3
Isolation Identification and Characterization of *Cystobasidium oligophagum* JRC1: A Cellulase and Lipase Producing Oleaginous Yeast

Continuous increase in the population and demands for the transportation fuel has urged the scientists across the globe to find alternative fuel resources, of which the biomass-derived fuels holds the special place [Sitepu *et al*., 2014a]. Yeasts like *Y. lipolytia, L. starkeyi, R. glutinis*, etc., which can accumulate cellular lipids more than 20 percent of their cell dry weight (CDW) (or biomass production) are known as oleaginous yeasts [Ratledge, 2004]. Oleaginous yeasts have been extensively studied for biodiesel production in the past few years. Yeasts primarily accumulate lipids in the form of triacylglycerol (TAG), comprising of saturated and unsaturated chains of fatty acids.

Various oleaginous yeasts of the genera like *Rhodotorula*, *Cryptococcus*, *Yarrowia*, *Lipomyces*, *Rhodosporidium*, are recently reported to accumulate higher amount of lipids on the pretreated lignocellulosic substrates, low-cost agro-industrial waste (paper mill, crude glycerol), and other renewable feedstocks such as animal fat, municipal solid waste, etc. [Leiva-Candia *et al*., 2014]. An enzymatic pre-treatment of cellulosic biomass to release utilizable hexose and pentose sugars usually precedes the oil production step by yeasts. Fewer reports discuss the lipid accumulation directly on the cellulosic substrate. Consolidated Bioprocessing (CBP) for the accumulation of lipids from cellulosic materials without the use of additional cellulase enzymes will be very advantageous [Lynd *et al*., 2005]. There are many fungi as well as bacteria, which are reported to be the excellent cellulase producers; limited studies are reported, however, for cellulolytic yeasts. Cellulose utilization involves a multi-enzyme complex consisting of three major enzymes (i) endo-1, 4 β-D-glucanase (or CMCase) (ii) cellobiohydrolase (or exoglucanase) and (iii) β-glucosidase. Certain genera of the yeast such as *Rhodotorula*, *Cryptococcus, Candida, Sporobolomyces,* and *Aureobasidium* are reported to be the producer of different types of cellulases [Baldrian and Vendula, 2008; Strauss *et al*., 2001].

So far there is only one report on the oleaginous yeast accumulating lipids using carboxymethylcellulose (CMC) as a carbon source [Kanti and Sudiana, 2015] to the best of our knowledge. However, a systematic and quantitative estimation of cellulolytic activity, along with lipid accumulation, is missing in the majority of these studies. In addition to the use of the cellulosic substrate, we wanted the same yeast to produce lipase, a potential transesterification catalyst. Lipase holds a special interest in next-generation biodiesel synthesis. Studies are underway to design efficient lipases and utilize them for transesterification instead of conventional catalysts to produce biodiesel [Aguieiras *et al*., 2015].

A successful attempt to isolate oleaginous cellulolytic yeast with both intracellular and extracellular lipase activity was made. The isolation of the yeast was followed by its identification, characterization and lipid profiling. Isolation of such an organism opens up the possibility of intracellular FAEE (fatty acid ethyl ester) synthesis directly by a few more experimental interventions. This study thus opens up the new prospect of the utilization of

cellulosic or industrial wastes directly for the production of biodiesel with desirable fuel properties.

3.1 ISOLATION AND SCREENING OF THE OLEAGINOUS YEAST

The soil samples rich in cellulosic wastes such as decaying leaves, vegetable wastes, etc. were collected from the five different regions around the local area, Jodhpur (co-ordinates: 26^0 16′ 18.2′′ N, 73^0 01′ 59.2′′ E). Samples were collected in the sterile tubes and stored at 4 °C until further use. One gram of the homogenized sample was aseptically suspended in 100 mL of the sterilized saline solution (0.85% w/v NaCl). 1 mL of the dilution obtained was transferred into 100 mL of YPD medium in a flask (g/L): yeast extract, 10; peptone, 20; glucose, 10; and incubated at 30°C and 120 rpm (pH 6.0). Ampicillin (100 µg/mL) was added to avert bacterial contamination.

Around 139 yeast colonies were isolated on YPD agar supplemented with ampicillin. The isolates obtained were further plated on the lipid production medium (g/L): Glucose, 40; KH_2PO_4, 7; NaH_2PO_4, 2; $(NH_4)_2SO_4$, 2; $MgSO_4.7H_2O$, 1.5; yeast extract, 1; agar powder, 20. Vitamins (mg/L): D-biotin, 0.002; inositol, 2; niacin, 0.4; Para-aminobenzoic acid (PABA), 0.2; thiamine, 0.4; pyridoxine HCl, 0.4; calcium pantothenate, 0.4; folic acid, 0.002; riboflavin, 0.2;, at30 °C (pH 5.5). The yeast colonies growing on lipid production medium were further screened for lipid accumulation using the nile red fluorescence microscopy protocol, as described in the subsequent sections. The yeast colonies which were showing significant lipid droplets inside the cells were further grown in the production medium, and the lipid yields were determined. The oleaginous yeasts obtained from the above step were cultured in the medium (g/L): CMC, 10; yeast extract, 0.6; KH_2PO_4, 7; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 1 and agar 20 (pH 6.0) [Goldbeck *et al.,* 2012], and incubated at 30 °C. The colonies which showed growth were replica plated. Extracellular cellulase enzyme activity was confirmed using a 0.2% congo red dye method [Teather and Wood, 1982].

The yeast cultures which were found to be oleaginous, and expressing cellulolytic activity, were further tested for lipase activity. The lipase activity was identified by the zone of clearance using tributyrin as a substrate along with glucose. The selected yeast cultures were spreaded on petri-plates containing (g/L): glucose, 10; tributyrin, 10; yeast extract, 10; peptone, 20; agar, 20, at pH 6.0 [Sztajer *et al.,* 1988] and were incubated at 30 °C. The zone of clearance around the colony was observed to confirm the lipase activity. The selected yeast was tested for its enzyme activity and lipid accumulation with the course of time. The yeast was further subjected to identification and studying its potential to assimilate different carbon sources and lipid accumulation. The lipid extract and biodiesel properties for the selected yeasts were studied using various analytical tools (Figure 3.1).

