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Assessing Oil Accumulation in the Oleaginous Yeast Cystobasidium oligophagum JRC1 Using Dairy Waste Cheese Whey as a Substrate

Research on renewable energy resources is gaining momentum, considering the limited stocks of fossil reserves [Kumar, 2018]. Third-generation biofuels have emerged as promising alternatives to the existing transport fuel. However, the process and the production cost are still higher when compared to conventional fossil fuels [Leong *et al*., 2018]. Oil production from the oleaginous yeasts is one of the alternatives which circumvent some of the drawbacks associated with third generation biofuels [Shields-Menard *et al*., 2018]. The second generation biofuels depend on the lignocellulosic wastes [Leiva-Candia *et al*., 2014; Patel *et al*., 2017b; Wang *et al*., 2017]. However, these wastes need elaborated pre-treatment before they serve as a feedstock. Waste streams originating from food industries offer readily utilizable substrates for lipid accumulation. Cheese whey is one such waste that can be used as a suitable feedstock for lipid accumulation in oleaginous microbes.

Cheese whey is a greenish-yellow liquid residue resulting from the cheese-making process of dairy industries. The chemical composition of the cheese whey varies with the type of cheese. It is typically composed of 92-95 % water. The solid content is 5-8 %, of which lactose accounts for 60-80 %, proteins account for 10 %, and trace elements, vitamins, fat, and other important elements account for the remaining 10 %. It contains 50-102 g/L chemical oxygen demand (COD) and 27-60 g/L biological oxygen demand (BOD) [Carvalho *et al*., 2013]. The global production of the cheese whey in the year 2000 was in the order of 140 million tons [F.A.O., 2002]. Cheese whey is nutrient-rich and has high utilization potential, yet nearly 50 % of the waste is discarded untreated. Disposing of untreated cheese whey leads to environmental problems like eutrophication, loss of agricultural land, etc., [Seo *et al.,* 2014; Taskin *et al.,* 2015]. In order to address this, several processes have been developed by researchers to utilize the lactose and protein part of the cheese whey and convert them into important chemicals and biomolecules like ethanol, pigments, enzymes, lipids, single-cell proteins, etc.[Guimaraes *et al.,* 2010].

Cheese whey has also been used as a substrate for lipid accumulation in oleaginous microbes. Some of the oleaginous yeasts were found to grow and accumulate lipid on the pre-treated cheese whey substrates [Seo *et al.,* 2014; Taskin *et al*., 2015; Arous *et al*., 2016, 2017]. Since lactose is a major component of cheese whey, the oleaginous organisms should have a lactose utilization pathway. Lactose assimilation ability is not so common amongst yeasts [Domingues *et al*., 2010]. Cheese whey, therefore, needs pre-treatment before its utilization as feedstock. The pre-treatment includes deproteinization, alkali treatment, hydrodynamic cavitation (HC) treatment, etc., [Seo *et al*., 2014; Arous *et al*., 2016]. Oleaginous yeast, which can accumulate lipids directly on untreated cheese whey, is desirable.

In this study, the oleaginous yeast *C. oligophagum* JRC1 was cultivated on cheese whey. The yeast was found to utilize lactose in the previous study. The yeast was grown on different concentrations of both deproteinized and untreated cheese whey, and the lipid accumulation

potential and COD removal were assessed. The oil composition was determined to check its suitability for biodiesel production. Figure 4.1 shows the overall schematics of the study.

