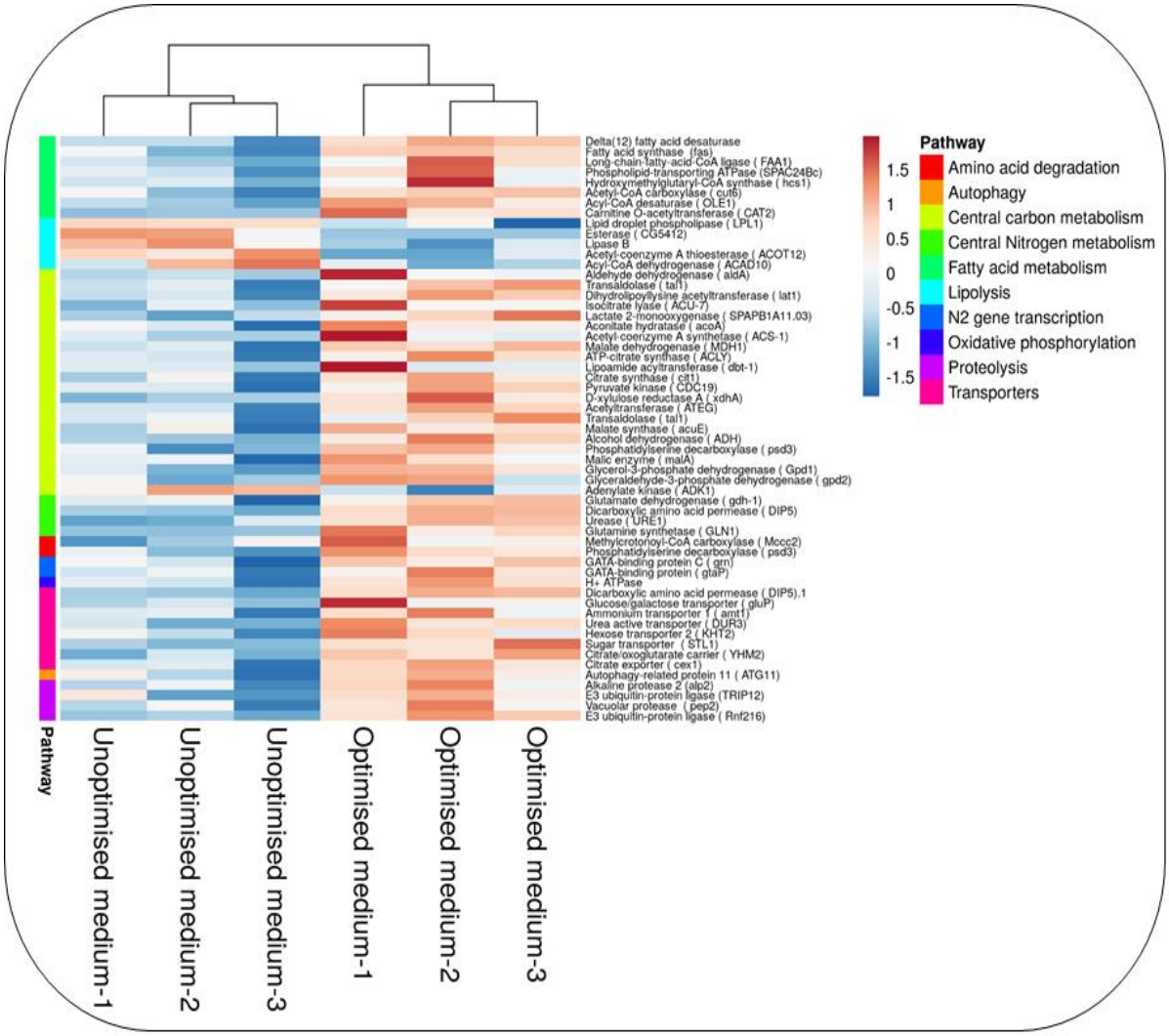


Figure-4.2.2: Differentially expressed genes involved in major lipid and their related central metabolism in oleaginous *R. pacifica* INDKK between unoptimized N+ sufficient and optimized N^- - limited media were represented as heat map constructed using ClustVis server. (Color codes blue and red of the heat map are the scaled normalized expression values and colour codes for pathway were assigned by ClustVis server)

In another study, the role of GATA factors involved in lipid accumulation was studied in *Y. lipolytica* [295].

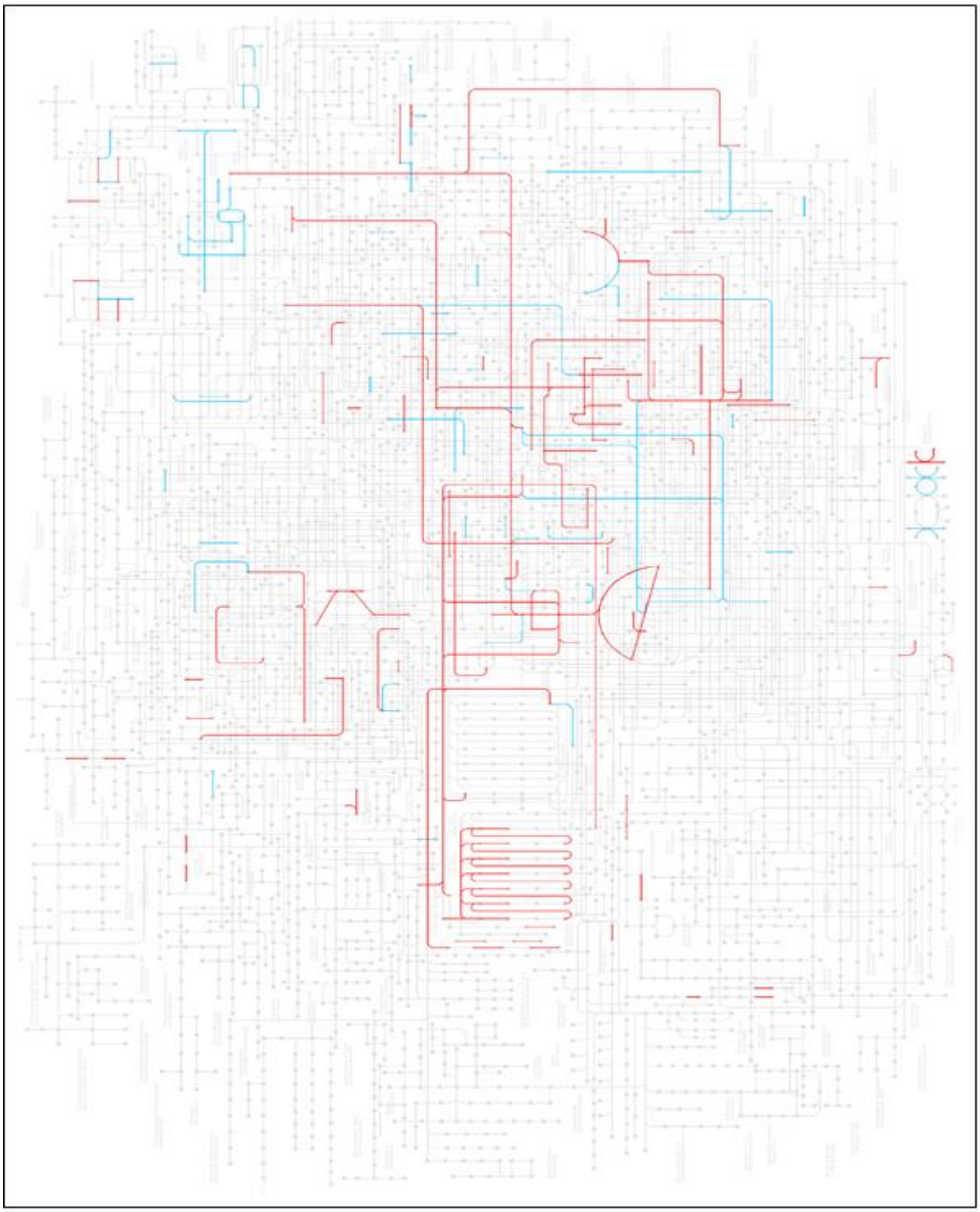


Figure-4.2.3: Differentially expressed cellular genes were represented using KEGG mapper (COLOR) and the overexpressed genes in optimized N^- - limited medium are represented in red while downregulated genes in blue relative to unoptimized N^+ - sufficient medium.

In *R. pacifica* INDKK, a total of 9 putative GATA transcription factors were identified that can bind to [AT]GATA[AG] motifs present upstream of genes of nitrogen metabolism. Out

of which, 2 GATA transcription factors, GATA-binding factor C (grn) and GATA zinc finger domain-containing protein (gtaP) were found to be overexpressed (0.36-fold, P-value = 0.02) under optimized N^- condition. Therefore, characterizing the role of GATA factors under N limiting conditions at the transcriptional regulatory level would be helpful to gain insights into molecular mechanism of regulation between nitrogen metabolism and fatty acid biosynthesis.

Various cellular metabolic processes were altered under N limitation [245] such as the central nitrogen metabolism genes (NCR) comprised of NAD-specific glutamate dehydrogenase (0.38-fold, P-value = 0.007), Glutamate synthase [NADH] (0.42-fold, P-value = 0.0000001) are known to be hub of cellular nitrogen utilization and their transcripts were highly upregulated [140]. Also, the genes involved in urea transport (DUR3) (2.38-fold, P-value = 0.0005) and urea degradation (URE1) (0.42-fold, P-value = 0.0001) were upregulated. The autophagy related gene (ATG11) and regulatory components of proteosome machinery genes such as (alkaline protease (alp2), E3 ubiquitin ligase (TRIP12, Ruf216), vacuolar protease (pep2) were upregulated (P-value < 0.05) as reported previously [140]. The vacuolar amino acid permeases (pep-2) (0.28-fold, P-value = 0.004), involved in vacuolar protein degradation were also activated. Also, amino acid degrading enzymes such as methylcrotonoyl Co-A carboxylase (Mccc2), phosphatidylserine decarboxylase (psd3) were downregulated. Furthermore, ammonium transporter (Amt1) (0.48-fold, P-value = 0.029) were also highly upregulated (Figure-4.2.3). Hence, nitrogen limitation induced various cellular pathways such involved in importers of nitrogenous molecules, nitrogen assimilating genes, secondary nitrogen source utilizing genes, vacuolar amino acid degrading genes.

The genes involved in central carbon metabolism such as aldehyde dehydrogenase (aldA), transaldolase (tal1), dihydrolipoyllysine acetyltransferase (lat1), isocitrate lyase (ACU-7), lactate 2-monooxygenase (SPAPB1A11.03), aconitate hydratase (acoA), acetylCoenzyme -A

synthetase (ACS-1), malate dehydrogenase (MDH1), ATP-citrate synthase (ACLY), lipoamide acyltransferase (dbt-1), citrate synthase (cit1), pyruvate kinase (CDC19), D-xylulose reductase A (xdhA), acetyltransferase (ATEG), transaldolase (tal1), malate synthase (acuE), alcohol dehydrogenase (ADH) are known to be involved in central pathways such as glycolysis, TCA cycle, pentose phosphate pathway (PPP) are known to generate precursors such as pyruvate, oxaloacetate , malate , citrate, acetyl coA, NADH/NADPH, for fatty acid biosynthesis were upregulated (P-value < 0.05).The V-type ATPase involved in oxidative phosphorylation or electron transport chain (ETC) was upregulated (0.42-fold, P-value = 0.009).Other transporter related genes like sugar transporter (STL1) (0.88-fold, P-value < 0.05), hexose transporter (KHT2) (0.46-fold, P-value = 0.027) that uptake hexoses into cells and mitochondrial dicarboxylate amino acid transporter (psd3) (0.68-fold, P-value = 0.014) were upregulated. Other fatty acid precursor carriers such as citrate exporter (cex1) (0.25 -fold, P-value = 0.045), citrate/oxaloacetate carrier (YHM2) (0.3-fold, P-value = 0.001), were found to be more active under N- limiting condition.

Based on the differential gene expression data obtained under N- and N+ media, it was found that central carbon pathways were highly upregulated to increase precursor flux of pyruvate, acetyl Co-A, NADH/NADPH towards fatty acid biosynthesis. Also, the fatty acid catabolic pathways such as beta oxidation and lipases were also down regulated to increase the cellular concentration. Under nitrogen limitation, the central nitrogen metabolism was driven to increase the cellular nitrogen concentration by activating GATA transcription factors, alternative nitrogen transporters such as urea, ammonia, amino acid catabolism and proteolysis.

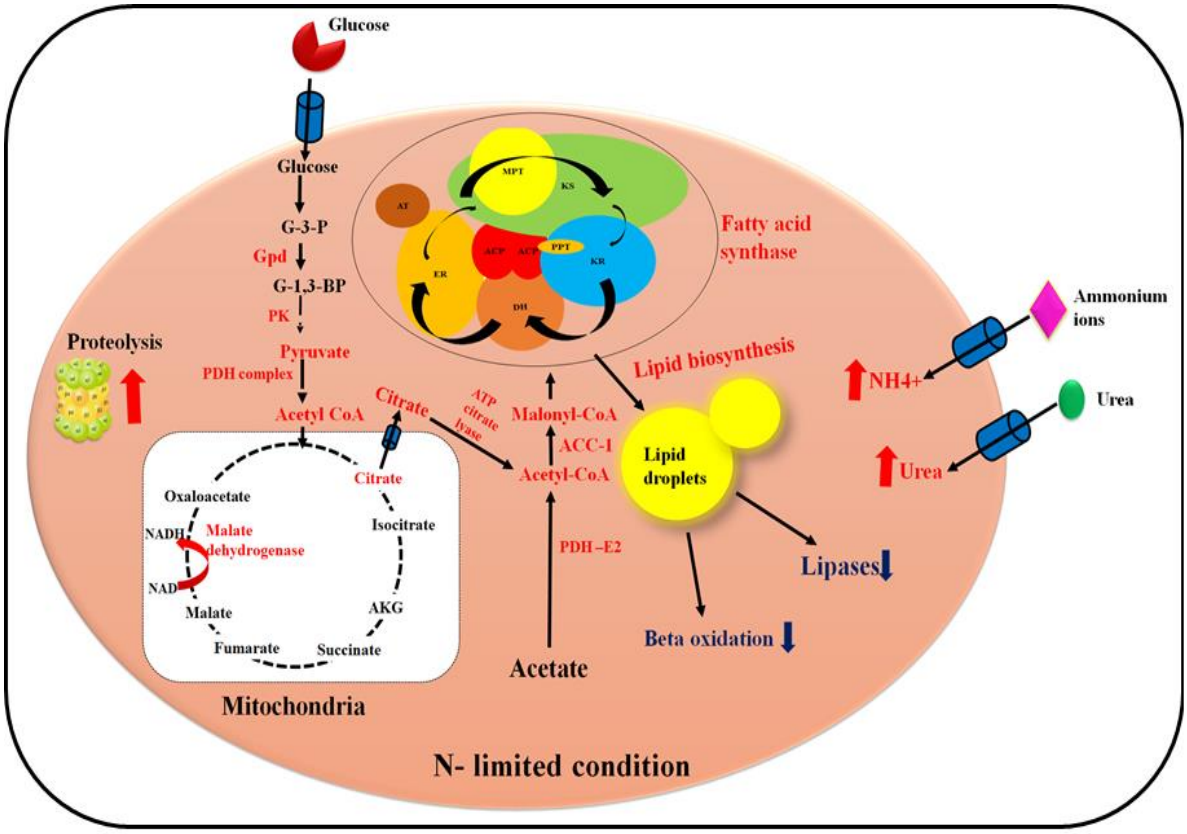


Figure-4.2.4: Reconstructed pathway of lipid production including glycolysis, TCA cycle, fatty acid synthesis and lipolysis, β -oxidation in *R. pacifica* INDKK is depicted. Also, metabolic reactions, the upregulated and downregulated genes under nitrogen limitation are represented in red and blue, respectively. Fatty acid synthase system (ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; ER, enoyl reductase; KR, ketoacyl reductase; KS, ketoacyl synthase; MPT, malonyl / palmitoyl transferase; PPT, phosphopantetheine transferase), GPD- Glyceraldehyde 3 phosphate dehydrogenase, PK-pyruvate kinase, PDH - Pyruvate dehydrogenase complex, E-2 component.