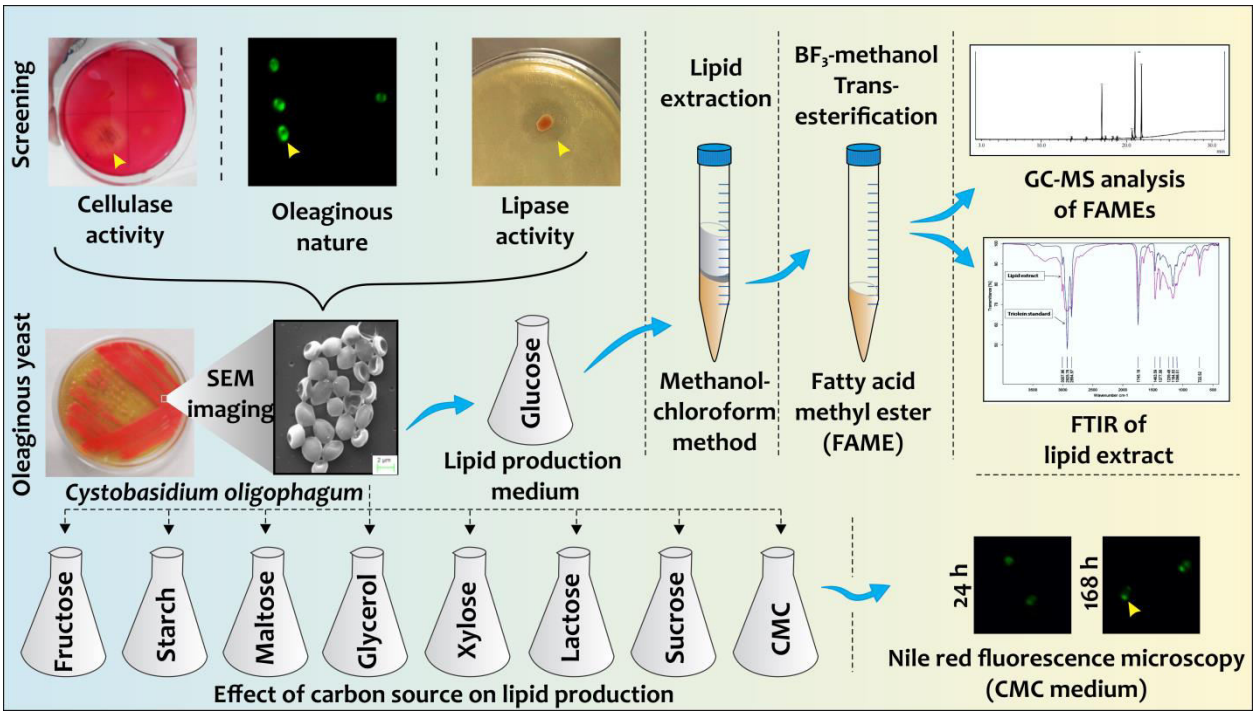

Figure 3.1: Schematic of the isolation and characterization studies of *Cystobasidium oligophagum* JRC1

3.2 RESULTS AND DISCUSSION:

3.2.1 Isolation and screening of oleaginous yeast

23 of the 139 were found to be oleaginous yeast. The oleaginous yeasts showed the accumulation of lipid in the form of intracellular droplets. Among the 23 yeast colonies, 9 yeast colonies were showing a high amount of lipid accumulation by fluorescence microscopy. Lipid outputs were determined for these 9 colonies and it was found that 4 out of 9 yeasts accumulated higher lipid content (42, 39, 38 and 32% respectively). Previously, many strategies have been reported to isolate oleaginous yeasts from natural environments. In one of the studies, 79 yeasts out of 479 microbial colonies isolated from the Himalaya region, were identified to be oleaginous [Patel *et al.*, 2014]. Generally, 3–10% of the randomly selected yeast population is found to be oleaginous, which was also the case in our study [Sitepu *et al.*, 2014a].

3.2.2 Screening of oleaginous yeasts for cellulase and lipase activity

The selected oleaginous yeast colonies were further subjected to congo red screening on a CMC medium. Out of four selected cultures, two yeasts were found to produce a zone of clearance, which indicated that the yeasts were expressing cellulolytic activity. Further, the selected yeasts were subjected to qualitative analysis for lipase. One of the two yeast isolates showed a zone of clearance on a tributyrin agar plate. At the end of the screening, successful isolation of one yeast strain was done. Isolation of oleaginous cellulolytic yeast with both intracellular and extracellular lipase activity has been attempted for the first time in literature.

3.2.3 Identification and characterization of the isolate

Only one of the yeast isolates fulfilled the screening criteria. The identification of the yeast strain was performed by Polymerase chain reaction (PCR) amplification of the partial 18s rRNA gene and sequencing of it. The isolate was identified to be closely related to *C. oligophagum* {formerly called *Rhodotorula oligophaga* with a sequence id: AB702968.1 of 1750 bp [Satoh *et al.*, 2013]}. The sequence was submitted to Genbank and accession No. KX668182 was assigned for the partial sequence of the 18s rRNA gene (843 bp). A recent report explained

that *R. oligophaga* and other few types of yeast were proposed to be transferred to the *Cystobasidium* genus from the *R. minuta* clade, and it was renamed as *C. oligophagum* [Yurkov *et al.,* 2015]. Phylogenetic tree for the partial 18s rRNA gene sequence of *C. oligophagum* JRC1 and other related strains was constructed by MEGA 6 software (sum branch length = 0.08313443) (Figure 3.2). Morphological characterization showed that cells are ellipsoidal (4–5 µm × 3–4 µm), occurring as a single cell or parental bud pairs. After 3–4 days of incubation at 28 °C, the culture turns slightly pink to dark pink in color (pigment production). No hyphae or pseudohyphae were noticed even in older cultures. It could assimilate glucose, sucrose, fructose, maltose, lactose, D-xylose, glycerol, and CMC. It grew optimally in the temperature range of 25–30 °C, growing slowly at 31–33 °C, and no growth was observed beyond 33 °C. The lineage of the identified yeast strain was; fungi - Dikarya - Basidiomycota – Pucciniomycotina - Cystobasidiomycetes - Cystobasidiales - Cystobasidiacea - *C. oligophagum* JRC1 (Basionym: *R. oligophaga*). The scanning electron microscopy was performed for morphological studies (Figure 3.3).

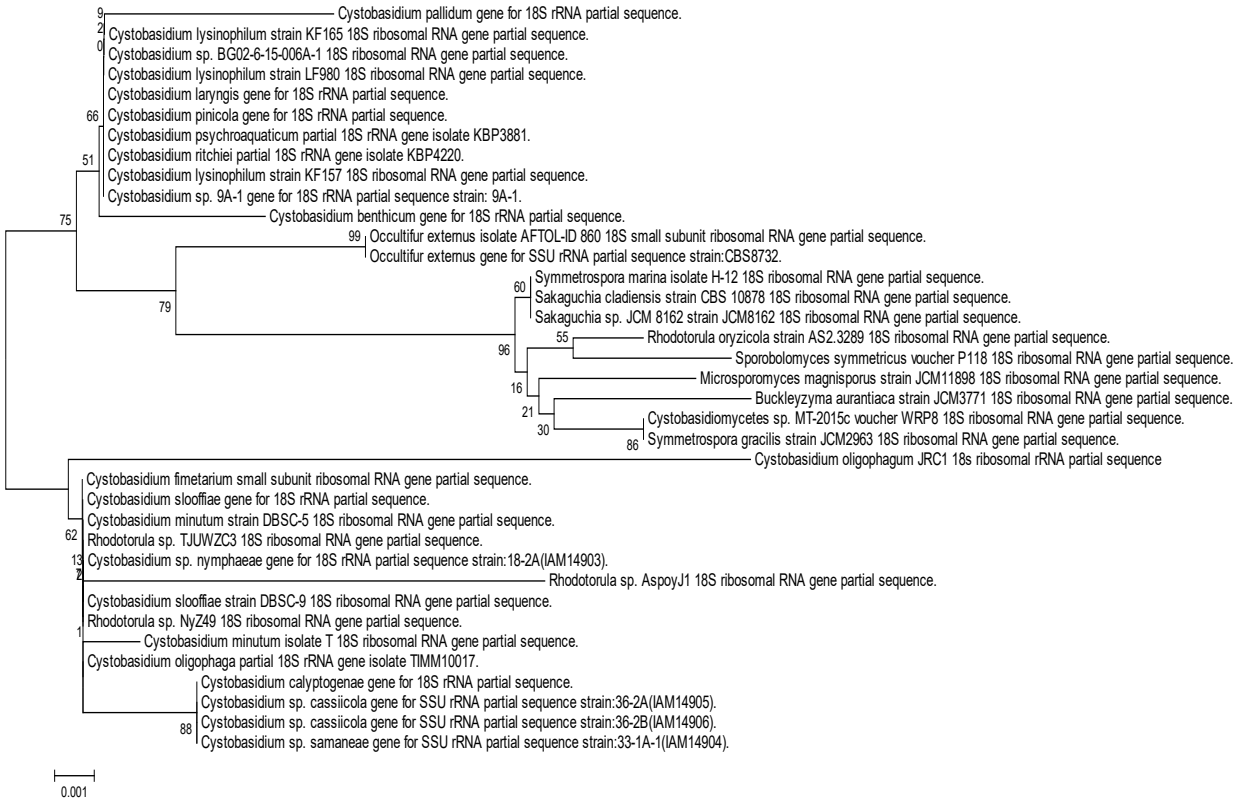

Figure 3.2: The phylogenetic tree of *C. oligophagum* JRC1**:** Constructed by the neighbour-joining method (MEGA 6.0 software) (sum branch length= 0.08313443)**.**