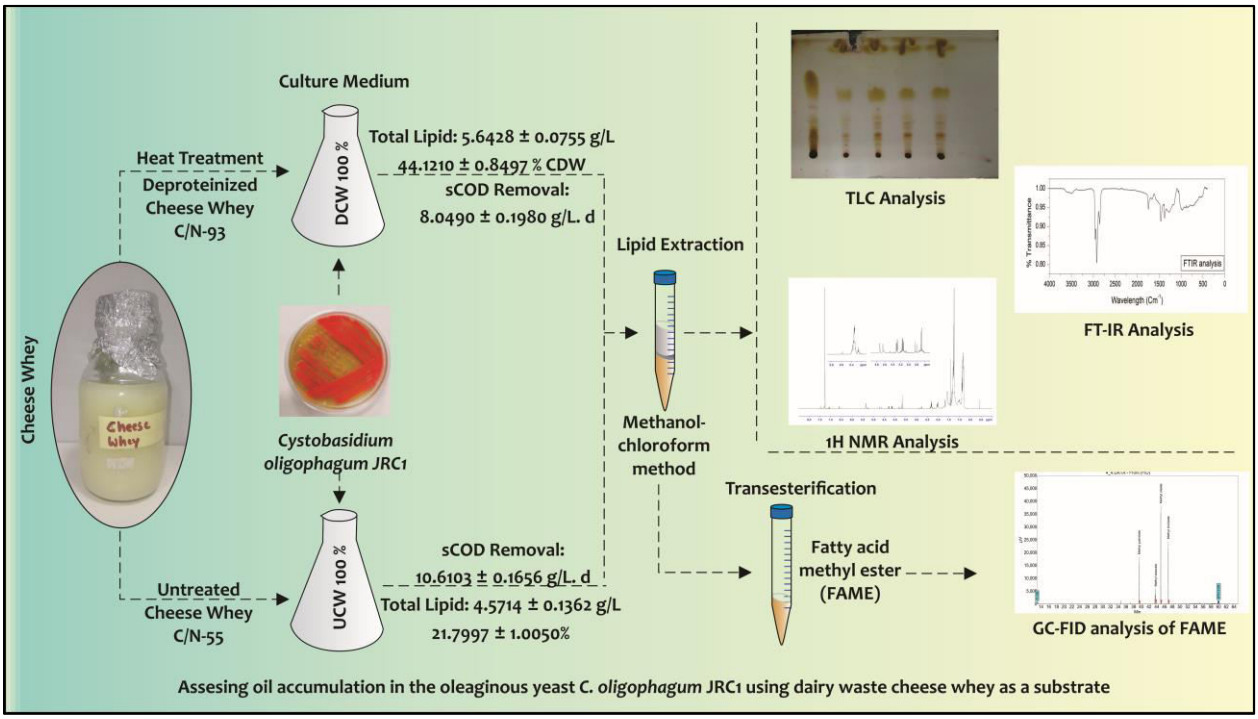


Figure 4.1 The schematic of the study

4.1 CULTIVATION OF *C. OLIGOPHAGUM* JRC1 ON THE DAIRY WASTE CHEESE WHEY

4.1.1 Cheese whey collection and characterization

The cheese whey from the cheddar cheese whey plant was collected from the nearby dairy industry and stored at − 20 °C in the sterile container until further use. The effluent was characterized for some basic parameters such as pH, chemical oxygen demand (COD), total reducing sugars, and total Kjeldahl nitrogen (TKN).

4.1.2 Yeast strain and media conditions

Oleaginous yeast *C. oligophagum* JRC1 originally isolated from the site rich in cellulosic waste was used for the study. The yeast was cultivated on two different media. In one of the medium, the cheese whey was deproteinized using a heat treatment method. It involved heating at 121 ^{0}C for 15 min. The proteins which precipitated were separated using centrifugation followed by filtration using Whatman filter paper. The filtrate so obtained was called deproteinized cheese whey (DCW) [Arous *et al*., 2016]. The other medium involved direct utilization of the cheese whey without any pre-treatment. This was called untreated cheese whey (UCW). The DCW and UCW both were utilized at 25, 50, 75 and 100 % (v/v) concentration. The dilutions were done with distilled water. The shake flasks experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL media supplemented with minerals at the concentration of (g/L): KH_2PO_4, 7; NaH_2PO_4, 3; $MgSO_4$, 3. Other trace elements were added at the concentration of (g/L): $CuSO_4$ $5H_2O$, 0.0001; $MnSO_4$ $5H_2O$, 0.0001; $ZnSO_4$ $7H_2O$, 0.001; CO $(NO_3)_3$ $3H_2O$, 0.0001. All of the DCW and UCW mediums thus prepared were

sterilized by autoclave (121 ^{0}C, 15 psig, 15 min). *C. oligophagum* JRC1 was grown on 100 mL YPD medium (yeast extract, 10; Peptone, 20; and glucose, 10 g/L) at 28 ^{0}C and 150 rpm and 5 mL of the culture during log phase of growth (approximately 1.74 x 10^8 cells/mL) was aseptically centrifuged at 8000 rpm for 10 min separating the cells and supernatant. The supernatant was discarded, and the cells were re-suspended in sterile saline (0.85 % NaCl w/v) and centrifuged again. This step was repeated twice. The washed cells were finally re-suspended in 5 mL sterile saline and introduced into the autoclaved cheese whey media aseptically. All the flasks were incubated at 28 ^{0}C and 150 rpm (Figure 4.1).