Therefore, the crosstalk between lipid metabolism and other central metabolic pathways in *R. pacifica* INDKK was uncovered as depicted in the figure 4.2.4. Therefore, the evidence of crucial cues explaining the oleaginicity were unfolded in this study which highlights the outlook of oleaginicity in *R. pacifica* INDKK.

4.2.2.3 CONCLUSION

In this DGE study under N^- medium to N^+ medium, a total of 1228 genes which were differentially expressed involved in central carbon, nitrogen, energy and lipid metabolic pathways were identified. The key enzymes of lipogenesis, FAS and ACC were found to be

upregulated and lipolytic genes such as beta oxidation and lipases were downregulated under N^- medium implying the key role nitrogen in lipid synthesis. Also, nitrogen responsive genes such as GATA factors and nitrogen transporters are also found to be upregulated under N^- medium. Therefore, DGE clues obtained aid in designing genetic modification strategy for enhanced lipid production in *R. pacifica* INDKK.

4.3 Metabolic engineering of *Rhodotorula pacifica* INDKK for lipid production improvement

Based on the lipid related metabolic pathways clues obtained from omics, ACC-1 was found to be overexpressed under N^- conditions which performs rate determining reaction in the synthesis of fatty acids by irreversible malonyl CoA formation reaction from acetyl CoA to improve lipid production. Another enzyme DGA-1 converts diacylglycerol to triacylglycerol. Previous studies have reported that overexpression of DGA-1 improves lipid production in yeast, DGA-1 was selected. Hence, the impact of ACC-1 and DGA-1 overexpression on lipid production in *R. pacifica* INDKK was studied.

4.3.1 MATERIALS AND METHODS

4.3.1.1 Strain and culture conditions

YPD medium was used for growth of *R. pacifica* INDKK, YEP medium (YE (0.1 % w/v) , peptone (0.2 % w/v) , NaCl (0.05 % w/v)) for growing *Agrobacterium tumefaciens* EHA105 [296]. 20 X AB salts stock was made using NH_4Cl (0.2 % w/v), $MgSO_4.7H_2O$ (0.06 % w/v), KCl (0.03 % w/v), $CaCl_2.2H_2O$ (0.02 % w/v), $FeSO_4.7H2O$ (0.005 % w/v), pH 7.1). Induction medium (IM) having 40 mM 2-(N-morp holino), Ethanesulfonic acid (pH 5.3), AB salts, acetosyringone (200 mM) , glycerol (0.5 % w/v) , 10mM glucose, and [297]. Antibiotics such as kanamycin (50 mg/mL); cefotaxime (600 mg/mL), nourseothricin (NAT) (100 mg/mL for liquid medium and 200 mg/mL for solid medium).

4.3.1.2 Plasmid

The ACC-1 and DGA-1 containing binary plasmid pGI2-880-ACC1-DGA1 which can replicate both in a*grobacterium* and yeast was procured from Dr. C.V. Rao, chemical and biomolecular engineering group, University of Illinois.

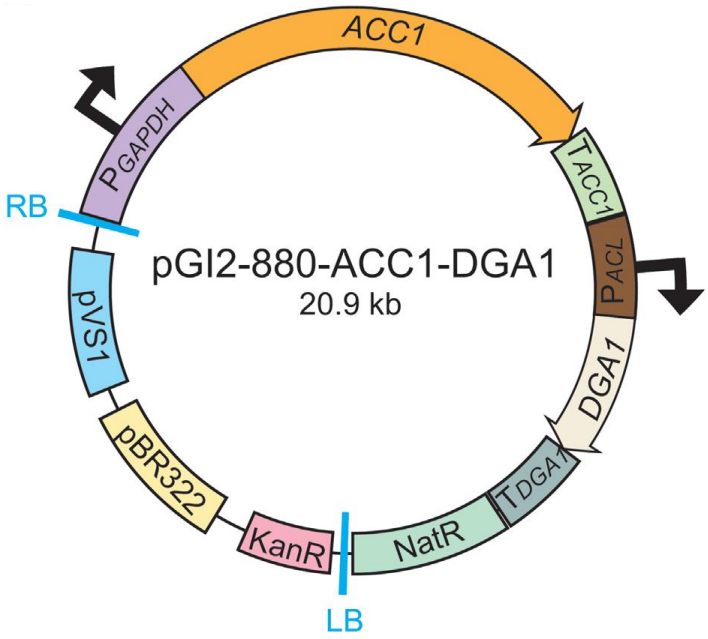


Figure-4.3.1: Diagrammatic representation of binary plasmid having ACC1 and DGA1 between the T-DNA`s left border (LB) and right border (RB) of *Agrobacterium tumefaciens*.

4.3.1.3 *Agrobacterium tumefaciens* mediated transformation (ATMT)

The binary plasmids was introduced into *Agrobacetrium tumefacians* EHA105 using freeze-thaw method [298]. Primary culture was cultured by inoculating *A. tumefaciens* in 5 mL of YEM + Rifampicin (Rif) and grow at 28 °C for 12-16 h. 2 mL of primary culture was added into 50 ml YEM + Rif at 250 rpm, 28 °C until OD_{600} reaches 0.5-1.0 (12 h). The culture was chilled on ice and centrifuged at 3000 xg for 5 min at 4 °C and resuspended in 1 mL of 20 mM $CaCl_2$ (0.03 g CaCl2 in 10 ml autoclaved water, filter) and centrifuged at 300 xg for 5 min at 4 °C and cell pellet was resuspended in l mL of 20 mM CaCl2. About ~ 1 µg of pGI2-880-ACC1-DGA1 plasmid DNA was added into the 100 µL of competent cells. Cells were frozen by placing them in liquid nitrogen and immediately thaw cells by transferring to 37 °C water bath for 5 min. Cells were resuspended in 1 mL of YEM medium and cultured at 28 °C for 4 h. Cells (200 µL/plate) were spread on YEM agar plates supplemented with kanamycin. *A. tumefaciens* EHA105 transformed with the binary plasmid pGI2-880-ACC1-DGA1 was cultured in 5 mL YEM medium + kanamycin until OD reached ~ 1 in 100 mL shake flask at 28 °C. The cells were centrifuged and resuspended in IM at OD_{600nm} of 0.1 then grown until

OD_{600nm} reaches 0.3 to 0.5. *R. pacifica* INDKK was grown in YPD medium until early exponential phase and 0.5 OD of cells were taken then mixed with 1:1 ratio of induced *A. tumefaciens* cells total volume of 1 mL, plated on IM plates and incubated at 25 °C for 48 h. The cells grown on membrane were spread on the YPD plate having NAT and cefotaxime and incubated for 96 h at 30 °C. Colonies obtained were restreaked on YPD plates containing NAT and cefotaxime for 5 generations to select stable transformants.

4.3.2 Results and Discussion

4.3.2.1 Initial characterization of *R. pacifica* INDKK

Yeast belonging to *Rhodotorula* species are well known producers of high lipid titers [8,96,120,299]. Following multiple screening tests, *R. pacifica* INDKK was found to accumulate highest lipid. Also, ploidy determined using fluorescence-activated cell sorting (FACS) and found to be diploid. Furthermore, under optimized condition of glucose (5.8 % w/v) concentration and ammonium sulphate (0.14 % w/v), *R. pacific* INDKK could produce lipid titers of ~ 14.5 g/L after 192 h duration in shake flask study. TLC studies have shown lipids produced were predominantly triglycerides. Hence, *R. pacifica* INDKK has shown the potential to produce triglycerides on glucose at high titers.

4.3.2.2 ACC1 and DGA1 over-expression in *R. pacifica* INDKK

Unlike conventional yeast such as *S. cerevisiae*, The absence of robust genome modifying tools for *Rhodotorula* species has been a hurdle so far [163]. Therefore, rational genetic modification techniques are required to develop *Rhodotorula* yeast for biotechnological application. The first transformation of *R. toruloides was* using polyethylene glycol (PEG) mediated protoplast transformation in 1985 but was ineffective and unstable[308,309].The electroporation mode of transformation [305,306] and chemical transformation by lithium acetate/PEG also been used [310].

Table-4.3.1: Recent studies on genetic modification of *Rhodotorula* yeast

Method	Strain	Integration	Expressed gene	Deleted gene	Selective marker	Transformation efficiency	Refs
Protoplast/PEG	*R. toruloides*	Random	*RtPAL*	-	Phenylalanine	1000 transformants/*m*g DNA	[163]
ATMT	*R. toruloides*	Random	*RtGFP*	-	Hygromycin	1000 transformants/plate	[300]
ATMT	*R. toruloides*	Targeted	*HPT3*	*KU70*	Hygromycin	5.2 %	[301]
ATMT	*R. toruloides*	Targeted	*HPT3*	*CAR2*	Hygromycin	75.3 %	[301]
ATMT	*R. toruloides*	Random	*HYG*	-	Hygromycin	1000transformants/10^5inputcells	[302]
ATMT	*R. toruloides*	Random	*NAT*	-	Nourseothricin	140 transformants/10^5 input cells	[302]
ATMT	*R. toruloides*	Random	*BLE*	-	Bleomycin	70 transformants/10^5 input cells	[302]
ATMT	*R. toruloides*	Targeted	*HYG*	*CRT*	Hygromycin	2%	[303]
ATMT	*R. toruloides*	Random	*ACC1, DGA1*	-	*Nourseothricin*	-	[192]
ATMT	*R. mucilaginosa*	Random	-	*CAR1*	*Nourseothricin*	-	[304]
Electroporation	*R. gracilis*	Targeted	*ShBLE*	*RgURA3*	Zeocin	40 CFU/*m*g DNA	[305]
Electroporation	*R. toruloides*	Random	*BLE*	-	Bleomycin	1000 CFU/*m*g DNA	[306]
LiAc/PEG	*R. toruloides*	Random	*ShBLE*	-	Zeocin	25 transformants/*m*g DNA	[307]
LiAc/PEG	*R. toruloides*	Random	*ShBLE*	-	Zeocin	–	[307]
LiAc/PEG	*R. toruloides*	Targeted	*ShBLE*	*URA3*	Zeocin, 5-FOA	–	[307]

The CRISPR/Cas9 system for *Rhodotorula* has been under development stage and very few studies have been reported [311,312]. While ATMT is most commonly used and efficient

method for *Rhodotorula* yeast [163,167,271,302,303,313]. Therefore, ATMT method was considered for transformation of *R. pacifica* INDKK in this study.

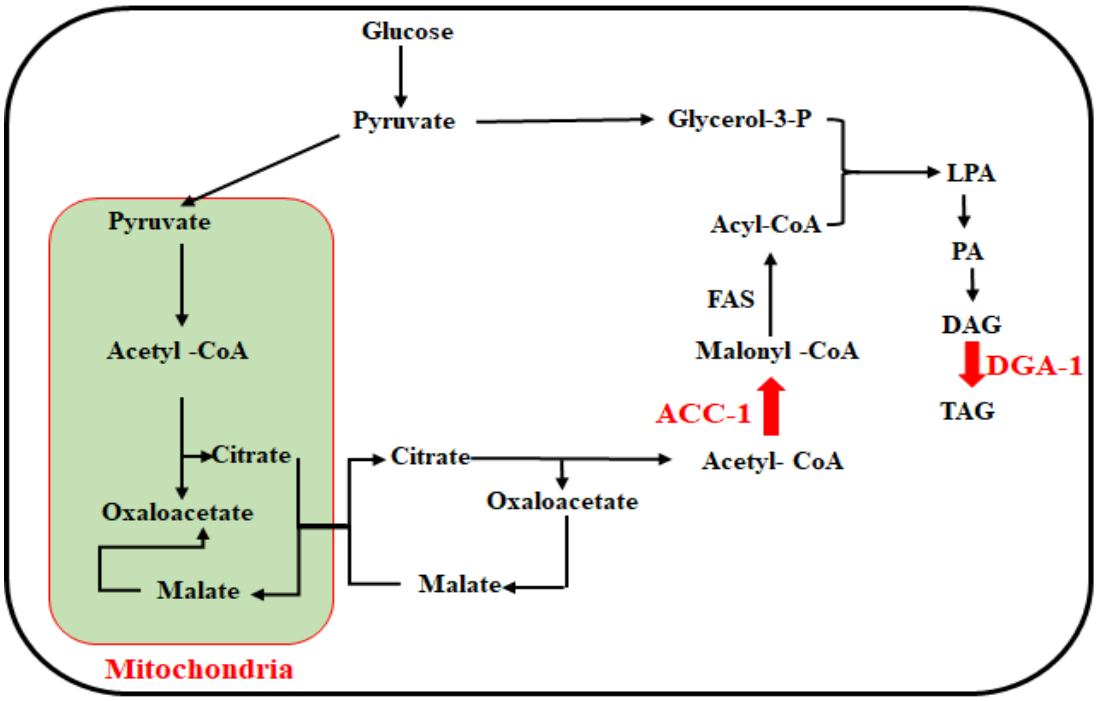


Figure-4.3.2: "Push-pull" strategy for over expression of ACC-1 and diacylglycerol DGA-1

The impact of overexpression of ACC-1 and DGA1 on lipid production in yeast complex was studied previously by several researchers. In one study, overexpression of ACC-1 and DGA-1 in *Y. lipolytica* improved lipid production by 41.1% [314] . Similarly, overexpression of ACC-1 and DGA-1 in *R. toruloides*, both ACC-1 and DGA-1 in *R. toruloides* improved lipid production by ~ 10 fold [271]. Since, the deletion of genes in *Rhodotorula* yeast is not efficient [163], hence the strategy of "push-pull" strategy of [35,271] for over expression of ACC-1 and DGA-1 was considered to further improve lipid production (Figure 4.3.2).