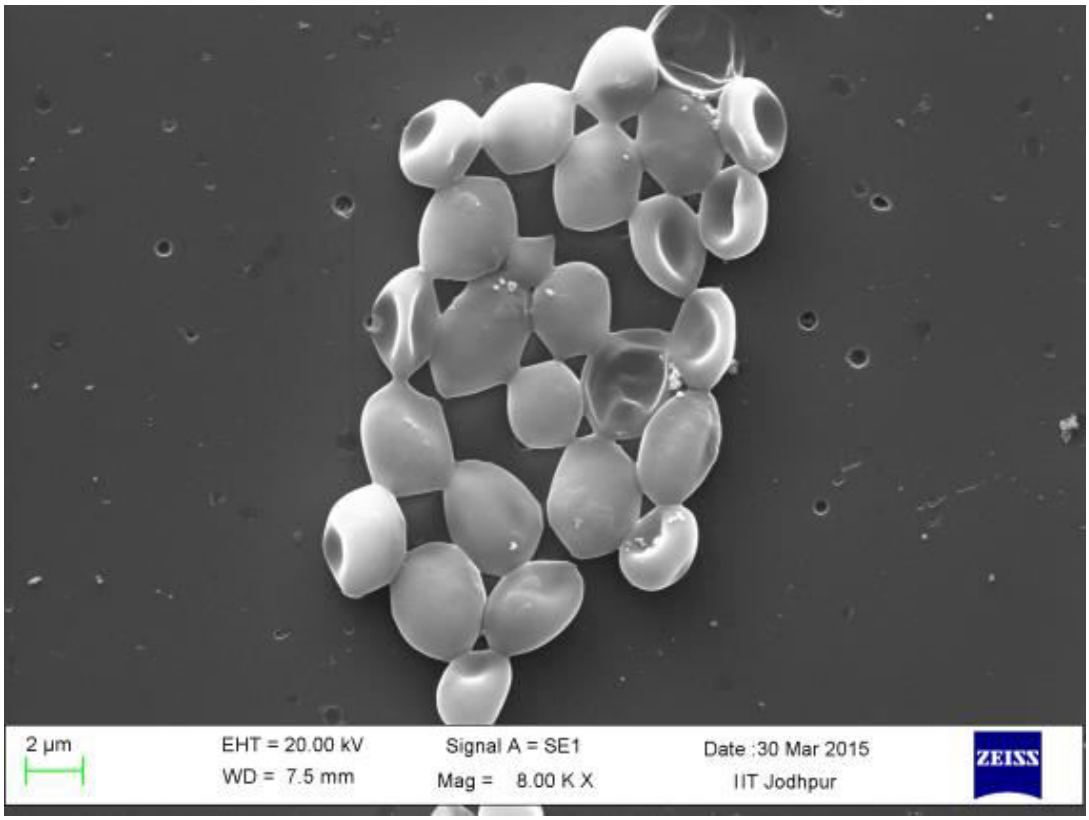

Figure 3.3: Scanning electron microscopy (SEM) image of *C. oligophagum* JRC1

3.2.4 Nile red fluorescence microscopy

Fluorescence images were acquired for the yeast grown in glucose medium at various time intervals. It was found that up to 96 h, no or small size of droplets were present. After 120 h, the lipid droplets of considerable size were observed inside the cells.

3.2.5 Batch cultivation of *C. oligophagum* JRC1 for total cellulase production

The cellulase enzymes include the endoglucanase (CMCase), exoglucanase (cellobiohydrolase/FPase), and β-glucosidase. The assays for all three enzymes, as well as protein concentration (100 mL medium) at 28 °C, were recorded after every 12 h, as shown in Figure 3.4. When the culture was cultivated on the CMC medium, the highest specific enzyme activity for endoglucanase (CMCase), exoglucanase and β-glucosidase achieved were 2.2701 ± 0.0070 , 1.2602 ± 0.0400 and 0.9801 ± 0.0400 IU/mg respectively (enzyme activity 0.0722 ± 0.0026, 0.0404 ± 0.0013, 0.0314 ± 0.0014 IU/mL respectively) after 72 h of incubation.

To date, very few studies are reported on the production of cellulases by yeasts. *R. muciliginosa* was reported to produce endoglucanase as well as β-glucosidase [Baldrian and Vedula, 2008; Strauss *et al*. 2001]. In a recent report, marine yeast *Aureobasdium pullulans* produced a maximum CMCase activity of 4.51 IU/mg in an optimized medium [Rong *et al.,* 2015]. β- glucosidase activity of the given yeast isolate was comparable with the recently reported yeast *Pseudozyma brasiliensis* (~0.14 IU/mL) [Neto *et al*., 2015]. *C. flavus* and *R. muciliginosa* species have also been reported to secrete endoglucanase enzyme naturally [Oikawa *et al.,* 1998]. In one of the studies, the gene encoding endoglucanase from *C. flavus* yeast was cloned and expressed in the *S. cerevisiae* for CBP of cellulosic biomass to bioethanol [Hatano *et al*., 1994]. The ability to produce of cellulase, as well as oleaginous nature, create a unique

combination that makes the *C. oligophagum* JRC1 yeast a strong candidate for CBP of cellulosic waste to biodiesel.