4.2 RESULTS AND DISCUSSION

4.2.1 Cheese whey waste characterization

The physical and chemical composition of the cheese whey from dairy industries may vary with the variation in the process, final product, and operation methods. In general, the sCOD of the cheese whey falls in the range of 50-102 g/L. The primary constituents are lactose and fats [Carvalho *et al*., 2013]. Both DCW and UCW were rich in sCOD with the values in the order of 66.3528 ± 1.5159 and 85.5392 ± 1.3232 g/L for DCW and UCW, respectively. The total reducing sugars obtained were 39.6308 ± 0.6768 and 56.4756 ± 1.7523 g/L for DCW and UCW, respectively. The pH values were 6.6 and 6.8 for DCW and UCW, respectively. This is consistent with the pH values observed with other types of cheese whey substrates [Carvalho *et al*., 2013]. TKN concentrations were 0.7110 ± 0.0240 and 1.5400 ± 0.09899 g/L for DCW and UCW, respectively, which was within the general range of 0.01- 1.70 g/L for cheese whey [Prazeres *et al*.,2012]. The COD/N ratio was 93 and 55 for DCW and UCW, respectively.

4.2.2 Lipid production in deproteinized cheese whey in a shake flask experiments

Very few yeasts possess lactose permease and β-galactosidase required for lactose metabolism [de la Fuente and Sols, 1962; Domingues *et al.,* 2010]. Due to this reason, the growth of oleaginous yeast on cheese whey medium is limited. The yeast *C. oligophagum* JRC1 accumulated lipids in the medium containing lactose as the only carbon source (chapter 3). This trait helped us extrapolate its utility for the valorization of industrial waste, such as cheese whey, which is primarily composed of lactose. Cheese whey was deproteinized to increase C/N ratio, and untreated cheese whey was also used to assess the growth and lipid accumulation by the yeast.

The reducing sugar, CDW, and lipid production for all four concentrations (25, 50, 75, and 100 % (v/v) DCW) were recorded for every 24 h, as shown in Figure 4.2. Different concentrations were tried to know the optimum concentration for yeast growth and lipid production. The approximate initial C/N ratio was calculated using the COD/N ratio, as previously reported [Saenge *et al.,* 2011b]. The initial TKN concentrations were 0.1830 ± 0.0028, 0.4200 ± 0.0042, 0.5490 ± 0.0141 and 0.7110 ± 0.0240 g/L (Initial C/N ratio: 99, 98, 98, 93) for 25, 50, 75 and 100 % (v/v) DCW media respectively. As shown in Figure 4.2, the initial reducing sugar concentrations were 10.1746 ± 0.1918, 19.1012 ± 0.5968, 28.2004 ± 0.9117, and 39.6308 ± 0.6768 g/L for 25, 50, 75 and 100 % (v/v) DCW media respectively. The levels of reducing sugars dropped faster during the first few hours and stabilized at 96 h. This was followed by the lipid accumulation phase. Sugar consumption rate increased with the increase in the concentration of cheese whey from 25 to 100 % (v/v). The CDW achieved also followed a similar trend with the highest concentration achieved at 100 % DCW. The values were 4.9285 ± 0.1133, 8.4523 ± 0.2450, 11.3571 ± 0.2142, 12.7904 ± 0.0786 g/L respectively. The lipid production were 2.5857 ± 0.0377, 4.1190 ± 0.0501, 5.2952 ± 0.4126 and 5.6428 ± 0.0755 g/L (52.4842 ± 01.5383, 48.7481 ± 01.1386, 46.6368 ± 3.7473 and 44.1210 ± 0.8497 % of CDW w/w) after 168 h for 25, 50, 75 and 100 % (v/v) DCW media respectively.