4.3.2.3 Antibiotic sensitivity of *R. pacifica* INDKK

To measure the sensitivity of *R. pacifica* INDKK, single colonies were streaked on YPD plates supplemented with 50 µg/mL, 100 µg/mL, 200 µg/mL NAT incubated at 30 °C for 5 days and found 50 µg/mL NAT inhibiting the growth.

4.3.2.4 Transformation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens EHA105 transformation was performed by using robust , conventional freeze thaw method [298]. *A. tumefaciens* cells were grown until actively dividing log phase and the culture was chilled and treated with $CaCl_2$ to make them competent.

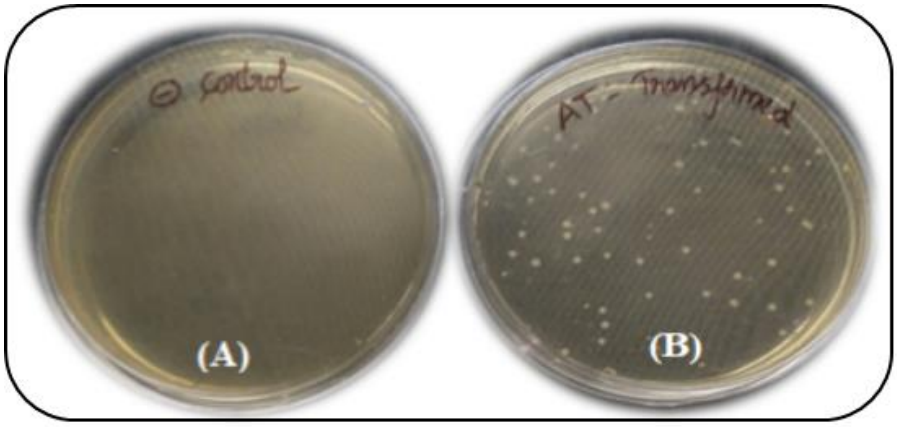


Figure-4.3.3: (A) Negative control (B) Transformed colonies appeared having plasmid pGI2-880-ACC1-DGA1 on YEM Kan+ plates after 48h of incubation at 28 °C

The competent cells were mixed with ~ 1 µg of pGI2-880-ACC1-DGA1 plasmid DNA and frozen in liquid nitrogen and immediately thaw cells by to 37 °C water bath for 5 min. Cells were revived within 1 mL of YEM medium and incubated at 28 °C for 4 h. Cells (200 µL/plate) were spread on YEM agar plates supplemented with kanamycin. Colonies appeared on KAN plate after 48 h at 28 °C was selected and screened using colony PCR.

4.3.2.5 *Agrobacterium* mediated transformation of *R. pacifica* INDKK

O.2 OD_{600nm} of overnight cultured *A. tumefaciens* strain in YEM medium diluted into 5mL of IM [315] with and without 0.2 mM acetosyringone (AS) and then grown for ~ 5 to 6 h time period to reach a final OD_{600} of 0.5~0.6 (~2×10^9 cells/ml as determined by plate growth count). Different (5×10^6, 10×10^6, 50×10^6 and 100×10^6) cells from early log phase *R. pacifica* INDKK grown in YPD medium were aliquoted, centrifuged and washed twice with IM. Four different ratios of *R. pacifica* INDKK to *A. tumefaciens* cells (1:4, 1:1, 1:40, 1:20) were taken and mixed well in IM. Only *R. pacifica* INDKK without *Agrobacterium* cells

were taken as a negative control. 200 µL volume from each mixed cultures was spread onto nylon membrane laid on IM plate with AS (200 Mm). After incubation at 25 ℃, the cells from the nylon membrane were resuspended in 2 ml sterile MQ and 400 µL was spread onto the YPD selection plate with NAT (200 µg/mL) and cefotaxime (600 µg/mL) and incubated at 30°C for 3 to 5 days.

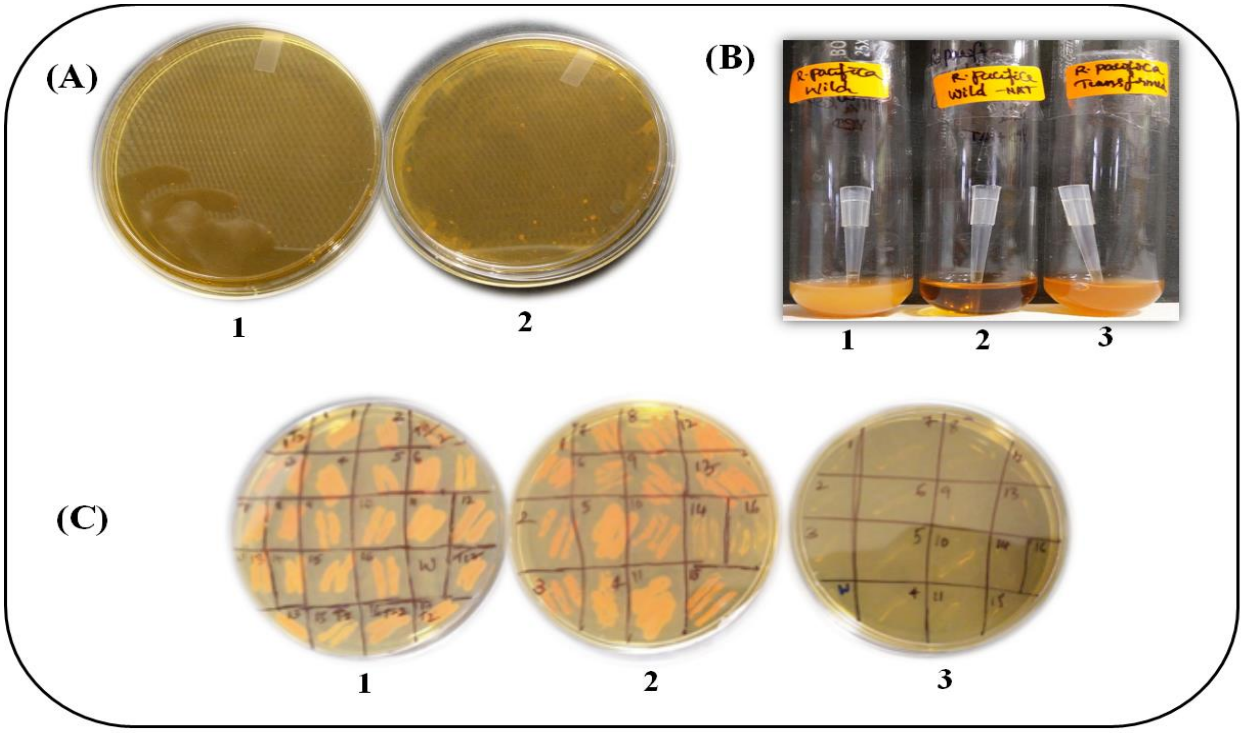


Figure-4.3.4: (A) Yeast selected on YPD plates (200 µg/mL) NAT (200 µg/mL) and Cefotaxime (600 µg/mL)**:** 1. Transformed colonies 2. Negative control. (B) Transformed and wild type colonies grown on YPD liquid medium: 1. Wild type *R. pacifica* INDKK on YPD medium, 2. Wild type *R. pacifica* INDKK on YPD medium with NAT (100 µg/mL), 3. Transformed *R. pacifica* INDKK on YPD medium with NAT (100 µg/mL) + Cefotaxime (300 µg/mL)**.** (C) Transformed colonies streaked on YPD plates with NAT (200 µg/mL) and Cefotaxime (600 µg/mL) – 1. Generation-1, 2. Generation-2, 3. Generation-3.

Then, individual colonies were picked and repeatedly streaked onto new YPD selection plate for further purification of single and stable transformant for 5 generations. It was observed that the transformed colonies could grow until 2 generations and have shown retarded growth or no growth completely after 2 generations. This might be due to generation of unstable

transformants by random integration of T-DNA in the genome of yeast as observed during ATMT transformation [309].

4.3.3 CONCLUSION

Agrobacterium mediated transformation of *R. pacifica* INDKK was attempted and genetically unstable transformants were obtained. Therefore, advanced genetic transformations method, CRISPR CAS9 is being developed for transformation of *R. pacifica* INDKK.

SUMMARY

Depletion of finite fossil fuels and raised concern on global warming due to greenhouse gases, forces to look for substitute energy sources such as lipid-based biofuels [316]. The vegetable oil derived biodiesel competes between food crops and fuel. Therefore, novel routes of sustainable biofuel production employing microbial cell factories on low cost substrates such as like usage of lignocellulosic materials are being highly explored [317]. Though SCOs are produced by several oleaginous microbes including yeast, fungi, microalgae, bacteria but yeast serves as the most promising unicellular microorganism for SCO production [9–11]. They have the capability to assimilate various low cost substrates like agro-industrial wastes [12–14]. Several unconventional yeast genera namely *Rhodosporidium, Lipomyces, Trichosporon, Yarrowia, Rhodotorula, Cryptococcus and Candida* have been employed for lipid production both on synthetic sugars and agro-industrial wastes. Despite many robust oleaginous yeasts were identified by researchers, yet no very few industrially robust strains have been developed so far. Therefore, development of the existing oleaginous yeast to produce the desired SCO titers for economically feasible microbial lipid bio refinery is the highly on demand need of the hour.

The innate maximum SCO production potential of oleaginous yeast can be achieved by making subtle changes in the various media components using RSM. Further, to reach higher scale production titers at desired levels, its metabolic networks are investigated using omics study and followed by genetic engineering. The efficiency of lipid production by yeast depends on optimal levels of media components such as carbon and nitrogen sources , C/N ratio [105], incubation time, temperature and pH [106,107]. Response Surface Methodology (RSM) [122] to achieve multi-factorial medium optimization has been preferred over OFAT [112,123–126]. Due to metabolic differences observed among different strains, omics studies are regarded as indispensable tools which provide useful information regarding genetic

makeup of microbes and facilitate in genetic modification. Yet the genetic modification tools for unconventional oleaginous yeast is not well established. However, several metabolic engineering efforts were made in unconventional oleaginous yeast such as *Rhodotorula*, *Rhodosporidium*, *Lipomyces*, *Trichosporon,* and *Candida* [9,318].. Therefore, to develop a sustainable SCO biorefinery, robust oleaginous yeast which can metabolize low-cost agro-industrial wastes are required.

In this study, search of robust oleaginous yeast, 57 oleaginous yeasts were screened and selected *R. pacifica* INDKK as it has shown promising oleaginous characteristics such as high lipid titers, high resistance to pretreatment generated inhibitors, ability to grow on both C5, C6 sugars and along with lignocellulosic materials. Its lipid titers were further improved to maximum lipid titer of 14.65 g/L by OFTA and RSM. SCOs production level requirements in the context of feasible biodiesel production are based on previous technoeconomic analysis are lipid titer (≥ 70 g/L), lipid yield (≥ 0.2 g/g of sugar, lipid productivity (≥ 5 g/ (l.h)), specific lipid productivity (≥ 0.6 g/g of DCW) [216,319–321]. *R. pacifica* INDKK could achieve near feasible lipid yield (≥ 0.25 g/g of sugar, specific lipid productivity (≥ 0.68 g/g of DCW), with less lipid productivity (≥ 0.077g/ (l.h)) and lipid titer (≥ 14.65 g/L) at shake flask fermentation which can be improved by performing high scale fermentation. Furthermore, in order to understand genetical precursors responsible for lipid production in *R. pacifica* INDKK, genomics and transcriptomics analysis were carried out. Through genomics, genes and reconstructed metabolic pathways related to lipid, central carbon, nitrogen and energy metabolism pathways were identified. While the transcriptomics study has revealed differential gene expressions pertaining to glycolysis, TCA cycle, glyoxylate cycle, pentose phosphate pathway, electro transport chain, proteolysis, autophagy, fatty acid metabolism was identified. With the aid of the clues obtained through omics study and literature, two genes ACC1 and DGA1 were selected for overexpression using

Agrobacterium mediated transformation to further improve the lipid titers. Although, the transformation was achieved, however due to genetic instability, the clones couldn't survive after 2 generations.

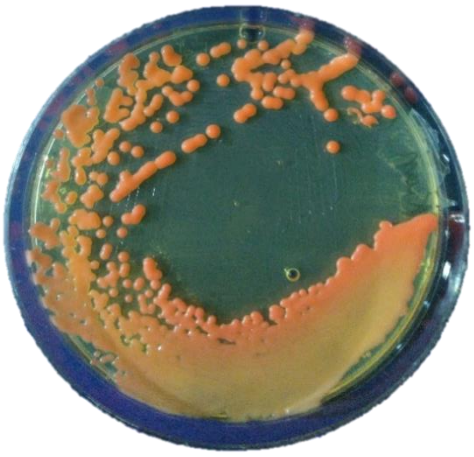
Hence, the molecular insights obtained through this study shows a better direction to develop *Rhodotorula pacifica* INDKK into a suitable host for sustainable SCO production. Hence, our future goal is to perform high scale fermentation to reach feasible SCO titers, study protein - metabolic level changes by proteomics and metabolomics study to understand the lipogenesis. Furthermore, this study would also help to develop genetic engineering strategies in *R. pacifica* INDKK to achieve industrial level SCO titers in future.

APPENDICES

2.2.4 Optimization process for pre-treatment and saccharification of Pongamia shells

Time	Treat-ment	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Total sugars (g/L)	HMF (g/L)	Furfural (g/L)	Acetic acid (g/L)
60 min	0.5% H_2SO_4	1.43	0	1.71	3.14	0	0.056	0.68
	1% H_2SO_4	1.89	1.89	3.82	9.53	0.012	0.056	2.15
	1.5% H_2SO_4	2.17	7	2.23	11.40	0.012	0.037	3.44
	2% H_2SO_4	2.22	21.52	5	28.74	0.041	0.039	6.04
90 min	2% H_2SO_4	8.19	2.06	33.61	5.06	0.07	0.81	8.19
	0.5% NaOH	-	0.01	-	0.01	0.003	-	0.71
	2% NaOH	-	0.7	-	0.7	0.01	-	0.52
Saccharification of 20% biomass (2% NaOH treated) @ 50°C, 150 rpm								
Time	**Enzyme load**	**Glucose (g/L)**	**Xylose (g/L)**	**Arabinose (g/L)**	**Total sugars (g/L)**	**HMF (g/L)**	**Furfural (g/L)**	**Acetic acid (g/L)**
72 h	5 FPU	9.61	7.6	0.09	17.3	-	-	0.02
	10 FPU	14.32	10.17	0.11	24.51	0.003	-	0.05
	15 FPU	21.1	14.18	0.2	35.48	0.01	-	0.04
	20 FPU	28.05	18.13	0.29	46.47	0.013	-	0.046

2.2.1 Screening and molecular identification of the selected yeast isolate



Selected lab isolate: *Rhodotorula pacifica* INDKK

List of primers used

2.1.4: ITS sequencing and phylogenetic analysis

Forward Primer: TCCGTAGGTGAACCTGCG

Reverse Primer: TCCTCCGCTTATTGATATGC

4.1.1.3: Sanger sequencing for genomic DNA purity check

Forward Primer: TCCGTAGGTGAACCTGCGG

Reverse Primer: TCCTCCGCTTATTGATATATGC

4.1.2.2 DNA Illumina library and quality control (QC)

The Barcode sequence "NB06: GACTACTTTCTGCCTTTGCGAGAA" used for DNA library preparation.