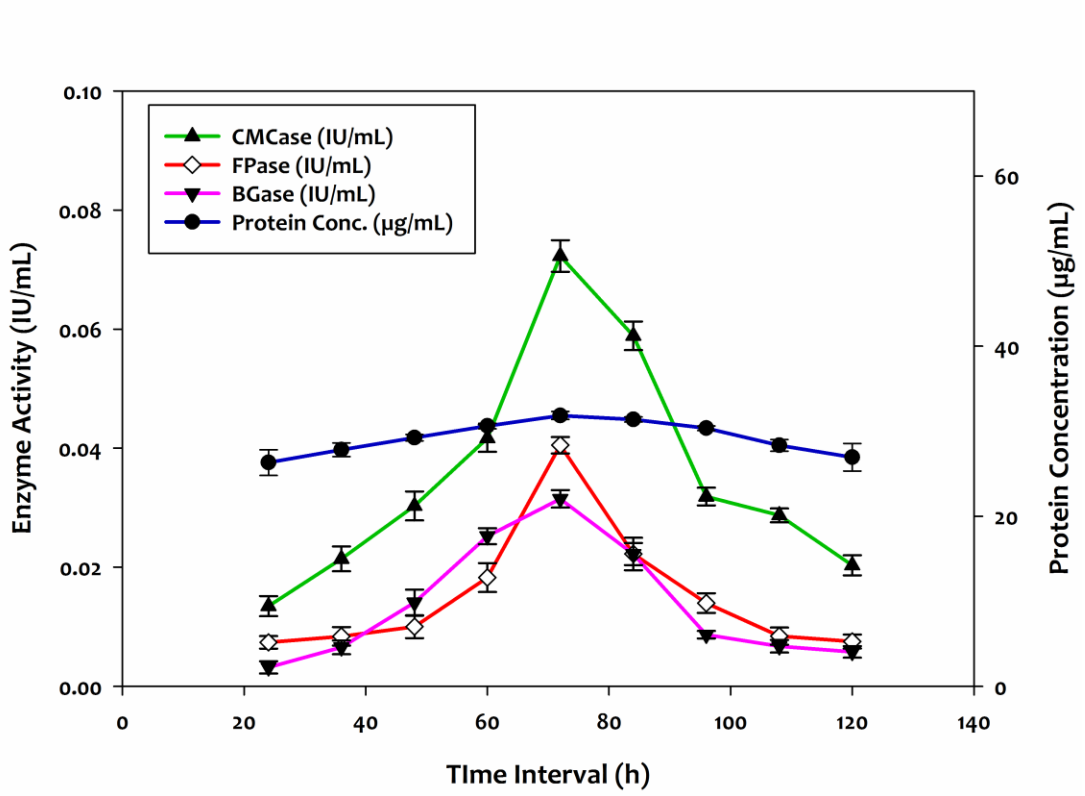

Figure 3.4: Cellulase activity profile of *C. oligophagum* JRC1 with time: Showing profile of endoglucanase, exoglucanase and *β*-glucosidase activity (IU/mL) and total protein (µg/mL) with time in medium containing CMC as a carbon source

3.2.6 Batch cultivation of *C. oligophagum* JRC1 for lipase production

Lipases are either extracellular or intracellular. As shown in Figure 3.5, the highest extracellular lipase activity achieved was 0.1418 ± 0.0090 IU/mL, and specific enzyme activity was 2.8835 ± 0.1667 IU/mg after 84 h of incubation. The intracellular crude lipase was recovered by sonication, and *p*NPP assay was performed, as shown in Figure 3.5, the highest activity achieved was 0.0851 ± 0.0073 IU/mL and specific enzyme activity was 2.1637 ± 0.0433 IU/mg. The efficiency of ultrasonication is affected by medium composition, dissolved gas, temperature, etc., [Chakma and Moholkar, 2013, 2016]. Thus ultrasonication was carried out at conditions used in the previous studies [Patel *et al.*, 2015]. Several yeasts such as *L. starkeyi*, *Y. lipolytica*, etc. have been reported to produce extracellular as well as intracellular lipase and their utilization as a catalyst for biodiesel (FAME) production has also been studied [Ribeiro *et al.*, 2011]. The kinetics of lipase-catalyzed transesterification can be enhanced by combining it with physicochemical methods such as ultrasonication [Malani *et al.*, 2014]. Further experimental interventions will be needed to utilize the enzymatic machinery of the isolated yeast in the present study for FAEE/Biodiesel synthesis *in vivo* or *in vitro*.

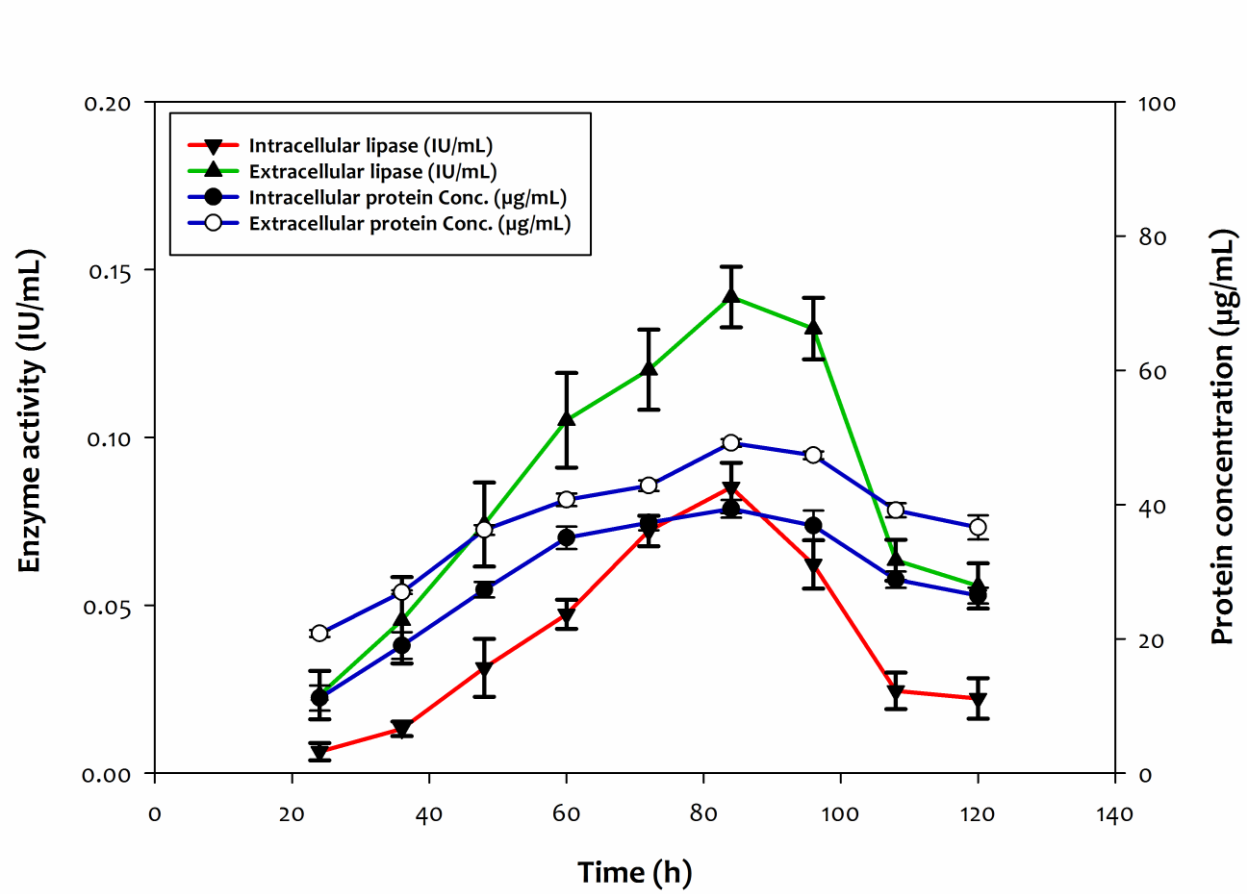

Figure 3.5: Lipase activity profile of *C. oligophagum* JRC1 with time: Showing extracellular and intracellular lipase activity (IU/mL) and protein concentration (µg/mL) with time in medium containing tributyrin and glucose as a carbon source

3.2.7 Batch cultivation of C. *oligophagum* JRC1 for lipid production and extraction

The optimization of the cultivation conditions in the lipid production medium was done at various temperatures and pH values. After the experiments, the temperature 28 °C and pH 6.0 was found to be giving the highest lipid production in a glucose medium (Figure 3.6 A and 3.6 B). The biomass production, lipid content and the lipid output (at 28 °C and 6.0 pH) were monitored at every 24 h intervals till 192 h, and their time courses were recorded as shown in Figure 3.6 C and 3.6 D. At 72 h the biomass production, lipid output, and lipid content were 5.3100 ± 0.9497 g/L, 1.2683 ± 0.2621 g/L and 23.79 % respectively. In the post stationary phase, the lipid accumulation phase started, and the lipid content increased steadily from 96 h to 192 h. At 168 h, the highest biomass production, lipid output, and lipid content were 12.4533 ± 0.9743 g/L, 4.9193± 0.5267 g/L, and 39.4445 ± 1.1995 % respectively. The profiles of residual sugars, as well as nitrogen (ammonical nitrogen) available in the medium, were obtained every 24 h (Figure 3.6 C and 3.6 D).