Cheese whey contains minimum levels of certain vitamins, which might have been useful for yeast growth [Carvalho *et al.,* 2013]. The C/N ratio is an important parameter for lipid accumulation. The nitrogen limiting condition (higher C/N ratio) triggers the tri-acylglycerol (TAG) accumulation in the oleaginous yeasts [Sitepu *et al.,* 2013] by channeling the excessive carbon into lipid bodies in the form of TAG [Ageitos *et al*., 2011]. However, the biochemical response is not solely influenced by C/N ratio but also influenced by factors such as the type of carbon sources, nitrogen sources, growth factors, and culture conditions (pH and temperature) [Papanikolaou and Aggelis, 2011]. The lipid accumulation in oleaginous yeast is induced at a C/N ratio > 20. In both UCW and DCW, the C/N was > 20 [Papanikolaou and Aggelis, 2011]. However, the difference between C/N of 100 % (v/v) UCW and DCW was close to 38, and this led to a significant increase in oil accumulation in DCW. The lipid content increased from 21.7997 ± 1.0050 to 44.1210 ± 0.8497 % of CDW. Similar results have been documented in earlier reports [Annamalai *et al.,* 2018]. Also, when comparing different concentrations of DCW and UCW, the difference in the C/N ratio was not significant as it was just diluted in the buffer. Therefore, the differences in the cellular yield and lipid productivity can be attributed to the concentration of carbon source and other nutrients available for yeast growth. Since the concentration in the 100 % (v/v) cheese whey was the highest, it supported the highest lipid productivity in both DCW as well as UCW.

All the results obtained in the present study were compared with the literature, as shown in Table 4.1. The lipid productivity increased from 0.01530 ± 0.0002 for 25 % (v/v) DCW to 0.0335 ± 0.0004 g/L. h for 100 % DCW. Yeast such as *D. etchellsii, Wickerhamomyces anomalus, Y. lipolytica and C. curvatus* etc. have been reported to accumulate lipids on DCW medium [Arous *et al.,* 2016; Arous *et al.,* 2017b; Seo *et al.,* 2014; Taskin *et al.,* 2015]. However, *D. etchellsii* could not assimilate lactose and utilized the DCW medium only as a nitrogen source and accumulated only 0.40 ± 0.05 g/L of lipid [15.90 ± 0.93 % of CDW)] [Arous *et al.,* 2016]. Also, *W. anomalus* accumulated 0.65 ± 0.01 g/L (24.00 ± 0.24 % of CDW) after 96 h of incubation [Arous *et al.,* 2017], while *C. oligophagum* JRC1 in the present study accumulated much higher lipid content with a value of 2.0904 ± 0.0297 g/L (27.3002 ± 1.5232 % of CDW) at 96 h of incubation in 100 % (v/v) DCW medium. The growth of *C. curvatus* was supported after the pre-treatment of cheese whey which involved alkaline treatment combined with hydrodynamic cavitations (HC) [Seo *et al.,* 2014]. In another study, *C. curvatus* NRRL Y-1511 yeast supported higher growth on ricotta cheese whey obtained after acid, salt, and deproteinization pretreatment [Carota *et al.,*2017]. Oleaginous yeast *Y. lipolytica* B9 strains grown in the non-sterile DCW medium and under optimized conditions achieved a lipid content of 2.73 ± 0.13 g/L (39 % of CDW). External supplementation of lactose and ammonium sulfate increased the lipid content to 4.29 g/L (58 % of CDW) [Taskin *et al*., 2015]. In the present study, the yeast could accumulate lipids higher than the previous reports and up to 5.6428 ± 0.0755 g/L (44.1210 ± 0.8497 % of CDW) lipid after 168 h of the incubation. The lipid productivities realized in this study are higher than most of the yeasts except for *C. curvatus* (Table 4.1). Although, longer incubation period leads to slightly low lipid productivity that can be addressed by optimizing the bioprocess for various parameters such as feeding strategy, C/N, etc. The lipid production was achieved without any external addition of C or N sources, which accentuates that the given yeast is the better candidate for cheese whey utilization.