Illumina Universal Adapters:

5'-
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA
TCT-3'

Index Adapter:

5'-
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCT
TG3'

4.1.1.4 DNA Illumina library preparation

Index sequences used for DNA Qubit concentration check and quality control

Index 1: D704, "GAGATTCC"

Index 2: D505, "AGGCGAAG"

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PUBLICATIONS

1. **Kumar, K.K**., Deeba, F., Sauraj *et al*. Harnessing pongamia shell hydrolysate for triacylglycerol agglomeration by novel oleaginous yeast *Rhodotorula pacifica* INDKK. *Biotechnol Biofuels***13,** 175 (2020). (Impact factor: 6.48)

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(Impact factor: 5.81)

Book chapters

1. Deeba, F., **Kumar, K.K.** and Gaur, N.A. (2020). Overview of Microbial Production of Omega-3-Polyunsaturated Fatty Acid. In Nutraceutical Fatty Acids from Oleaginous Microalgae (eds A.K. Patel and L. Matsakas).

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Biotechnology for Biofuels

RESEARCH

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Harnessing pongamia shell hydrolysate for triacylglycerol agglomeration by novel oleaginous yeast *Rhodotorula pacifica* INDKK

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Abstract

Background: To meet the present transportation demands and solve food versus fuel issue, microbial lipid-derived biofuels are gaining attention worldwide. This study is focussed on high-throughput screening of oleaginous yeast by microwave-aided Nile red spectrofluorimetry and exploring pongamia shell hydrolysate (PSH) as a feedstock for lipid production using novel oleaginous yeast *Rhodotorula pacifica* INDKK.

Results: A new oleaginous yeast *R. pacifica* INDKK was identified and selected for microbial lipid production. *R. pacifica* INDKK produced maximum 12.8 ± 0.66 g/L of dry cell weight and 6.78 ± 0.4 g/L of lipid titre after 120 h of growth, showed high tolerance to pre-treatment-derived inhibitors such as 5-hydroxymethyl furfural (5-HMF), (2 g/L), furfural (0.5 g/L) and acetic acid (0.5 g/L), and ability to assimilate C3, C5 and C6 sugars. Interestingly, *R. pacifica* INDKK showed higher lipid accumulation when grown in alkali-treated saccharified PSH (AS-PSH) (0.058 ± 0.006 g/L/h) as compared to acid-treated detoxified PSH (AD-PSH) (0.037 ± 0.006 g/L/h) and YNB medium (0.055 ± 0.003 g/L/h). The major fatty acid constituents are oleic, palmitic, linoleic and linolenic acids with an estimated cetane number (CN) of about 56.7, indicating the good quality of fuel.

Conclusion: These results suggested that PSH and *R. pacifica* INDKK could be considered as potential feedstock for sustainable biodiesel production.

Keywords: Pongamia shell hydrolysate, *Rhodotorula pacifica* INDKK, Microwave-aided Nile red screening, Triacylglycerol, Biodiesel

Background

Global population is increasing exponentially and likely to reach ~ 8.6 billion by 2030, raising big concerns about energy [1]. Demand for plant oils has also been shooting up in parallel since they have many industrial applications like epoxy biopolymers [2], drug delivery systems [3], bio-lubricants [4], pharmaceuticals [4] and biodiesel [5]. However, plant oil-derived biodiesel production has raised many questions related to the sustainable use of food crops for cleaner energy production [6]. Therefore, oleaginous microbes having fatty acid profile similar to vegetable oils are considered as suitable alternative for biodiesel [7]. Among oleaginous microbes, micro-algae are being widely used for lipid production, but it requires vast region of land for large-scale cultivation, longer incubation time and specific light exposure [8]. Currently, oleaginous yeasts are of special interest as they can produce high lipid titres in short duration and require limited space [9]. Additionally, yeast has the ability to utilize various renewable carbon sources along with the potential to grow at low pH, which prevents bacterial contamination [10]. Together, these characteristics facilitate the

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development of oleaginous yeast-based technologies for economically attractive industrial process.

Presently *Rhodotorula*, *Rhodosporidium, Lipomyces* and *Trichosporon* are considered as potential yeast for lipid-based biodiesel production. Yeast strains belonging to these genera can accumulate intracellular lipids more than 60% of their dry cell weight (DCW), displayed tolerance to pre-treatment-derived inhibitors along with the ability to assimilate wide range of carbon sources [10]. Consequently, screening and identification of oleaginous yeast isolates from unexplored natural habitats are still relevant [10]. However, the cost of microbial oil production is exorbitant due to the high substrate cost [11]. Therefore, to establish a sustainable microbial lipid production, extra endeavours are requisite such that yeast efficiently utilize renewable and low-cost carbon sources [12]. In recent years, inexpensive lignocellulosic carbon sources like rice straw hydrolysate [13], elephant grass hydrolysate [14], sugarcane bagasse hydrolysate [15], groundnut shell hydrolysate [16], wheat straw [17] and waste office paper hydrolysates [18] have been used for microbial lipid production. But, the conversion of these renewable feedstock like lignocellulosic materials into lipids in a cost-effective manner is a key challenge [19]. The common steps involved in converting lignocellulosic materials to microbial lipids for biodiesel production include: hydrolysis of lignocellulosic materials into fermentable sugars; utilization of released sugars by microbes for lipid production; and biodiesel production from lipids. However, during thermo-chemical pre-treatment such as acid/alkali and steam explosion process, hydrolysates are laden with weak acids, furans and phenolic compounds like 5-hydroxymethylfurfural (5-HMF), furfural and acetic acid which inhibit yeast growth and lipid accumulation. Therefore, for removal of pre-treatment-generated inhibitors, detoxification methods like treatment with activated charcoal and over liming [20] are employed to facilitate efficient fermentation of hydrolysate sugars into lipids. Various studies focus on the utilization of the detoxified hydrolysate for the yeast lipid production. Patel et al. [21] explored lignocellulosic wastes such as *cassia fistula* for biodiesel production using *Rhodosporidium kratochvilovae*. Huang et al. [22] explored the rice straw hydrolysate which was detoxified by activated carbon for lipid production by *Trichosporon fermentans* with lipid titer of 12.1 g/L after 10 days fermentation. Recently, Liu et al. [17] reported lipid production on wheat hydrolysates using different oleaginous yeasts. Moreover, lignocellulosic hydrolysates contain wide range of C5 and C6 sugars including glucose, xylose and arabinose along with the inhibitors. Therefore, yeast isolates utilizing C5 and C6 sugars derived from low-cost feedstock along with the potential to tolerate high concentration of pre-treatment derived inhibitors are much essential for lipid production.

In this regard, *Pongamia pinnata* was explored as source of substrate for microbial lipid production. It is a non-edible oilseed tree, which belongs to Leguminosae family and grows in the semiarid regions such as Asia, Australia and Florida. It was estimated that one hectare of land can produce approximately 6.8 tons of seeds in shell which can generate 1.12 tons of oil, 1.9 tons of meal, 2.67 tons of pod shells and others [23]. The air-dried pongamia shells (PS) consist of 46.02% carbon, 0.23% nitrogen, 42.46% oxygen, 5.58% hydrogen and 5.7% ash [24]. However, the PS are generally discarded or burned after oil extraction from seeds. Recently, pongamia shells have been utilized as fuel briquettes [24], but their utilization as feedstock for biodiesel production remain unexplored. The compositional analysis of widely available waste PS unveils its potential application for lipid production by microbes.

Utilizing low-cost materials required development of more efficient process like employing trans-esterification process to produce a high-quality FAME biodiesel. FAME production process involves some advantages due to low energy utilization, flexibility in feedstock consumption, reduced capital cost and faster reaction by employing accelerated trans-esterification at lower temperature. However, renewable or green diesel produced via hydro-processing of vegetable oils involves costly additional steps of isomerization and cracking at higher temperature and pressure [25]. Interestingly, any alteration in the fatty acid profile influences the biodiesel properties during trans-esterification process. The relative fatty acids composition in oleaginous yeasts was found to be C18:1 > C16:0 > C18:2 = C18:0 that can be altered depending on the feedstock provided and their growth conditions [26]. The biodiesel quality is also affected by the refining process, production process and post-production parameters. Hence, international standards namely European (EN 14214), American Society for Testing and Materials (ASTM D6751) and Indian standards (IS15607-05) have been set up to monitor the parameters and quality of biodiesel. The important parameters for potential biodiesel are cetane number (CN), high heating value (HHV), cold filter plugging point (CFPP, °C), oxidative stability (OS, h), viscosity (mm^2/s), iodine value (IV, $mgI_2/100$ g), density (kg/m^3) and saponification value (SV, mg KOH/g oil). The oil composition and biodiesel properties were evaluated in this study to ensure the biodiesel quality and compared to the ASTM, IS and EN biodiesel standards specifications.

The aim of this study was to explore an inexpensive and renewable raw material pongamia shell hydrolysate (PSH) for yeast lipid production. First, the high

lipid-accumulating oleaginous yeast was screened using microwave-aided Nile red spectrofluorimetry method. Alkaline pre-treatment, acid pre-treatment, enzymatic hydrolysis, detoxification and lipid production by yeast fermentation in PSH were then conducted and optimized. To the best of our knowledge, this is the first report on lipid production from waste PSH using yeast fermentation. This study provides valuable information for researchers on microbial lipid production using PSH as sole carbon source.

Results

Screening and molecular identification of the selected yeast isolate

In this study, we collected a pool of potential oleaginous yeast isolates (57) including strains procured from collection centres in India (NCIM and MTCC) as well as by screening samples from various sites of biomass degradation (Additional file 1: Table S1). Next, molecular identification of new yeast isolate was carried out by PCR amplification of the ITS region (using genomic DNA template) followed by phylogenetic relationship analysis. Top hits of the BLAST analysis showed 99% identity with yeast belonging to *Rhodotorula* species such as *Rhodotorula* sp. SY-96 (Acc. No. AB026006.2 of 1194 bp), *Rhodotorula* sp. LH23 (Acc. No. HQ832796.1 of 643 bp), *Rhodotorula pacifica* (Acc. No. AB193175.1 of 1194 bp) and *Rhodotorula pacifica* AUMC 10761 (Acc. no. KY495729.1 of 614 bp), indicating that selected isolate might belong to *Rhodotorula* species. Phylogenetic relationship with oleaginous yeasts was established by MEGA X software [27]. The evolutionary background of the taxa was determined by the maximum likelihood method [28]. The bootstrap consensus of tree was obtained from 1000 replicates and units of branch length are represented by number of nucleotide substitutions per site. As shown in Fig. 1, four clades were formed; clade-1 belongs to *R. toruloides* strains. Clade-2 includes *Yarrowia lipolytica.* Our lab isolate was grouped with *R. pacifica* strains in clade-3. Indicating evolutionary closeness to *R. pacifica*. Clade-4 comprised strains belonging to *R. mucilaginosa*. The ITS sequence was submitted in NCBI as *R. pacifica* INDKK with GenBank Accession No. MN560184. The evolutionary hierarchy of *Rhodotorula pacifica* INDKK was Eukaryota > Fungi > Dikarya > Basidiomycota > Pucciniomycotina > Microbotryomycetes > Sporidiobolales > Sporidiobolaceae > *Rhodotorula pacifica* INDKK.

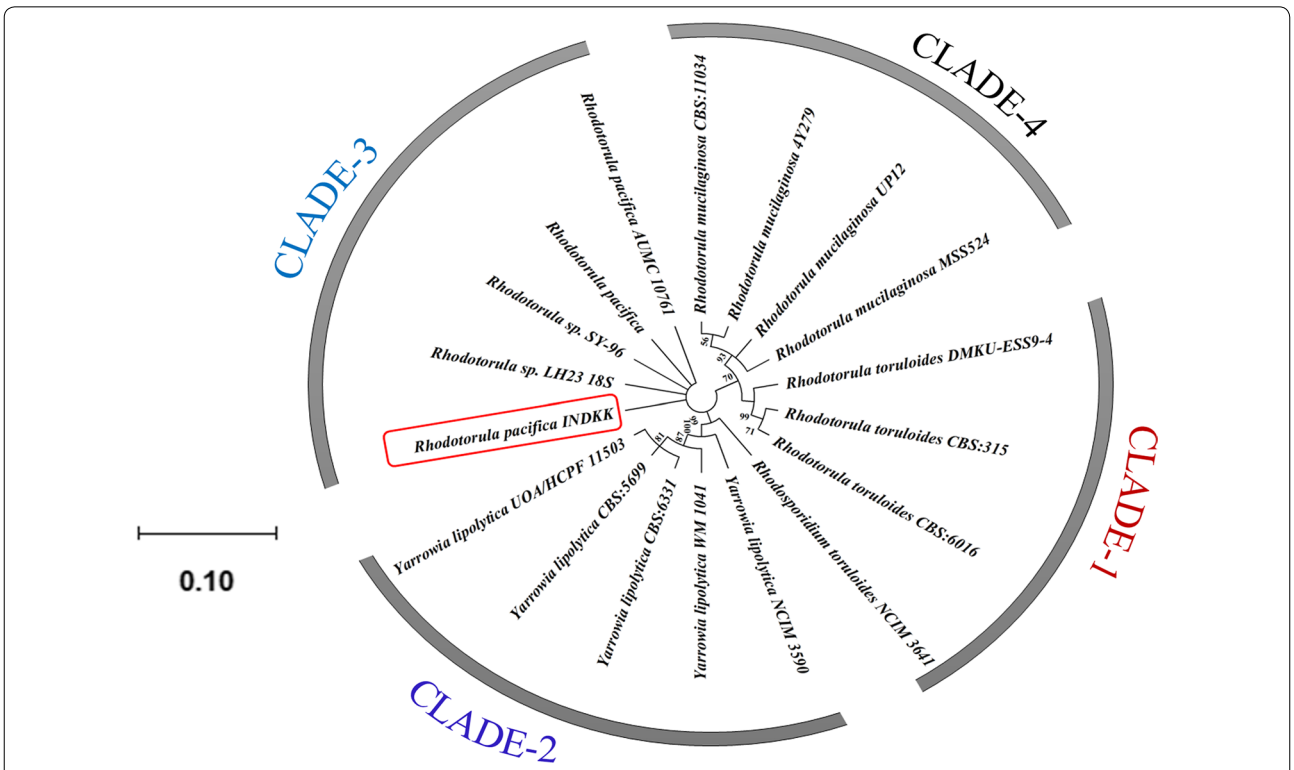

Fig. 1 Phylogenetic tree of *Rhodotorula pacifica* INDKK (Acc. No. MN560184) showing evolutionary history constructed by MEGA X software using the maximum likelihood method. Bootstrap values greater than 50% are represented as numbers above the branches