Most of the oleaginous yeasts accumulate lipid in the post stationary phase. Generally, the incubation period for achieving maximum lipid content varies among different yeasts (120-192 h). Various other genera like *Candida*, *Cryptococcus, Rhdosporidium, Trichosporon, Yarrowia, Rhodotorula etc.*, of the yeast species have also been reported to accumulate lipids at different levels (values of which are mentioned in Table 3.1 for comparison). The results for lipid productivity in the case of glucose as a carbon source were comparable with *C. viswanathii* Y-E4, *R. toruloids*, and *C. vishniacci* and lower than *Y. lipolytica* and *R. graminis* [Ayadi et al., 2016;

Deeba *et al.*,2016; Fontanille *et al.*, 2012; Galafassi *et al.*, 2012a; Patel *et al.*,2014]. The lipid productivity was comparable with *C. viswanathii* Y-E4, *R. babjevae* [Ayadi *et al.*, 2016; Munch *et al.*, 2015] and lower than *R. graminis* [Galafassi *et al.*, 2012] while using glycerol as a carbon source. The lipid productivity using xylose and starch as carbon sources were comparable with other yeasts such as *C. viswanathii* and *C. terricola* respectively [Ayadi *et al.*, 2016; Tanimura *et al.*, 2014]. Further optimization of the culture conditions and cultivation strategies using these carbon sources can enhance the lipid productivity from the yeast isolate.

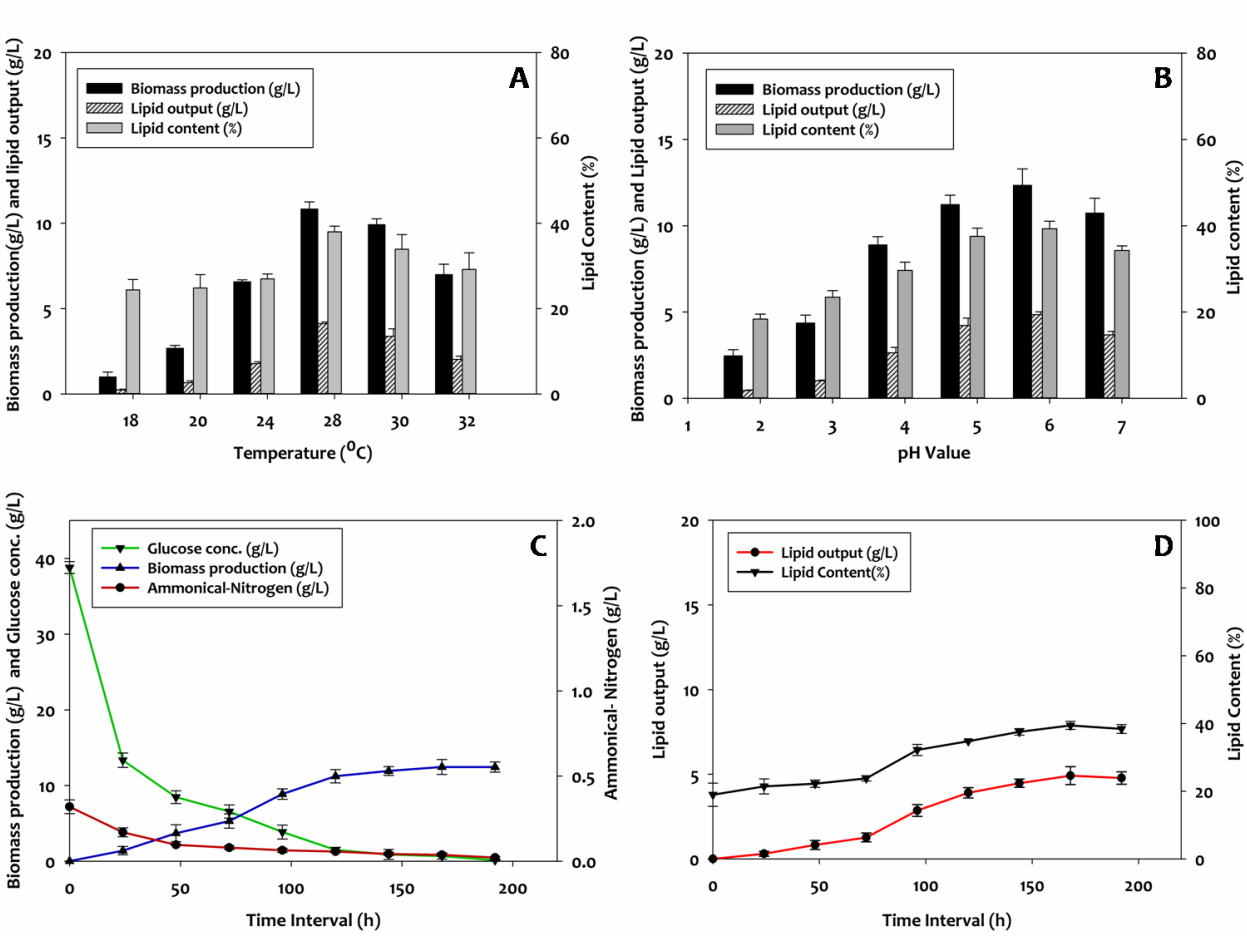

Figure 3.6: Lipid production from *C. oligophagum* JRC1: (a) Effect of temperature on lipid production: Profile of biomass production (g/L), lipid output (g/L), and lipid content (%) of *C. oligophagum* JRC1 in lipid production medium at various temperatures (B) Effect of pH on lipid accumulation: Profile of biomass production (g/L), lipid output (g/L), and lipid content (%) of *C. oligophagum* JRC1 in lipid production medium at various pH ranging from pH value 2–7 (C) Profile of substrate utilization; biomass production (g/L), residual glucose (g/L), ammonical nitrogen (g/L) of *C. oligophagum* JRC1 with time in lipid production medium (D) Profile of lipid output (g/L) and lipid content (%) of *C. oligophagum* JRC1 with time in lipid production medium

Table 3.1: Summary of the lipid production parameters for some of the oleaginous yeasts reported