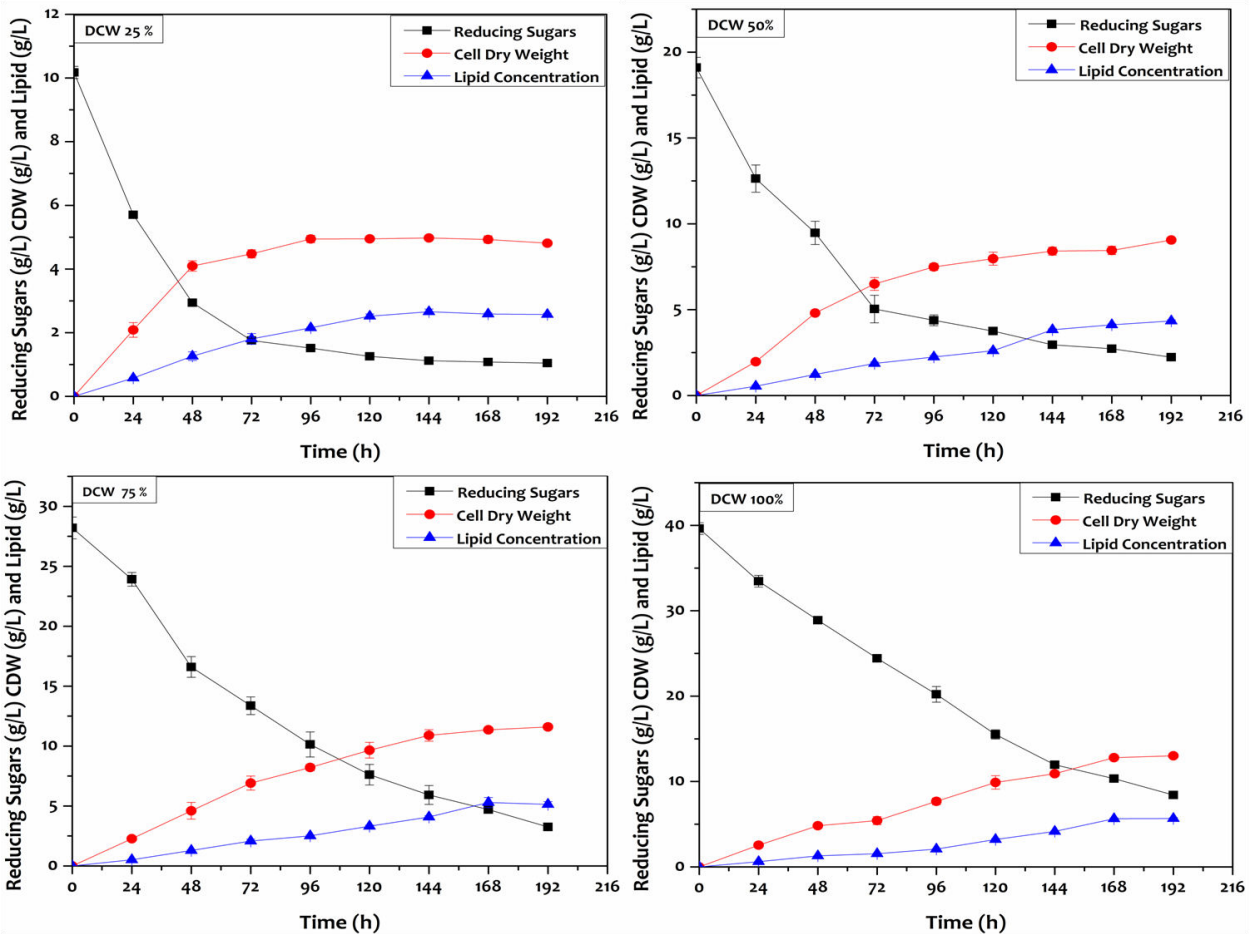


Figure 4.2 Lipid production with time on Deproteinized cheese whey (DCW): Showing 25 % DCW, 50 % DCW, 75 % DCW and 100 % DCW as the time course of lipid production