Selection of high triacylglycerol (TAG)-accumulating yeast isolates

Yeast isolates accumulating more than 20% TAG are considered as good candidates for microbial lipid-based fuel production [29]. For screening high lipid-accumulating strain, cells were grown in nitrogen (N)-limiting yeast nitrogen base (YNB) medium [30]. Initially, conventional Nile red staining-based spectrofluorimetry method [31] was tested with known high and low TAG accumulating yeast strains, *R. toruloides* (NCIM-3641) and *Saccharomyces cerevisiae*, respectively. Surprisingly, no emission was found at ~ 580 nm (corresponding to neutral lipids) and the emission at ~ 620 nm, (corresponding to polar lipids) was not able to distinguish between oleaginous and non-oleaginous yeast [32]. Hence, we optimized microwave-aided Nile red staining for yeast generating emission peak at ~ 580 nm and clearly differentiating the relative fluorescence unit (RFU) values of *R. toruloides* (NCIM-3641) and *S. cerevisiae* (Additional file 2: Figure S1). The 6 strains, *Rhodotorula pacifica* INDKK (lab isolate), *Rhodosporidium toruloides* (NCIM-3641), *Rhodosporidium kratochvilovae* (MTCC-248), *Rhodotorula rubra* (NCIM-3260), *Rhodotorula glutinis* (NCIM-3168), *Rhodosporidium dibovatum* (NCIM-3658) showed higher or comparable RFU value (Fig. 2a) to *Yarrowia lipolytica* (NCIM-3590), a previously reported high lipid-accumulating yeast isolate [33]. The high lipid accumulation in these isolates was further confirmed by lipid droplet (LD) size measurement using confocal microscopy (Additional file 3: Figure S2). DCW (g/L), lipid titre (g/L) and lipid content (%) of these isolates were also determined gravimetrically and *R. pacifica* INDKK showed maximum lipid titre (1.8 g/L) followed by *R. kratochvilovae* (1.6 g/L), *R. toruloides* (1.4 g/L) and *R. rubra* (1.2 g/L) at 72 h as shown in Fig. 2b. Gravimetric lipid analysis of the selected strains showed good correlation with microwave-aided Nile red staining (correlation coefficient $R = 0.94$), suggesting the reliability of microwave-aided Nile red spectrofluorimetry for different yeast species and genera (Fig. 2c). Moreover, quantitative analysis of lipid was performed by TLC. The chromatogram showed highest lipid accumulation by *R. pacifica* INDKK with TAG (67.92%), MAG (12.11%), DAG (10.29%) and FFA (9.67%). Notably, TAG content in *R. pacifica* INDKK was 1.46-fold higher than *Yarrowia lipolytica* (NCIM-3590) (Additional file 4: Figure S3).

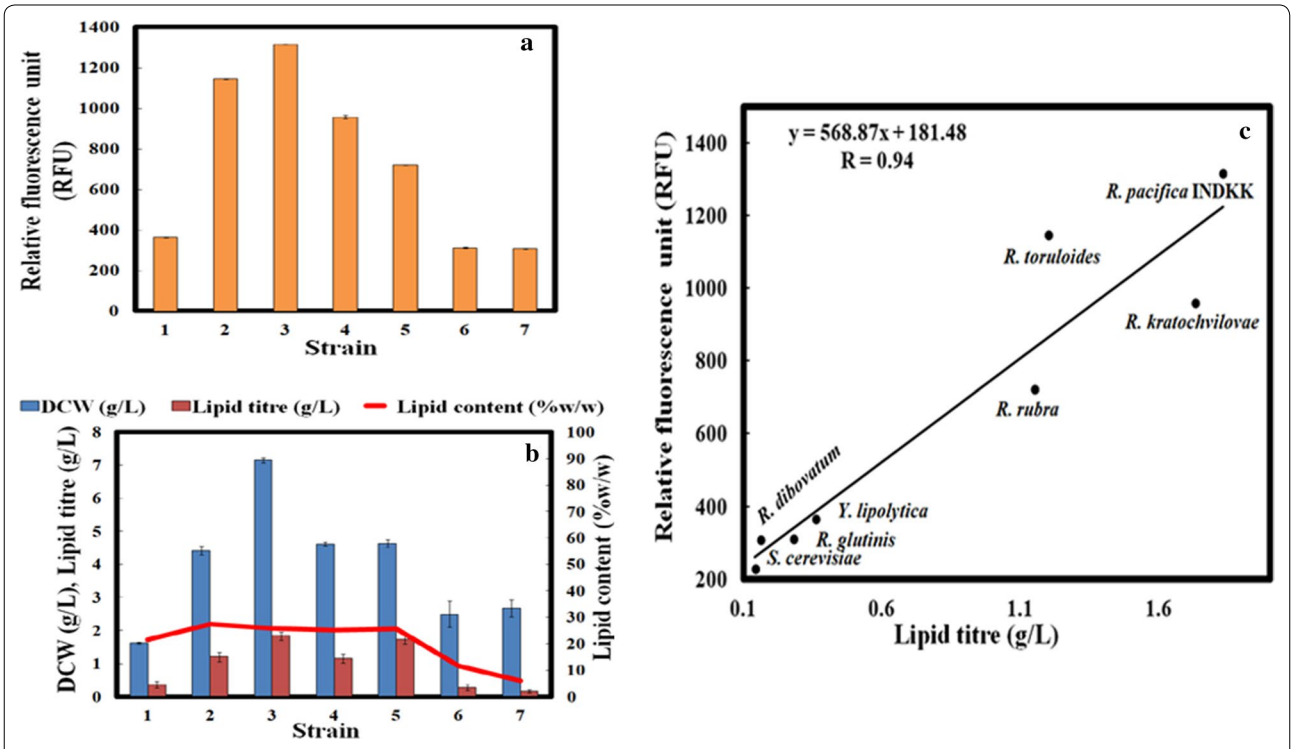

Fig. 2 **a** RFU values of (1) *Y. lipolytica as control,* (2) *R. toruloides* (NCIM-3641), (3) *R. pacifica* INDKK, (4) *R. kratochvilovae* (MTCC-248), (5) *R. rubra* (NCIM-3260), (6) *R. glutinis* (NCIM-3168), (7) *R. dibovatum* (NCIM-3658) from Nile red spectrofluorimetry. **b** Comparison of DCW (g/L), lipid titre (g/L) and lipid content (% w/w) of six selected strains with (1) *Y. lipolytica as control,* (2) *R. toruloides* (NCIM-3641), (3) *R. pacifica* INDKK, (4) *R. kratochvilovae* (MTCC-248), (5) *R. rubra* (NCIM-3260), (6) *R. glutinis* (NCIM-3168), (7) *R. dibovatum* (NCIM-3658) after 72 h (one-way ANOVA test, $P < 0.05$). **c** Correlation plot showing liner correlation between lipid titres (g/L) from gravimetric analysis and RFU values of *S. cerevisiae*, *Y. lipolytica* (NCIM-3590), *R. toruloides* (NCIM-3641), *R. pacifica* INDKK, *R. kratochvilovae* (MTCC-248), *R. rubra* (NCIM-3260), *R. glutinis* (NCIM-3168) and *R. dibovatum* (NCIM-3658)

We further performed kinetic studies with four isolates showing > 20% w/w of lipid content in gravimetric analysis (*R. pacifica* INDKK, *R. toruloides, R. kratochvilovae, R. rubra)*. Kinetics of growth and lipid accumulation among these isolates revealed lowest DCW (6.88 ± 0.22 g/L), lipid titre (3.47 ± 0.05 g/L), lipid productivity (0.024 ± 0.05 g/L/h) and slow glucose utilization rate (0.17 g/L/h) by *R. toruloides* at 120 h (Fig. 3a). However, *R. pacifica* INDKK showed maximum DCW (12.8 ± 0.66 g/L), lipid titre (6.8 ± 0.4 g/L) and lipid productivity (0.056 ± 0.4 g/L/h) with glucose consumption rate of 0.25 g/L/h (Fig. 3b). Although glucose consumption rate (0.025 g/L/h) of *R. kratochvilovae* is similar to *R. pacifica* INDKK, it showed a lower lipid titre (6.32 g/L) (Fig. 3c). *R. rubra* also showed low lipid titre (5.35 g/L) and poor sugar consumption rate (0.02 g/L/h) as compared to *R. pacifica INDKK* (Fig. 3d). Together, among all the tested isolates *R. pacifica* INDKK showed maximum potential for lipid accumulation and was selected for further analysis in this study.

R. pacifica INDKK assimilated wide range of sugars and displayed inhibitor-tolerant phenotypes

Hydrolysates of lignocellulosic biomass contain mixture of C5 and C6 sugars and toxic inhibitors generated during pre-treatment such as furfural, acetic acid and 5-HMF [34]. These inhibitors reduce cell growth as well as lipid yield and productivity [35]. Therefore, yeast isolates capable of assimilating wide range of sugars (C5 and C6) along with enhanced tolerance to pre-treatment inhibitors are very important for economical microbial lipid production [36]. Interestingly, *R. pacifica INDKK* was able to grow on all the tested C6, C5 and C3 sugars except cellobiose and rhamnose (Fig. 4a). Moreover, the presence of lignocellulosic hydrolysate inhibitors such as 5-HMF (2 g/L), furfural (0.5 g/L) and acetic acid (0.5 g/L) in culture media did not show any significant effect on *R. pacifica INDKK* cell growth (Fig. 4b–d).

Microbial lipid production using PS

Pongamia tree bears non-edible fruits whose shells after oil extraction from the seeds are generally discarded or burned [37]. Compositional analysis showed that PS contains 56.8% w/w holocellulose, 12% w/w cellulose and 8%

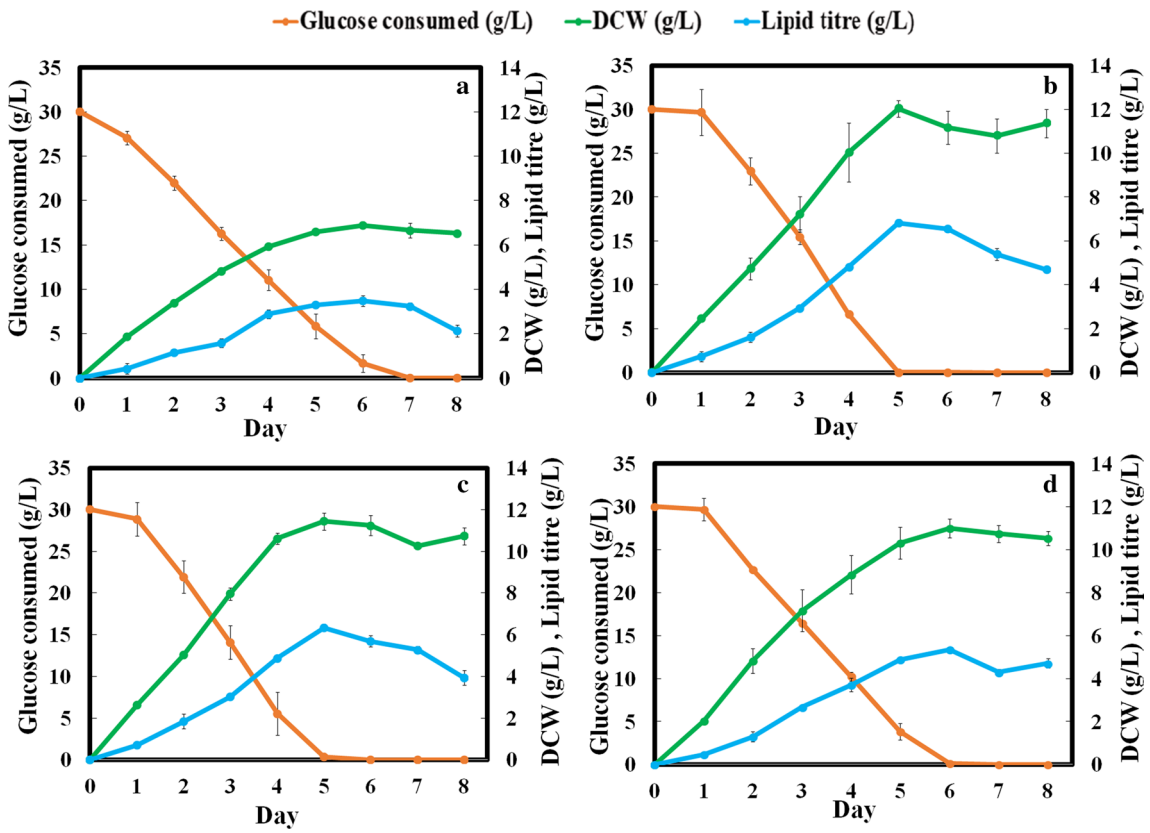

Fig. 3 Time-course study of DCW, lipid titre and glucose consumption by selected oleaginous yeasts in YNB medium. **a** *R. toruloides* (NCIM-3641); **b** lab isolate *R. pacifica* INDKK; **c** *R. kratochvilovae* (MTCC-247); **d** *R. rubra* (NCIM-3560) (one-way ANOVA test, $P < 0.05$)

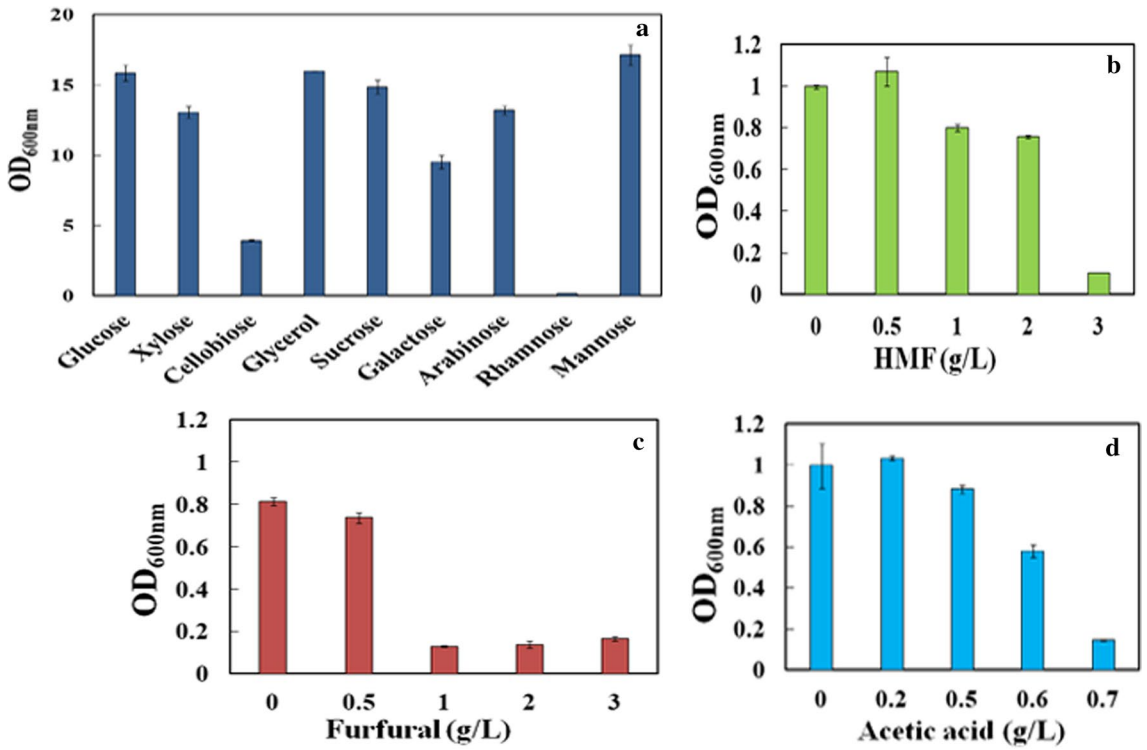

Fig. 4 **a** Growth study of *R. pacifica* INDKK in different C6, C5 and C3 carbon sources and tolerance study in pre-treatment-generated inhibitors; **b** 5-HMF; **c** furfural and **d** acetic acid

w/w of xylan (Table 1). However, PS has not been considered as source of carbon and nitrogen for microbial cell growth thus far. In this regard, we explored hydrolysate of *Pongamia pinnata* shells for growth and lipid production by our newly isolated yeast isolate *R. pacifica INDKK*.