Organism	Carbon source	Type of culture	Biomass production (g/L)	Lipid output (g/L)	Lipid content (%)	Lipid productivity g/L.d	Reference
Candida viswanathii Y-E4	Glucose Xylose Glycerol	Batch-7-L stirred tank bioreactor	13.62 12.19 9.61	3.45 3.24 2.50	25.33 26.57 32.72	0.69 0.64 0.50	[Ayadi *et al.*, 2016]
Cryptococcus vishniaccii	Glucose	Batch Shake-flask	13.59	5.5	40.44	0.91	[Deeba *et al.*, 2016]
Rhodosporidium babjevae	Glycerol	Batch Shake-flask	9.9	2.4	24.1	0.48	[Munch *et al.*, 2015]
Rhodosporidium diobovatum	Glycerol	Batch Shake-flask Batch reactor	14.1 13.6	7.1 6.9	50.3 50.7	1.42 0.98	[Munch *et al.*, 2015]
Rhodosporidium kratochvilovae HIMPA1	Glucose	Batch Shake-flask	14.46	6.2	41.92	0.88	[Patel *et al.*, 2014]
Yarrowia lipolytica MUCL28849	Glucose + acetic acid	Batch 0.5 L bioreactor	30.83	12.36	40.69	4.56	[Fontanille *et al.*, 2012]
Rhodotorula graminis	Glucose Glycerol	Batch Shake-flask	15.75 16.25	N.M. N.M.	45.00 53.00	2.40 3.12	[Galafassi *et al.*, 2012]
Cryptococcus terricola JCM 2452	Starch Glucose	Batch Shake-flask	4.88 N.M.	3.02 N.M.	61.96 61.53	0.302 N.M.	[Tanimura *et al.*, 2014]
Rhodosporidium toruloides	Glycerol + surfactants	Batch Shake-flask	10.7	7.1	66.00	1.25	[Xu *et al.*, 2016]
Cryptococcus curvatus	Crude glycerol	Batch Shake-	29.2	7.7	26.0	N.M.	[Leiva-Candia *et al.*,

ATCC20509		flask					2015]
Lipomyces starkeyi DSM 70296	Crude glycerol	Batch Shake-flask	16.6	2.6	16.0	N.M.	[Leiva-Candia *et al.*, 2015]
Rhodosporidium toruloides DSM 4444	Crude glycerol	Batch Shake-flask	28.5	7.6	27	N.M.	[Leiva-Candia *et al.*, 2015]
Candida freyschussii ATCC 18737	Glycerol	Batch Bioreactor	13.9	4.6	33	3.6	[Raimondi *et al.*, 2014]
Trichosporon oidesspathulata	Glycerol	Batch-5-L Bioreactor	11.3	5.01	44.30	N.M.	[Kitcha and Cheirsilp, 2013]
Candida ortholopsis	CMC	Batch Shake-flask	N.M.	N.M.	63.75	N.M.	[Kanti and Sudiana, 2015]
Cystobasidium oligophagum JRC1	Glucose CMC Glycerol Starch Xylose	Batch Shake-flask	12.45 6.25 6.48 4.72 11.09	4.91 2.28 2.72 1.96 4.02	39.44 36.46 42.04 41.54 36.24	0.70 0.32 0.38 0.28 0.57	Present study

(Where N.M.: Not mentioned; CMC: Carboxymethylcellulose)

3.2.8 Effect of carbon source on lipid production

The yeast *C. oligophagum* JRC1 showed a wide range of substrate utilization when grown on the different types of carbon sources like maltose, fructose, sucrose, lactose, starch, CMC, xylose, and glycerol. In all the experiments the biomass production (g/L), lipid output (g/L) and lipid content (%) were measured as shown in Figure 3.7. By using individual carbon sources the highest biomass production obtained with glucose (12.4533 ± 0.9743 g/L) followed by fructose (11.5000 ± 0.7903 g/L), xylose (11.0901 ± 0.7597 g/L), lactose (9.7466 ± 0.8348 g/L),

sucrose (8.9833 ± 0.0306 g/L), glycerol (6.4803 ± 0.5790 g/L), CMC (6.2533 ± 0.7867 g/L), starch (4.7233 ± 0.5596 g/L) and maltose (2.2666 ± 0.4561 g/L). The lipid output (g/L) was highest for glucose (4.9193± 0.5267 g/L) followed by xylose (4.0203 ± 0.3202 g/L), fructose (2.7931 ± 0.2064 g/L), glycerol (2.7270 ± 0.3089 g/L), CMC (2.2828 ± 0.3374 g/L), lactose (2.1328 ± 0.1380 g/L),starch (1.9621 ± 0.2290 g/L), sucrose (1.9515 ± 0.1774 g/L) and maltose (0.5999 ± 0.1464 g/L). Thus, the variation was observed in the accumulation of lipid as the carbon source was varied. The lipid content (% w/w), on the other hand was highest for glycerol (42.0403 ± 1.7136 %) followed by starch (41.5491 ± 0.3469 %), glucose (39.4445 ± 1.1995 %), CMC (36.4615 ± 1.4997 %), xylose (36.2410 ± 1.0975 %), maltose (26.3114 ± 1.8228 %), fructose (24.2933 ± 0.9087 %), lactose (21.9134 ± 0.6388 %) and sucrose (21.7229 ± 1.7760 %). Table 3.2, summarizes different lipid production parameters obtained on individual carbon sources. Calculations were done as per the definitions are given by Sitepu *et al*, 2014 [Sitepu *et al*., 2014a].

Although the lipid content (% w/w) in the case of starch and glycerol was observed to be higher than that of glucose, the lipid productivity was low, which demands further optimization studies. The ability of the isolate to utilize different types of carbon sources reflects upon its ability to utilize industrial wastes such as those obtained from biodiesel transesterification plants, dairy industry, starch processing industries, sugar mill effluents, etc. Most of the basidiomycetes yeasts can grow and accumulate lipids on the pentose and hexoses (C5 as well as C6 sugars) [Sitepu *et al*., 2014a]. The different studies so far show that the yeasts of the genus like *Yarrowia, Rhodotorula, and Rhodosporidium* are reported to be grown on the carbon sources derived from the lignocellulosic biomass [Leiva-Candia *et al.,* 2014]. Also, glycerol a byproduct of the biodiesel industry, is widely employed as a substrate for lipid accumulation. *C. terricola* was reported to bean oleaginous yeast that can grow directly on starch wastes [Tanimura *et al.,* 2014]. The lipid profile of oleaginous yeast changes with the change in the carbon source and other conditions [Sitepu *et al*., 2013]. The fatty acid constitution is important as it affects the biodiesel properties and determines its usability as fuel. The carbon sources other than glucose are assimilated via different metabolic pathways and thus lead to different fatty acid composition in lipids [Fakas *et al*., 2009]. Leiva-Candia *et al*., 2014, have reviewed the effect of carbon sources derived from agro-industrial wastes on the yeast fatty acid profile. Yeast lipid profiles obtained from various carbon sources were found to be similar to palm oil or rapeseed oil (commonly used substrates for first-generation biodiesel). It was also found that the majority of the substrates lead to higher degree saturation in lipids, which leads to higher oxidation stability and better cetane number [Leiva-Candia *et al*., 2014]. Therefore, manipulation of culture conditions and/or genetic modification is needed to obtain the desirable fatty acid profile, which in turn determines the biodiesel quality.