4.2.3 Lipid production in untreated cheese whey in shake flask experiments

In another set of experiments, the cheese whey was utilized without any pre-treatment. In this medium, also no external carbon or nitrogen sources were added. The C/N ratio was lower in this case as expected. The initial C/N ratio was in the order of 59, 58, 56, and 55 for 25, 50, 75, and 100 % (v/v) UCW media, respectively. The reducing sugar, CDW, and lipid accumulations were monitored every 24 h for all four concentrations, and the profiles can be seen in Figure 4.3. The cellular growth was higher in UCW as compared to DCW. Also, the CDW increased from 25 % to 100 % (v/v) UCW. The initial reducing sugar concentrations were 13.8894 ± 0.1256, 25.9444 ± 0.3446, 41.8868 ± 0.2256 and 56.4756 ± 1.7523 g/L for 25, 50, 75 and 100 % (v/v) UCW media respectively. The dry weight achieved were 6.3809 ± 0.1091, 12.5571 ± 0.4098, 17.8714 ± 0.9072 and 20.9809 ± 0.3640 g/L and lipid content were 2.7523 ± 0.0297, 3.5952 ± 0.1248, 4.2000 ± 0.0571, and 4.5714 ± 0.1362 (43.1374 ± 0.2760, 28.6312 ± 0.3598, 23.5360 ± 1.0598 and 21.7997 ± 1.0050 % of CDW) at 168 h interval for 25, 50, 75 and 100 % (v/v) UCW respectively. The lipid productivity observed was 0.0163 ± 0.0001, 0.0214 ± 0.0007, 0.0250 ± 0.0003 and 0.0272 ± 0.0008 g/L. h for 25, 50, 75 and 100 % (v/v) UCW respectively. The lipid production in the present study was higher than Y. *lipolytica* NCIM 3589 [Katre *et al.*, 2012]. Since the C/N ratios were lower for UCW, the lipid content dropped in UCW compared to DCW. The ability of C. *oligophagum* JRC1 to grow on untreated cheese whey and without any nutrient addition indicated its utility for cheese whey valorization in a sustainable way. Also, to date, no other oleaginous yeast has been explicitly reported to grow and accumulate lipids on

untreated cheese whey. The cultivation process can be further optimized for various biochemical parameters to further increase lipid productivity.