PS were subjected to acid as well as alkali pre-treatments (Additional file 5: Table S2). Liquid fraction of acid treatment showed higher sugar concentration (37.38 g/L) while alkali-treated liquid fraction obtained negligible amount of fermentable sugars (0.7 g/L total sugars). Therefore, liquid fraction of acid treatment was detoxified by activated charcoal, which reduced acetic acid concentration from 5.61 ± 0.035 g/L to 0.11 ± 0.005 g/L, completely removed furfural and 5-HMF with slight reduction in sugar concentration (14.63%). The acid-treated and detoxified liquid fraction of PSH (AD-PSH) contains 31.91 ± 0.042 g/L of total sugars (0.45 g/L glucose, 29.01 g/L xylose and 2.45 g/L arabinose). Time course study revealed that *R. pacifica* INDKK consumed all the sugars after 120 h of growth and produced 10.63 ± 0.004 g/L of DCW with 4.48 ± 0.02 g/L of lipid titre and 0.037 ± 0.001 g/L/h of lipid productivity (Fig. 5. Panel-1 5a).

The solid fraction of both acid-treated and alkali-treated PS were subjected to enzymatic hydrolysis (as

Table 1 The pongamia shell hydrolysate (PSH) nutrient composition before and after consumption by *Rhodotorula pacifica* INDKK

Component	PS (dry)	Before cultivation	After cultivation
Cellulose (%)	12	–	–
Holocellulose (%)	56.8	–	–
Xylan (%)	8	–	–
Glucose (g/L)	–	28.05 ± 0.01	–
Xylose (g/L)	–	18.13 ± 0.04	0.05 ± 0.08
Arabinose (g/L)	–	0.29 ± 0.01	–
Total proteins (g/L)	–	0.33 ± 0.02	0.23 ± 0.005
Total nitrogen (g/L)	1.90	0.053 ± 0.01	0.037 ± 0.02
Calcium (mg/L)	–	107 ± 0.87	6.3 ± 2.28
Sodium (g/L)	–	1.08 ± 0.15	0.84 ± 0.28
Magnesium (g/L)	–	1.53 ± 0.003	0.22 ± 0.06
Phosphorous (g/L)	2.05	1.51 ± 0.001	0.36 ± 0.12
Potassium (g/L)	6.32	1.53 ± 0.005	0.38 ± 0.12
Manganese (mg/L)	–	0.37 ± 0.05	0.24 ± 0.07
Iron (mg/L)	–	0.067 ± 0.009	0.036 ± 0.021
Sulphur (g/L)	0.276	–	–
5-HMF (g/L)	–	0.013 ± 0.005	0.01 ± 0.01
Acetic acid (g/L)	–	0.046 ± 0.01	–

– not detected.

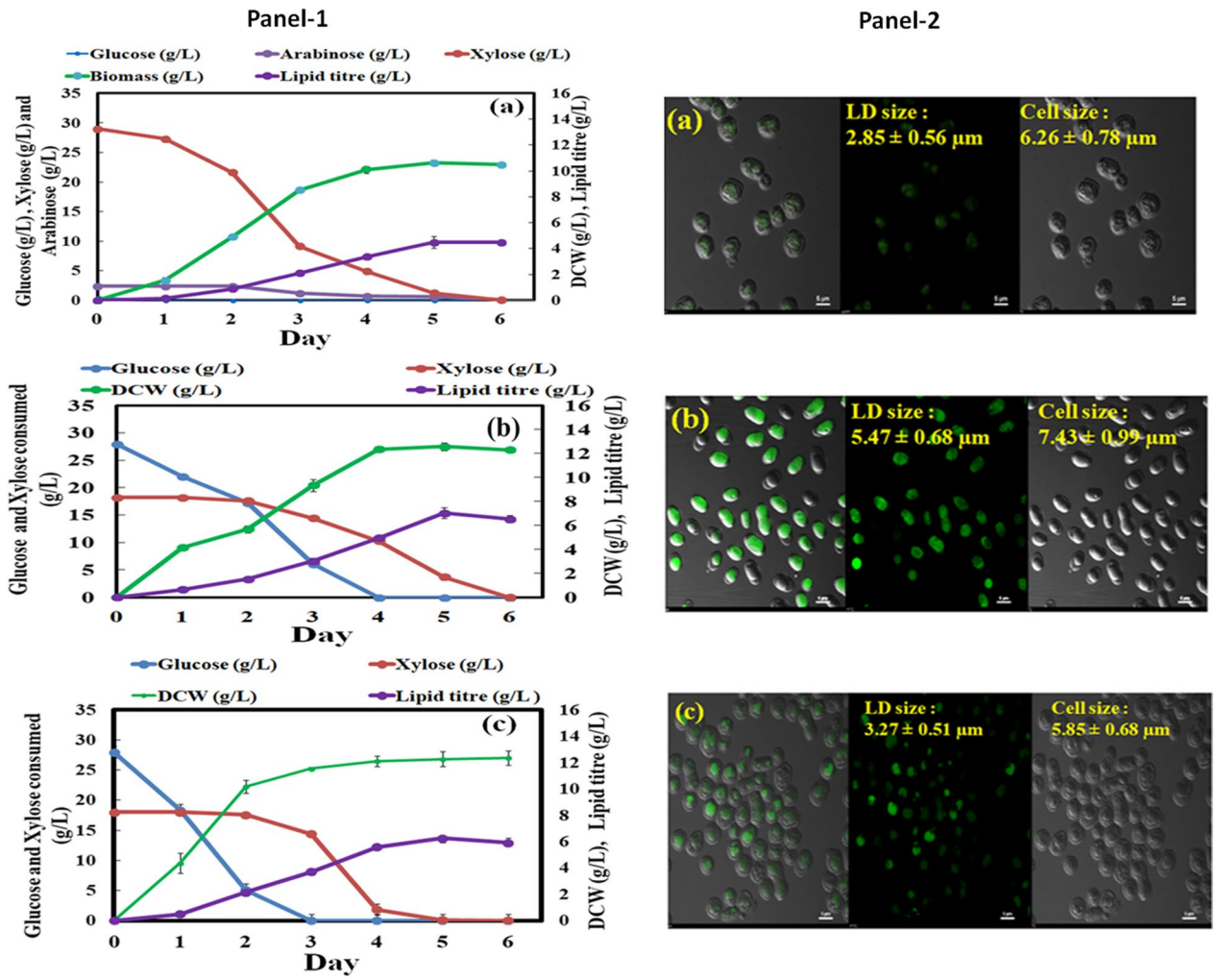

Fig. 5 Panel: 1 Time-course study of DCW, lipid titre and sugar consumption by *R. pacifica* INDKK in **a** AD-PSH **b** AS-PSH and **c** control (YNB) (one-way ANOVA test, $P < 0.05$). Panel: 2 Confocal microscopy of *R. pacifica* INDKK cells cultivated in **a** AD-PSH, **b** AS-PSH, and **c** control (YNB) after 120 h showing average LD size and cell size at 100×

described in "Methods"). The enzymatic saccharification of solid fraction from acid-treated PS yielded very less fermentable sugars (7.63 g/L) along with inhibitors such as acetic acid (0.3 g/L) and furfural (0.19 g/L) while enzymatic saccharification of solid fraction of alkali-treated PS resulted in high amount of total sugars (46.47 g/L), wherein glucose was the most abundant (28.05 ± 0.01 g/L) followed by xylose (18.13 ± 0.04 g/L) and arabinose (0.29 ± 0.01 g/L). As expected very low amount of inhibitors (5-HMF 0.013 ± 0.005 g/L, furfural 0 g/L and acetic acid 0.046 ± 0.01 g/L) was detected. Interestingly, trace elements such as Mn (0.37 ± 0.05 mg/L), K (1.53 ± 0.005 g/L), Ca (107 ± 0.87 mg/L), Fe (0.67 ± 0.009 mg/L), Na (1.08 ± 0.15 g/L) and Mg (1.53 ± 0.003 g/L) were also found in alkaline-treated saccharified PSH (AS-PSH), which could be helpful in cell growth and lipid accumulation. There was a drastic reduction (17-fold) in calcium ions which might be due to increase in calcium influx into the cell to cope up with the physiological stress conditions [38, 39]. Time course analysis of *R. pacifica* INDKK in AS-PSH showed that all the sugars (C6 and C5) were consumed after 120 h of growth and maximum 12.6 ± 0.5 g/L of DCW, 7.02 ± 0.7 g/L of lipid titre, 0.104 ± 0.004 g/L/h of biomass productivity and 0.058 ± 0.006 g/L/h of lipid productivity were achieved (Fig. 5, Panel-1 5b). Remarkably, the DCW and lipid productivities were 1.02 and 1.12-fold higher in AS-PSH as compared to YNB (0.101 ± 0.005 g/L/h of biomass productivity and 0.052 ± 0.003 g/L/h of lipid productivity) (Fig. 5, Panel-1 5c). Moreover, after 120 h

of growth 30.0% reduction in protein content and 94.11, 22.22, 85.62, 73.71, 75.16 and 35.13% utilization of trace elements corresponding to Ca, Na, Mg, P, K and Mn were also observed. The lipid accumulation in PSH batch-cultivated cells was also confirmed by confocal microscopy. The average cell size (7.43 ± 0.99 µm) and average LD size (5.47 ± 0.68 µm) was 1.67 and 1.27-fold higher in AS-PSH cultivated cells as compared to YNB medium, respectively (Fig. 5, Panel-2). However, cell size and LD size was 1.18 and 1.56-fold higher in AS-PSH grown cells as compared to AD-PSH (6.26 ± 0.78 and 3.49 ± 0.56 µm), respectively. Together, batch cultivation in AS-PSH significantly showed more biomass and lipid productivity as compared to AD-PSH (P value < 0.05).

Fatty acid profile and analysis of biodiesel properties

The fatty acid profile of lipids extracted from *R. pacifica* INDKK cultivated in AS-PSH and YNB were analysed by gas chromatography–mass spectroscopy (GC–MS) after transesterification (as described in "Methods"). The 91.77% of FAME yield was achieved after transesterification with stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3) and palmitic (16:0) fatty acids, which is desirable for biodiesel production [40].

The lipids extracted from AS-PSH-grown *R. pacifica* INDKK showed higher amount of C18:1 (52.58%) followed by C16:0 (28.85%), C18:2 (12.45%), C18:3 (1.37%), C14:0 (0.89%), C22:0 (0.46%) and C15:0 (0.1%) fatty acids as depicted in Table 2. The fatty acid profile of *R. pacifica* INDKK grown in AD-PSH also showed higher C18:1 (61.98%) followed by C18:2 (16.86%), C16:0 (14.45%), C18:0 (6.13%) and C18:3 (1.37%). Interestingly, C18:2, C18:3 and C22:0 fatty acids were not detected in YNB-grown *R. pacifica* INDKK. Moreover, saturated fatty acid (SFA, 30.19%) and monounsaturated fatty acid (MUFA, 52.58%) were higher in AS-PSH-grown cells as compared to YNB (SFA 10.95% and MUFA 43.61%). The CN in AS-PSH and YNB were 57.29 and 86.86, respectively. The CN of *R. pacifica* INDKK grown in AS-PSH is higher as compared to AD-PSH (CN 53.92). The result showed that IV of AS-PSH (73.58 g I_2/100 g) and YNB (39.02 g I_2/100 g) grown cells were in accordance with the standards of EN 14214. Compared to rape seed oil and jatropha oil, *R. pacifica* INDKK oil in AS-PSH-grown cells contains more MUFA, SFA and less polyunsaturated fatty acid (PUFA), conferring greater CN. Other estimated biodiesel properties such as kinematic viscosity (KV, 3.7 mm^2/s) and density (0.84 g/cm^3) were comparable to rape seed oil, jatropha oil and standard values specified by EN 14214, ASTM D6751 and IS 15607 (Table 2). The above speculated biodiesel property values obtained from AS-PSH grown *R. pacifica* INDKK satisfies the specifications precisely by EN-14214, ASTM-D6751 and IS-15607 standards, suggesting it as an ideal biodiesel feedstock.

Table 2 Comparative FAME profile and biodiesel properties of *R. pacifica* INDKK cultivated in PSH and YNB

Fatty acid/biodiesel properties	Rape seed oil methyl ester [30]	Jatropha oil methyl ester [30]	AD-PSH	AS-PSH	Control (YNB)	EN 14214	ASTM D6751	IS 15607
C14:0 (%)	ND	ND	ND	0.89	0.55	–	–	–
C16:0 (%)	11.9	14.9	14.45	28.85	10.4	–	–	–
C16:1 (%)	ND	1	ND	ND	ND	–	–	–
C18:0 (%)	4.1	6.1	6.13	ND	ND	–	–	–
C18:1 (%)	20.8	40.4	61.98	52.58	43.61	–	–	–
C18:2 (%)	53.8	36.2	16.86	12.45	ND	–	–	–
C18:3 (%)	9.3	0.3	ND	1.37	ND	–	–	–
C20:0 (%)	ND	ND	ND	ND	ND	–	–	–
C22:0 (%)	ND	ND	ND	0.46	ND	–	–	–
C24:0 (%)	ND	ND	ND	ND	ND	–	–	–
Iodine value (IV) (g of I_2/100 g)	107.76	98.02	86.29	73.58	39.02	120 (max.)	–	–
Cetane number (CN)	54.35	55.23	53.92	56.72	86.86	47	51	51
High heating value (HHV) (MJ/kg)	40.78	40.55	39.42	38.14	21.56	–	–	–
Density (g/cm^3)	0.80	0.88	0.87	0.84	0.47	–	0.86–0.90	0.86–0.89
Kinematic viscosity (KV) (mm^2/s)	4.4	4.48	3.90	3.7	2.13	1.9–6	3.5–5	2.5–6
Cold filter plugging point (CFPP)	ND	ND	−6.1	−5.97	−13.2	–	–	–

– No standard limit designated by biodiesel standards ASTM D6751, EN 14214 and IS 15607; *ND* not detected.