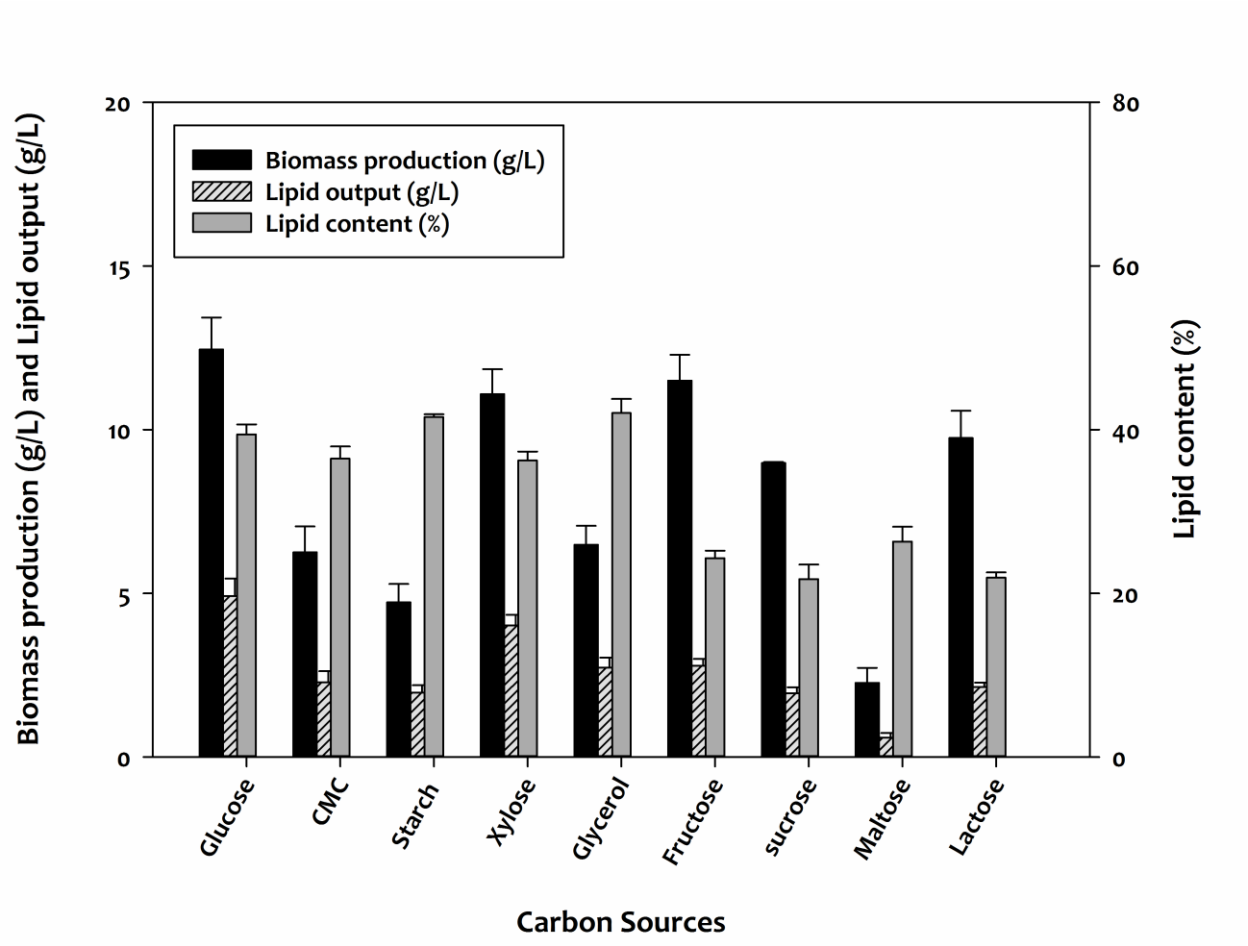

Figure 3.7: Effect of carbon sources on lipid accumulation: profile of biomass production (g/L), lipid output (g/L), and lipid content (%) of *C. oligophagum* JRC1 in lipid production medium containing various carbon sources.

Table 3.2: Summary of the various cell growth and lipid production parameters obtained for *C. oligophagum* JRC1 on different carbon sources.

Type of carbon source	**Biomass production (P_X)(cell g/L)**	**Biomass yield (Y_X) (cell g/g of carbon source)**	**Biomass conversion (cell g/100 g of carbon source)**	**Lipid content (C_L) (% w/w) (g lipid/100 g yeast)**	**Lipid yield/ Co-efficient (E_L) (g lipid/100 g of carbon source)**	**Lipid output (Y_L) (g lipid/L)**	**Lipid productivity (g/ L. d)**
Glucose	12.4533 ± 0.9743	0.3113 ± 0.0243	31.3033 ± 2.4359	39.4445 ± 1.1995	12.2983 ± 1.3169	4.9193 ± 0.5267	0.7027 ± 0.0752
CMC	06.2533 ± 0.7867	0.3126 ± 0.0293	31.2066 ± 3.9339	36.4615 ± 1.4997	11.4144 ± 1.6870	2.2828 ± 0.3374	0.3261 ± 0.0482
Starch	04.7233 ± 0.5596	0.1180 ± 0.0239	11.8083 ± 1.3991	41.5491 ± 0.3469	4.9053 ± 0.5727	1.9621 ± 0.2290	0.2803 ± 0.0327
Xylose	11.0900 ±	0.2772 ±	27.7250 ±	36.2410 ±	10.0508 ±	4.0203 ±	0.5743 ±

Type of carbon source	Biomass production (P_X)(cell g/L)	Biomass yield (Y_X) (cell g/g of carbon source)	Biomass conversion (cell g/100 g of carbon source)	Lipid content (C_L) (% w/w) (g lipid/100 g yeast)	Lipid yield/ Co-efficient (E_L) (g lipid/100 g of carbon source)	Lipid output (Y_L) (g lipid/L)	Lipid productivity (g/ L. d)
	0.7597	0.0189	1.8993	1.0975	0.8006	0.3202	0.0457
Glycerol	06.4803 ± 0.5790	0.1620 ± 0.0144	16.2008 ± 1.4476	42.0403 ± 1.7136	06.8175 ± 0.7723	2.7270 ± 0.3089	0.3895 ± 0.0441
Fructose	11.5000 ± 0.7903	0.2875 ± 0.0197	28.7500 ± 1.9759	24.2933 ± 0.9087	06.9827 ± 0.5162	2.7931 ± 0.2064	0.3990 ± 0.0294
Sucrose	08.9833 ± 0.0306	0.2245 ± 0.0176	22.4083 ± 0.7666	21.7229 ± 1.7760	04.8789 ± 0.4435	1.9515 ± 0.1774	0.2787 ± 0.0253
Maltose	02.2666 ± 0.4561	0.0566 ± 0.0114	05.6066 ± 1.1402	26.3114 ± 1.8228	01.4999 ± 0.3662	0.5999 ± 0.1464	0.0857 ± 0.0209
Lactose	09.7466 ± 0.8348	0.2436 ± 0.0208	24.3666 ± 2.0870	21.9134 ± 0.6388	5.3321 ± 0.3452	2.1328 ± 0.1380	0.3046 ± 0.0197

[Note: All carbon sources were added at 40 g/L, except CMC at 20 g/L. All the experiments were carried out at pH 6.0, and 28°C (cultivation time 168 h); CMC: Carboxymethylcellulose]

3.2.9 FT-IR and TLC analysis of the lipid extract

Transmission spectra for the extracted lipids from the yeast culture were obtained and showed similarity with triolein, used as a standard (Figure 3.8). The peak assignment to the respective functional group was done according to the previous reports [Patel *et al.,* 2014b]. The absence of the peaks in the lipid extracts from 4000 cm^{-1} to 3010 cm^{-1} pointed out that there is the absence of the free hydroxyl group (–OH) and an amine group. The three characteristic absorbance peaks at 3007 cm^{-1}, 2925 cm^{-1}, and 2854 cm^{-1} show the presence of stretching vibration of -CH, $-CH_2$, and $–CH_3$ respectively, while three peaks observed at 1463-1377 cm^{-1}, 1370-1098 cm^{-1} and 722 cm^{-1} were bending vibrations for $–CH_3$, $-CH_2$ and -CH respectively. One peak observed at 1745 cm^{-1} shows the presence of carbonyl (–C=O) group in the crude lipid extract. The TLC of the lipid extract was run against the triolein standard, which showed R_f values similar to it. It was noticeable that the lipid extracts in the present study showed only one band attributed to TAG, indicating that the lipid bodies of the isolate are essentially in the form of TAG. The composition on TAG, Diacylglycerol (DAG), and Monoacylglycerol (MAG) are species-specific [Sitepu *et al.,* 2014a].