Table 3 Biodiesel production from *R. pacifica* INDKK using PS as substrate

Pongamia shell powder used (%)	*R. pacifica* INDKK			
	DCW produced (g /L)	Lipids produced (g/L)	Biodiesel produced (g/L)	Transesterification efficiency (%)
20	12.8	6.8	6.24	91.77

It was estimated that 6.8 g microbial lipid was obtained from 200 g of dry PS (20% w/v), from which 6.24 g of biodiesel was produced (Table 3) in this study.

Discussion

Biodiesel-derived from lignocellulosic materials is often challenging and costly because of additional material processing steps such as biomass pre-treatment to release sugars and removal of pre-treatment-generated inhibitors in the hydrolysates that hinder fermentation. Therefore, use of potential oleaginous yeast that could simultaneously utilize mixed carbon sources and show tolerance to inhibitors will reduce the major obstacles of biodiesel production. Over the past few years many inhibitor-tolerant oleaginous yeast have been found, but their lipid production performances are still substandard. Hence, the quest for robust oleaginous yeast is still relevant. Yeast isolates isolated from sites of biomass degradation have shown great potential for TAG accumulation [41]. To untap the potential of oleaginous yeasts isolated from natural habitats related to lignocellulosic biodiesel production, 57 yeast isolates were screened.

High-throughput microwave-aided Nile red staining was found to be quick, effective and easy method for screening high TAG accumulating oleaginous yeasts as compared to other traditional methods for lipid estimation. This method clearly differentiates the RFU values between oleaginous and non-oleaginous yeast. Among 57 yeasts, 6 strains belonging to *Rhodotorula* and *Rhodosporidium* species showed higher RFU values. Further, gravimetric analysis data showed that four strains (*R. pacifica* INDKK, *R. toruloides, R. kratochvilovae, R. rubra*) displayed > 20% lipid content. Interestingly, when kinetic analysis was performed, our isolate *R. pacifica* INDKK displayed high lipid productivity with effective sugar utilization rate, which could be by stimulation of genes related to growth and lipid production [42]. Inhibitors present in lignocellulosic hydrolysates (acetic acid, 5-HMF, furfural, etc.) inhibit yeast growth. Acetic acid inhibits growth by repressing the expression of genes involved in nutrient transporters such as glucose transporters (HXT1 and HXT3) [43, 44]. 5-HMF inhibits dehydrogenases and glycolysis, whereas furfural reduces growth by inhibiting the key enzymes of carbon metabolism, increased production of radical oxygen species which damage DNA, protein and membranous structures [45].

Interestingly, increased growth was observed in 0.2 g/L concentration and displayed reduction of growth at 0.6 g/L of acetic acid. The increase in growth at 0.2 g/L could be due to the utilization of acetic acid as carbon source at this concentration. The yeast cells were also tolerant to 5-HMF (2 g/L) and furfural (0.5 g/L), beyond this concentration significant decrease in growth was observed. According to previously reported studies, most *Rhodosporidium* species could not tolerate furfural at 0.5 g/L concentration while *Rhodotorula glutinis* showed growth inhibition by 5-HMF > 0.5 g/L [36]. Therefore, in this study enhanced tolerance to inhibitors was observed by *R. pacifica INDKK*. It also showed ability to utilize all the tested C6, C5 and C3 sugars effectively. However, no significant growth was observed on rhamnose and cellobiose in comparison to glucose ($P < 0.05$). *R. pacifica INDKK* showed similar carbon source utilization profile as reported earlier in *Rhodosporidium mucilaginosa* [36]. The data elucidate that *R. pacifica INDKK* was tolerant to pre-treatment-generated inhibitors with potential to utilize different carbon sources present in lignocellulosic hydrolysates. The results were consistent with previously reported literature, wherein yeast such as *Trichosporon fermentans* and *Cryptococcus curvatus* were reported with similar potential but with low lipid productivity [6, 33].

Lignocellulosic lipid production is a multistep process where feedstock collection and valorization itself accounts for 70–80% biodiesel production cost. Therefore, we exploited abundantly available lignocellulosic waste PS as feedstock to reduce the major obstacle of biodiesel production using oleaginous yeast. Notably, when isolate *R. pacifica* INDKK was tested with AD-PSH and AS-PSH as carbon sources, we achieved lipid titre of 4.48 and 7.02 g/L at 30 °C in 120 h. The higher biomass and lipid was observed with AS-PSH, which could be due to preferable utilization of glucose as carbon source as compared to xylose. Also, AS-PSH contains more nutrients which supports the growth of *R. pacifica* INDKK. The gravimetric data were in co-relation to confocal microscopy study ($P < 0.05$). The lipid productivity of *R. pacifica* INDKK in AS-PSH (0.058 g/L/h) was higher than the previously reported oleaginous yeasts on different lignocellulosic hydrolysates such as 0.041 g/L/h on waste office paper enzymatic hydrolysate [18], 0.02 g/L/h on saccharified sweet sorghum juice [46, 47], and 0.029 g/L/h on corn stover enzymatic hydrolysate [48] as shown in Table 4. To the best of our knowledge, no yeast isolate reported has produced equivalent lipid titre to isolate *R.*

Table 4 Comparative study of *R. pacifica* INDKK with different oleaginous yeasts grown in lignocellulosic biomass

Strain	Substrate	Biomass (g/L)	Lipid titre (g/L)	Lipid content (%)	Lipid productivity (g/L/h)	References
Trichosporon fermentans	Saccharified sweet sorghum	15.51	1.8	11.6	0.020	[46]
Cryptococcus curvatus	Acid treated and saccharified waste office paper hydrolysate	11.48	4.95	43.11	0.041	[18]
Cryptococcus podzolicus SCTCC30292	Ammonium carbonate-steam explosion pre-treated and saccharified corn stover	10.56	5.03	47.6	0.029	[48]
Cryptococcus curvatus	Lime treated and saccharified sorghum bagasse hydrolysate	6	2.6	43.3	0.021	[53]
Rhodotorula pacifica INDKK	YNB	12.26	6.61	54.24	0.055	This study
Rhodotorula pacifica INDKK	Acid treated and detoxified PSH	10.63	4.48	42.14	0.037	This study
Rhodotorula pacifica INDKK	Alkali treated and saccharified PSH	12.56	7.02	55.89	0.058	This study

pacifica INDKK on AS-PSH with effective glucose utilization rate.

Hoekman et al. [49] reported that differences in degree of unsaturation and carbon chain length influence the properties and performance of biodiesel. For better OS, biodiesel should have high amount of SFA and MUFA, but low amount of multi-unsaturated FAME. While biodiesel should have low amount of long-chain SFA for good low-temperature performance [49], the fatty acid composition of *R. pacifica* INDKK was rich in C16:0 and C18:1 depicting improved biodiesel properties such as CN and OS [50, 51]. The biodiesel obtained must meet the fuel standards (EN 14214, ASTM D6751, and IS) specifications before using it as a pure fuel. The CN parameter of diesel engine determines the auto-ignition quality of the fuel [49]. The high MUFA content elucidates balance between CFPP and OS for better quality of biodiesel [52]. The biodiesel properties obtained were in range of the biodiesel standard specifications illustrating the vehicular quality. The KV and density were similar to rape seed oil and jatropha oil. Results indicated that high CN of biodiesel from *R. pacifica* INDKK cultivated on AS-PSH significantly affects engine performance and start of injection with improved ignition characteristic. Longer OS and low CFPP properties of biodiesel obtained lead to longer shelf life and good engine performance, respectively. Hence, biodiesel production from *R. pacifica* INDKK cultivated on PSH was environmentally friendly and cost-efficient. Therefore, this process could be considered as an important step for the development of a cost-effective biodiesel production process.

Conclusions

In this study, 57 yeast isolates were screened for lipid accumulation by microwave-aided Nile red spectrofluorimetry. Among them, novel oleaginous yeast isolate *R. pacifica* INDKK (Gen Bank accession No: MN560184) showed highest lipid accumulation, ability to grow on wide range of carbon sources and also displayed pre-treatment inhibitor-tolerant growth phenotypes. *R. pacifica* INDKK showed maximum DCW (12.8 ± 0.66 g/L), lipid titre (6.8 ± 0.4 g/L) and lipid productivity (0.056 ± 0.4 g/L/h) with glucose consumption rate of 0.25 g/L/h in YNB, among all the tested strains. *R. pacifica* INDKK can utilize wide range of C5 and C6 sugars and showed pre-treatment inhibitor-tolerant phenotypes. *R. pacifica* INDKK produced more DCW (12.56 ± 0.5 g/L), lipid titre (7.02 ± 0.4 g/L) and lipid productivity (0.058 ± 0.006 g/L) in AS-PSH as compared to AD-PSH (DCW, 10.63 ± 0.37 g/L; lipid titre, 4.48 ± 0.78 g/L; lipid productivity, 0.037 ± 0.006 g/L/h) and YNB (DCW, 12.26 ± 0.68 g/L; lipid titre, 6.65 ± 0.35 g/L; lipid productivity, 0.055 ± 0.003 g/L/h) grown cells. C18:1 (52.58%), C16:0 (28.85%) and C18:2 (12.45%) were major fatty acids detected in AS-PSH-grown cells, attributing better quality of the fuel. The present study demonstrates the lipogenic potential of *R. pacifica* INDKK for the production of biodiesel using PS as feasible feedstock.

Methods and materials

Media and other chemicals

PS were collected from International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi campus. All analytical reagents and solvents (chloroform, methanol, n-hexane, diethyl ether, glacial acetic acid, H_2SO_4) were of high-performance liquid chromatography (HPLC) grade. Nile red (9-diethylamino-5-benzo[α] phenoxazinone), Bodipy 493–503 nm, heptadecanoic acid (internal standard) and FAME external standard (Supelco 37 component FAME mix) for GC–MS analysis were procured from Sigma (USA). Standard for

thin-layer chromatography (TLC) (Triolein), sugars (glucose, xylose, arabinose) were procured from Hi-Media Laboratories (Mumbai, India). YNB, yeast extract and peptone were purchased from Difco (USA).

Screening and identification of oleaginous yeast

Yeast strains

Soil sample was collected from the Coringa mangrove forest, Kakinada, Andhra Pradesh (16.83139°N–82.33667°E), India. 10^{-1} to 10^{-6} serial dilutions were made in sterilized (0.9%) saline and seeded onto YPD agar plates (peptone, 2%; glucose, 2%; yeast extract, 1%; agar, 2%) supplemented with chloramphenicol (35 µg/mL) and neomycin (50 µg/mL). Plates were incubated at 30 °C for 72 h and colonies with yeast-like morphology were further streaked on YPD agar plates for pure single cell culture. Yeast strains were also procured from National Collection of Industrial Microorganisms (NCIM), Pune, and Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh.

Microwave-aided Nile red staining and screening of oleaginous yeast

Yeast isolates were screened for lipid accumulation by using microwave-assisted Nile red staining protocol [54] with modifications. Briefly, single colony culture of each strain was grown overnight in YPD medium at 30 °C and 200 rpm (pre-culture). The pre-culture was centrifuged and washed twice with Milli-Q (MQ) water, re-suspended in 100 mL YNB medium with glucose (3%) and $(NH_4)_2SO_4$ (0.5%) to optical density (OD) of 0.2 at 600 nm and incubated at 30 °C for 3 days at 200 rpm. Cells corresponding to OD 1 of the above grown cultures were centrifuged (5000 × *g*, 4 min) and re-suspended in 50 µL of dimethyl sulphoxide (DMSO) followed by microwave treatment (1250 watts power for 60 s). Cells were mixed with Nile red solution (10 µg/mL) and again subjected to microwave treatment (1250 watts power, 60 s). Four replicates of each treatment were prepared and relative fluorescence intensity (RFU) was measured at exciting and emission wavelengths of 475 and ~ 580 nm, respectively. Relative neutral lipid content was represented as RFU of LD [55].

ITS sequencing and phylogenetic analysis

The 18S rDNA sequence was PCR amplified from the genomic DNA by using ITS1-F (TCCGTAGGTGAACCTGCG) and ITS4-R (TCCTCCGCTTATTGATATGC) universal primers in a thermocycler (Eppendorf, Nexus GSX1, Germany) [56]. The reaction was carried out in 50 µL containing 1.0 µL of genomic DNA, 2.5 µL of forward primer, 2.5 µL of reverse primer, 1.0 µL of deoxyribonucleotide triphosphate (dNTP) mix, 5.0 µL of PCR Taq buffer, 0.5 µL of Taq DNA polymerase (G-Biosciences, USA) and 37.5 µL of sterile water. The PCR conditions: initial denaturation (95 °C for 5 min), 30 cycles of denaturation (95 °C for 30 s), annealing (52 °C for 30 s) and extension (72 °C for 1.0 min), and a final extension (72 °C for 5.0 min). The PCR products were cleaned up using Gene JET PCR Purification Kit (Thermo scientific, Lithuania) and sequenced (Invitrogen BioServices, India). The PCR-amplified DNA fragments were sequenced and used for BLAST (Basic Local Alignment Search Tool) analysis in NCBI (National Centre for Biotechnology Information) database and were aligned by ClustalW (a multiple sequence alignment tool). Phylogenetic tree was constructed by MEGA (molecular evolutionary genetics analysis) X software using sequences with shared similarity.

Cell growth in presence of different carbon sources and pre-treatment inhibitors

To know the carbon source utilization efficiency by *R. pacifica* INDKS, the experiment was conducted on individual carbon sources. For this, cells were grown in 100 mL YNB medium supplemented with 2% carbon source individually (sucrose, cellobiose, glucose, mannose, galactose, rhamnose, arabinose, xylose and glycerol) and incubated at 30 °C, 200 rpm for 3 days. The inhibitor tolerance profile was tested by growing cells in YNB with 2% glucose supplemented with varying concentrations of 5-HMF (0.5 to 3 g/L), furfural (0.5 to 3 g/L) and acetic acid (0.2 to 0.7 g/L). YNB without inhibitors was used as control medium for cell growth.