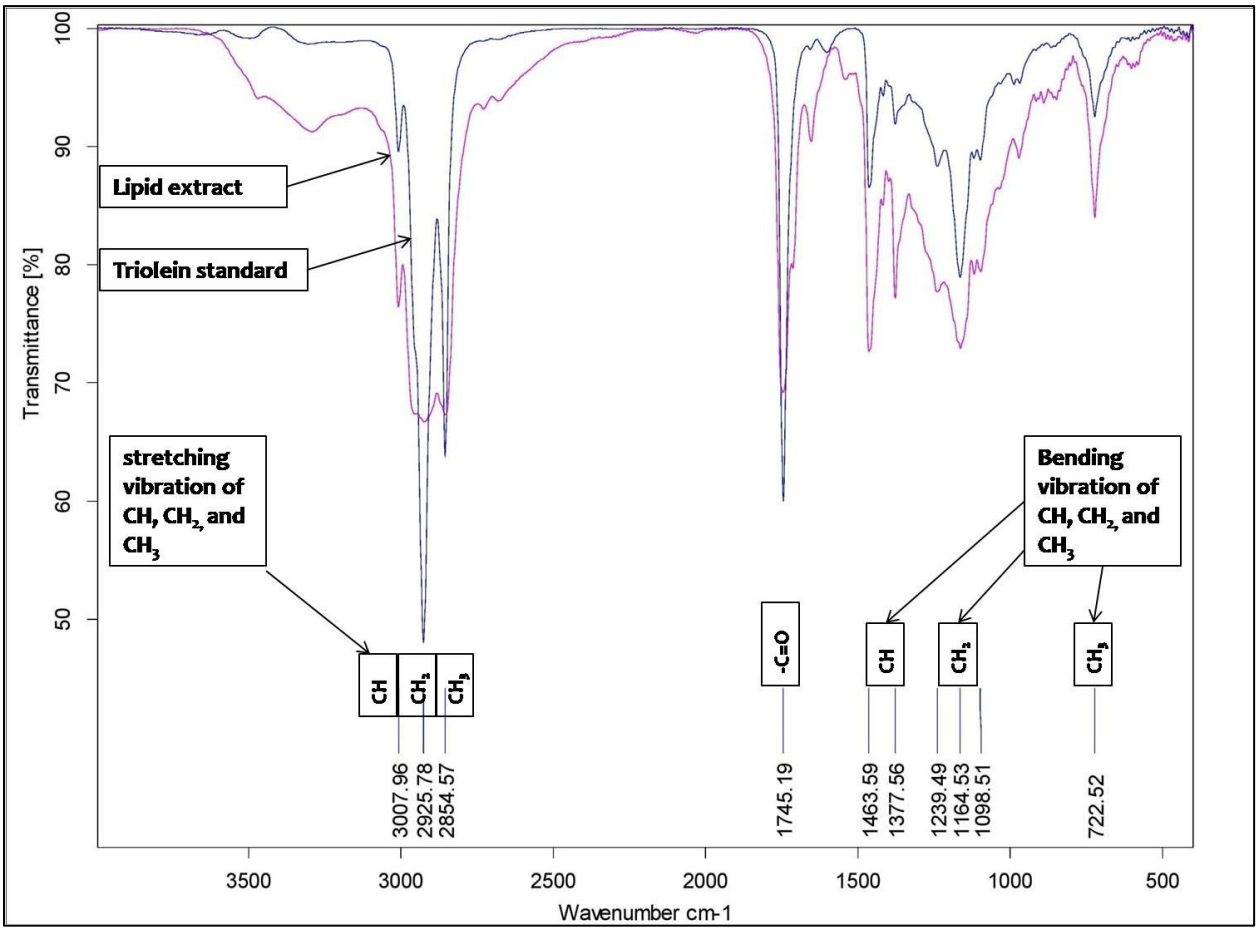

Figure 3.8: FT-IR analysis for lipid extract of *C. oligophagum* JRC1

3.2.10 Determination of FAME composition by GC-MS analysis

The GC-MS analysis of the Fatty Acid Methyl Ester (FAME) derived from the lipid extracts of *C. oligophagum* JRC1 cultivated in the glucose medium was performed. The palmitic acid ($C_{16:0}$) content was 26.0250 ± 0.6010 % higher than jatropha (14.66 %) and soyabean oil (11%) [Deeba *et al*., 2016]. While the palmitoleic acid ($C_{16:1}$), was found to be 0.4300 ± 0.0282 %. The stearic acid ($C_{18:0}$)content was found to be 4.1500 ± 0.1131 % which was also matching palm oil (4-6.3%) and jatropha oil (6.86 %), while the oleic acid ($C_{18:1}$) content was found to be 42.7950 ± 0.5161 %, which was higher than jatropha oil (39.08%), and other vegetable oils such as palm oil (37-53 %) and soyabean oil (23.4%) indicating a better lipid profile than other commonly used feedstock. Further, the linoleic acid ($C_{18:2}$) content was found to be 24.2200 ± 1.3010 %, which was lower as compared to jatropha (32.48%) and another similar type of oils [Deeba *et al*., 2016]. A small portion of linolenic acid ($C_{18:3}$) was observed at 0.8300 ± 0.0565 %. The myristic acid ($C_{14:0}$), pentadecanoic acid ($C_{15:0}$) and $C_{17:0}$, were present in an amount of 0.7250 ± 0.0070 %, 0.1900 ± 0.0141 %, and 0.2400 ± 0.0282 % respectively. The total saturated fatty acid (SFA) (31.3300 ± 0.7071%) was high as compared to other types of oils (jatropha (21.52 %) > soyabean oil (15 %) > sunflower oil (4.5 %) > rapeseed oil (6.6%) but lower than the palm oil (44.41 %).

Certain important properties of biodiesel, such as cetane number, oxidative stability, and kinematic viscosity, etc., depending on chain length and degree of saturation [Sitepu *et al*., 2014a]. The MUFA (Mono-Unsaturated Fatty Acid) and PUFA (Polyunsaturated fatty acid) were present in an amount of 43.2250 ± 0.4879 % and 25.0505 ± 1.2445 % respectively. According

to the reports, the lower degree of saturation is better for a melting point as well as kinematic viscosity [Sitepu *et al*., 2014a]. The overall FAME data shows that the extracted lipid from the yeast *C. oligophagum* JRC1 can be used for biodiesel production with appropriate fuel properties. The results show that it can be a better alternative to the vegetable and jatropha oils, thereby making it a suitable candidate for biodiesel production.

3.3 CONCLUSIONS

Oleaginous yeast capable of cellulase and lipase production was successfully isolated. Simultaneous expression of these traits indicated that in a single step, both the feedstock and catalyst can be obtained from renewable resources like cellulose. Further studies are needed to enhance the lipids through optimal cultivation strategies, to test lipid accumulation in real industrial wastes, to test the simultaneous utilization of C6 and C5 sugars, and to check resistance to phenolic and non-phenolic inhibitors. In vivo synthesis of FAEE can be attempted by over-expressing ethanol pathway and other genetic alterations, as previously reported for *E. coli* [Steen *et* al., 2010].

…