Biomass and lipid production on PSH

PS were washed thoroughly with water, dried in oven (60 °C for 48 h) and crushed in grinder. ~ 20 g of dried powder was subjected to dilute acid (2% v/v) as well as alkaline (2% v/v) treatments in autoclave at 121 °C for 90 min. The liquid fraction of acid treatment was detoxified by activated charcoal (15% w/v) at 30 °C for 3 h. Solid fraction of both acid and alkali treatments were neutralized and subjected to enzymatic saccharification using 20 FPU of cellulases/g (Sigma, USA) of biomass at 50 °C, 150 rpm for 72 h [57]. The hydrolysate was filtered and used as cultivation medium after supplementation of micro-nutrients such as NH_4SO_4 (0.5 g/L), $MgSO_4$ (1.5 g/L), KH_2PO_4 (1.5 g/L). In parallel, YNB supplemented with 46.5 g/L of sugars (glucose 28 g/L, xylose 18.18 g/L, and arabinose 0.3 g/L) was used as control for cell growth and lipid accumulation. Lipid production was carried out in Erlenmeyer flasks (250 mL) containing 100 mL medium by adding overnight grown cells of OD 0.2 and incubating at 30 °C, 200 rpm for 196 h with initial pH of 6.8. Cells were harvested by centrifugation, washed

with MQ water, lyophilized (Labconco, USA) and DCW (g/L) was determined. Lipids were extracted from lyophilized cells by using modified Bligh and Dyer method [58], lipid titre (g/L) was measured gravimetrically. Biomass productivity (g/L/h), lipid productivity (g/L/h) and lipid contents (%) were calculated as described previously [52].

Lipid analysis

Lipid analysis was performed by confocal microscopy (Nikon, India) after staining the cells with Bodipy dye (0.5 µg/mL DMSO) [21]. Cell sizes and LD sizes were measured by using Nikon software. TAG analysis was performed on TLC plates (Merck, India) with triolein as standard in hexane: diethyl ether: acetic acid (85:15:1, v/v/v) solvent system. TLC plates were immersed in methanolic-$MnCl_2$ solution, dried and heated at 120 °C (20 min) [59]. The TAG estimations were performed by using Image-J software.

Analytical methods

Sugar (glucose, xylose, arabinose) and inhibitor (5-HMF, furfural and acetic acid) concentrations in PSH were determined by using HPLC (Agilent 1260 Series) equipped with Aminex HPX-87H column (Bio-Rad, USA) and refractive index (RI) detector. The mobile phase H_2SO_4 (4 mM) at a flow rate of 0.3 mL/min at column temperature of 40 °C and the sugar as well as inhibitor were quantified by dividing the peak area of the sample with the peak area of standard (1.0 g/L) at specific retention time [28]. Trace elements were determined by inductively coupled plasma-induced ion chromatography–mass spectroscopy (ICP-MS) analysis (Agilent 7900) using argon as carrier gas and sample flow rate was 2.0 mL/min with approximately 2.5 min total analysis time per sample. The samples were acidified with nitric acid to pH below 2.0 and filtered through a 0.45-µm pore diameter membrane filter. The calibration curves were prepared by diluting ICP multi-element standard solution, including the blank [52]. The protein concentration was estimated by Bradford method [60]. Holocellulose, cellulose and xylan content were determined by using standard Association of Official Analytical Chemists (AOAC) methods of analysis [61]. The dried PS powder was also subjected to energy dispersive X-ray (EDX) elemental analysis.

Transesterification and GC–MS analysis

Transesterification was performed by previously described method [62] with some modifications. Briefly, lyophilized yeast cells and 6% methanolic-H_2SO_4 in 1:20 ratio were taken in teflon-sealed tube and heated at 80 °C for 1 h. FAMEs were extracted into hexane phase and analysed by GC–MS (7890A series) equipped with Omegawax (30 m × 0.25 mm ID, 0.25 µm thickness) and Agilent 7000 QQQ MS [63]. Identification and quantification of FAMEs were performed by NIST (National Institute of Standards and Technology) mass spectral database, AMDIS (Automated Mass Spectral Deconvolution and Identification System) and mass hunter software. Physical properties of biodiesel were computed by using previously reported experimental equations [49] and collated with rape seed oil methyl ester, jatropha oil methyl ester and to EN 14214, ASTM D6751 and Indian standards IS156907 [64].

Statistical analysis

All experiments were performed in minimum three replicates. Average values with standard deviation were mentioned. One-way ANOVA test with post hoc analysis by Tukey's test was performed using Microsoft Office Excel 2013 (Microsoft, USA) to analyse statistical significance of the results. Statistical differences at $P \leq 0.05$ were considered as significant.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13068-020-01814-9.

Additional file 1: Table S1. List of yeast strains screened in this study by microwave aided Nile red spectrofluorimetry and their corresponding Relative fluorescence intensity (RFU) values.

Additional file 2: Figure S1. Comparison between conventional and Microwave aided Nile red spectrofluorimetry.

Additional file 3: Figure S2. Confocal microscopy of selected 6 strains and *Y. lipolytica* (control) showing cell size and lipid droplet size stained with Bodipy.

Additional file 4: Figure S3. TLC And densitometric analysis of lipid extracts cultivated in YNB medium from selected 6 strains.

Additional file 5: Table S2. Optimisation of Pre-treatment and saccharification process for pongamia shell hydrolysate (PSH) preparation.

Abbreviations

PS: Pongamia shells; PSH: Pongamia shell hydrolysate; AS-PSH: Alkali-treated saccharified pongamia shell hydrolysate; AD-PSH: Acid-treated and detoxified pongamia shell hydrolysate; TAG: Triacylglycerol; LD: Lipid droplet; NCBI: National Centre for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; FAME: Fatty acid methyl esters; 5-HMF: 5-Hydroxyl methyl furfural; TLC: Thin-layer chromatography; NIST: National Institute of Standards and Technology; ASTM D6751: American Society for Testing and Materials;

IS156907: Indian standards; EN 14214: European; AMDIS: Automated Mass Spectral Deconvolution and Identification System; YNB: Yeast nitrogen base; YPD: Yeast extract peptone dextrose; RFU: Relative fluorescence unit; HPLC: High-performance liquid chromatography; ICP-MS: Inductively coupled plasma-induced ion chromatography–mass spectroscopy; GC–MS: Gas chromatography–mass spectroscopy; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; SFA: Saturated fatty acid; OS: Oxidative stability; CFPP: Cold filter plugging point; CN: Cetane number; HHV: High heating value; KV: Kinematic viscosity; IV: Iodine value.

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Authors' contributions
KKK and FD designed and performed all the experiments of this research work. NAG participated in experimental design, scientific suggestions and data analysis. S and YSN performed parts of the experiments and data processing. KKK, FD and NAG participated in data interpretation and manuscript writing. All authors have reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this manuscript and its additional files.

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Full Length Article

High density cultivation of oleaginous yeast isolates in 'mandi' waste for enhanced lipid production using sugarcane molasses as feed

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ABSTRACT

This study focuses on the use of synthetic and low cost carbon sources for enhanced biomass and lipid production from oleaginous yeast strains using batch and fed batch fermentation. Five strains were selected out of 20 strains which were all isolated from various habitats, on the basis of lipid accumulating potential using Sudan B black dye. The selected isolates were characterized and their lipid production was optimized by batch cultivation in waste extract. Gas chromatography analysis revealed that lipids from all isolated yeast strains contained mainly long-chain fatty acids such as oleic acid, palmitic acid, stearic acid and linoleic acid. On the basis of lipid profile and production, two most suitable oleaginous yeast strains were selected for further experiments, i.e. fed batch fermentation using glucose (control) and sugarcane molasses as feed. Using glucose as feed, 38.8 ± 0.9 g/L and 29.4 ± 0.2 g/L biomass dry cell weight (DCW) and total lipids were produced respectively, whereas with sugarcane molasses feed, 50 ± 1 g/L (DCW) biomass and 39.2 ± 0.6 g/L lipids were produced by *Rhodosporidium toruloides* ATCC204091. After transesterification the lipid profile was analyzed by GC–MS revealing suitability for use as a potential feedstock for biodiesel production.

1. Introduction

Oleaginous microorganisms produce lipids above 20%-50% of their dry cell weight. Wide ranges of fatty acids are produced from eukaryotic microorganisms; mostly triacylglycerides also known as TAGs [1,2]. A number of single-celled microorganisms, such as bacteria, yeast, microalgae and fungi that have potential of producing oils or lipids also called Single cell oil (SCO), under specific conditions. These single cell microbial oils can decrease the future demand of fossil fuels along with producing high lipid content [3]. TAGs are the most valuable form of lipids which are in recent demand for production of biodiesel worldwide. Chemically the lipids are basically composed of triglyceride molecules with three long chain fatty acids in which ester is bonded to a single glycerol molecule [4]. These fatty acids differ with the length of carbon chains, position, orientation and number of double bonds chains. Therefore, biodiesel is lower alkyl esters of long chain fatty acids produced or synthesized by fatty acid esterification or by transesterification with lower alcohol (methanol or ethanol) using acid or base as catalyst [5]. Furthermore, most oleaginous microorganisms' metabolize different carbon sources, demonstrating the potential to produce TAGs from lignocellulosic biomass and other cost effective sources [6-8].

Oleaginous yeast strains, such as *Rhodosporidium* and *Rhodotorula* store up to ~ 70% lipid with respect to their dry cell weight. Triacylglycerol's (TAGs) containing long-chain fatty acids are the major form of fatty acids found in these lipids which are also comparable to conventional vegetable oils used for biodiesel production [9]. The growth of oleaginous yeast for the production of lipid is mainly dependent on carbon-to-nitrogen (C/N) ratio of the culture medium with other factors like oxygen, pH, temperature etc. [10]. Huge amount of lipids are accumulated by oleaginous microorganisms when cultivation is carried out in excess sugar with nitrogen limited media [11,12].

However, the cost and production of microbial oils are high as compared to vegetable oils at present. To improve the quality and cost for production of microbial oils, inexpensive locally available media should replace synthetic fermentation media. Here we utilized a cost effective and carbon rich media i.e., 'mandi' waste extract [13] and sugarcane molasses as supplement, for production of lipid via batch and fed batch fermentation of yeast. Sugarcane molasses sugar contains up to 50% sucrose, and other nutrients including organic acids, vitamins

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CORRECTION

Correction to: Isolation and identification of carotenoid-producing yeast and evaluation of antimalarial activity of the extracted carotenoid(s) against *P. falciparum*

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There were two omissions in the initial online publication: author Shailja Singh was not listed as a corresponding author, and a reference citation was missing in the caption to Fig. 1b. The original article has been corrected.

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Article

Metabolomic Profiling Revealed Diversion of Cytidinediphosphate-Diacylglycerol and Glycerol Pathway towards Denovo Triacylglycerol Synthesis in *Rhodosporidium toruloides*

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Abstract: Oleaginous yeast *Rhodosporidium toruloides* has great biotechnological potential and scientific interest, yet the molecular rationale of its cellular behavior to carbon and nitrogen ratios with concurrent lipid agglomeration remains elusive. Here, metabolomics adaptations of the *R. toruloides* in response to varying glucose and nitrogen concentrations have been investigated. In preliminary screening we found that 5% glucose (*w*/*v*) was optimal for further analysis in *Rhodosporidium toruloides* 3641. Hereafter, the effect of complementation to increase lipid agglomeration was evaluated with different nitrogen sources and their concentration. The results obtained illustrated that the biomass (13 g/L) and lipid (9.1 g/L) production were maximum on 5% (*w*/*v*) glucose and 0.12% $(NH_4)_2SO_4$. Furthermore, to shed lights on lipid accumulation induced by nitrogen-limitation, we performed metabolomic analysis of the oleaginous yeast *R. toruloides* 3641. Significant changes were observed in metabolite concentrations by qualitative metabolomics through gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), which were mapped onto the governing metabolic pathways. Notable finding in this strain concerns glycerol and CDP-DAG metabolism wherein reduced production of glycerol and phospholipids induced a bypass leading to enhanced de-novo triacylglyceride synthesis. Collectively, our findings help in understanding the central carbon metabolism of *R. toruloides* which may assist in developing rationale metabolic models and engineering efforts in this organism.

Keywords: *Rhodosporidium toruloides*; triacylglycerol; metabolomics; biofuel

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1. Introduction

The red yeast *Rhodosporidium toruloides* has been recognized as a promising microbial cell factory for the production of functional lipids, oleo-chemicals, and biofuels [1,2]. Triacylglycerols (TAG) is the major form of neutral lipids (up to 70% of dry cell weight) accumulated in this yeast. This basidiomycetous fungus is a potential host for metabolic engineering to produce terpenes and fatty acids [3]. Interestingly, *R. toruloides* can metabolize challenging substrates including waste glycerol, biomass hydrolysates, xylose, and can naturally co-produce valuable compounds such as carotenoids and other useful enzymes relevant to the pharmaceutical and chemical industries [2–4].

The fundamental requisite for yeast-based biodiesel production meeting the criteria for sustainable biofuels is the optimization of conditions that up-regulate higher lipid productivity in fast-growing oleaginous yeast. A better understanding of such phenomena leads to more accurate bioengineering of industrially viable strains. A variety of biochemical approaches have been implemented in this direction to improve biomass and lipid synthesis. Indeed, different oleaginous yeasts were investigated for high cellular lipid

5

Overview of Microbial Production of Omega-3-Polyunsaturated Fatty Acid

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Abstract

Omega-3 polyunsaturated fatty acids (ω-3 PUFA) are significant in terms of health benefits which led to their higher consumption as dietary supplements. The accelerating demand of ω-3 PUFA has put an additional pressure on global fish stocks. Therefore, alternative and renewable source of ω-3 PUFA production particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been considered to meet the present demand. Many microorganisms rich in DHA and EPA present a promising source of ω-3 PUFAs. This chapter gives an overview of microbial production platforms for ω-3 fatty acids development. These microbes have been used as bio-factories for ω-3 PUFA commercially in recent years. Current biotechnological and potential metabolic engineering approach for enhanced amount of ω-3 PUFA in microorganisms has also been discussed.

Keywords: Microorganisms, omega-3 polyunsaturated fatty acids, anaerobic polyketide synthase pathway, aerobic desaturase and elongase pathway

5.1 Introduction

Omega-3 polyunsaturated fatty acids (ω-3 PUFA) are extensively distributed in nature and are essential constituents of the membrane that surround each cell in the human body [1]. The three major ω-3 PUFAs are docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and α-linolenic acid (ALA) [2]. They are the significant components required for

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10 Saccharide to Biodiesel

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CONTENTS

10.1 INTRODUCTION

Rapid population growth, unbalanced food supply and diminishing fossil fuel resources have caused the world energy threats (Patel et al., 2020). Biodiesel is found to be a sustainable alternative for fossil diesel and emits low level of greenhouse gases (Mahlia et al., 2020). The low CO_2 emission levels deprived of sulphur and other harmful elements are the key factors that make it green and environment-friendly (Hill et al., 2006).

Biodiesel production and consumption has increased by 14% from 2016 to 2020 globally, driven by biofuel policies in the USA, Argentina, Brazil, Indonesia and EU by Food and Agriculture Organization (FAO) and Organisation for Economic Co-operation and Development (OECD). This accounts for an increase in production from 33.2 billion litres in 2016 to 37.9 billion litres in 2020. Waste-derived biodiesel was expected to grow to 4.4 billion litres, which is 53% rise in production (*OECD/FAO | S&P Global Platts*, 2020). In one study, it was claimed that vegetable oil cost accounts for up to 77% of total expenses for biodiesel production at a smaller scale (Skarlis et al., 2018). It is also clear that as operating costs get higher, there is a decline in entrepreneur

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