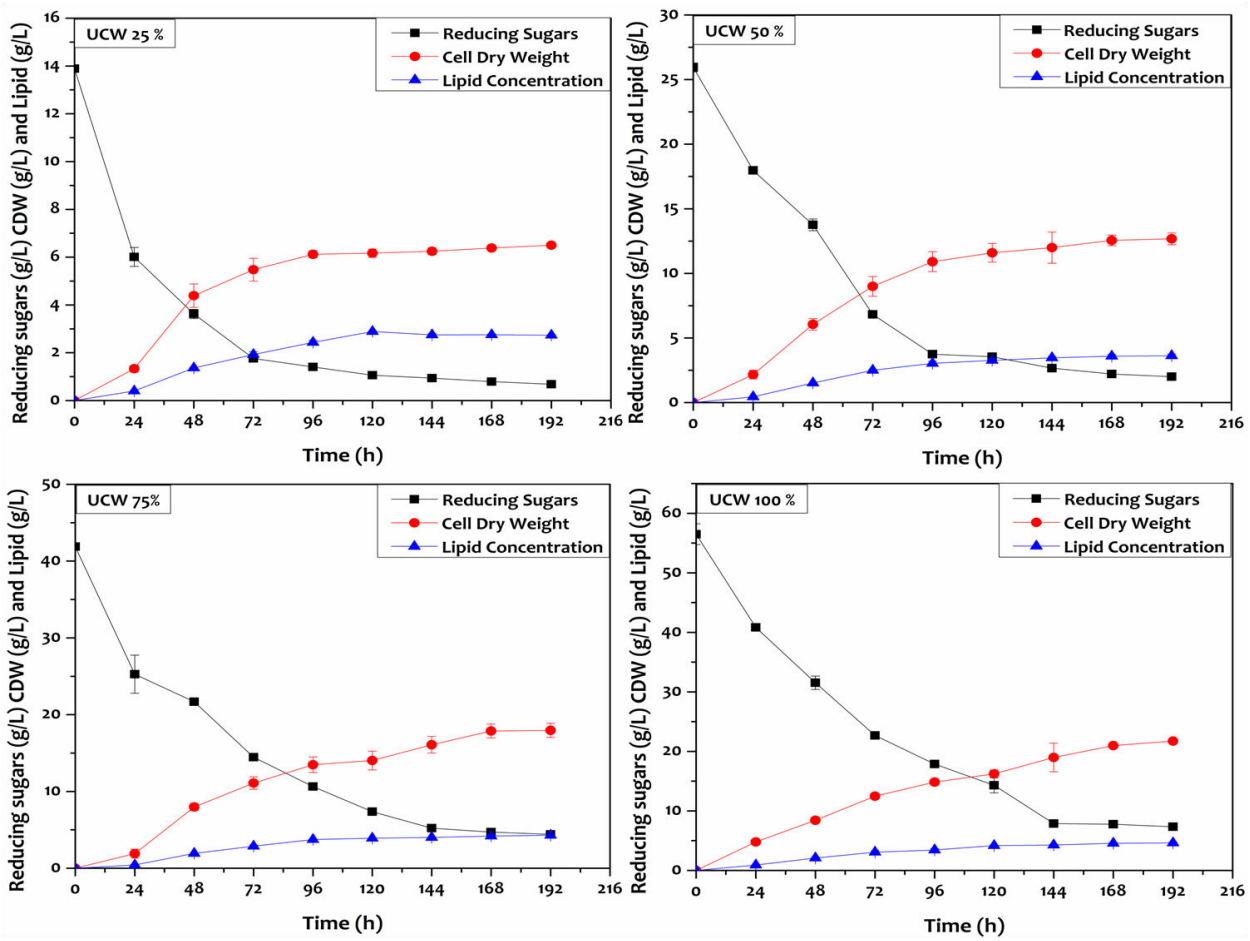


Figure 4.3: Lipid production with time on Untreated Cheese Whey (UCW): Showing 25 % UCW, 50 % UCW, 75 % UCW and 100 % UCW as the time course of lipid production

Table 4.1: Comparison of lipid production by oleaginous yeasts grown on different cheese whey sources

Organism	Types of the media	Culture mode	Incubation period (h)	Cell dry weight X (g/L)	Lipid production Y (g/L)	Lipid content (%)	Lipid productivity (g/L. h)	References
Debaryomyces etchellsii	Deproteinized cheese whey	Shake flask	96	2.80 ± 0.11	0.40 ± 0.05	15.90 ±0.93	0.0041	[Arous *et al.*, 2016]
Wickerhamomyces anomalus	Deproteinized Cheese whey	Shake flask	96	2.61 ± 0.03	0.65 ± 0.01	24.00 ± 0.24	0.0067	[Arous *et al.*, 2017]
Yarrowia Lipolytica B9	Deproteinized	Shake flask;	120	7.00 ±0.18	2.73 ± 0.13	39	0.0220	[Taskin *et al.*,

	cheese whey	non-sterile						2015]
Cryptococcus curvatus	Pretreated cheese whey (HC treatment)	Shake flask	24	7.2	4.68	65	0.195	[Seo *et al.*, 2014]
Cryptococcus curvatus NRRL Y-1511	Ricotta cheese whey	3-L stirred tank bioreactor	72	10.77 ± 0.21	6.83 ± 0.14	63.41	0.094	[Carota *et al.*, 2017]
Yarrowia lipolytica NCIM 3589	Cheese whey	Shake flask	72	2.6	0.33	13	0.004	[Katre *et al.*, 2012]
Cystobasidium oligophagum JRC1	DCW 25 % DCW 50 % DCW 75 % DCW 100 %	Shake Flask	168	4.9285 ± 0.1133 8.4523 ± 0.2450 11.3571 ± 0.2142 12.7904 ± 0.0786	2.5857 ± 0.0377 4.1190 ± 0.0501 5.2952 ± 0.4126 5.6428± 0.0755	52.4842 ± 1.5383 48.7481 ± 1.1386 46.6368 ± 3.7473 44.1210 ± 0.8497	0.0153 ± 0.0002 0.0245 ± 0.0020 0.0315 ± 0.0002 0.0335 ± 0.0040	(This study)
C.ystobasidium oligophagum JRC1	UCW 25 % UCW 50 % UCW 75 % UCW 100 %	Shake Flask	168	6.3809 ± 0.1091 12.5571 ± 0.4098 17.8714 ± 0.9072 20.9809 ± 0.3640	2.7523 ± 0.0297 3.5952 ± 0.1248 4.2000 ± 0.0571 4.5714 ± 0.1362	43.1374 ± 0.2760 28.6312 ± 0.3598 23.5360 ± 1.0598 21.7997 ± 1.0050	0.0163 ± 0.0001 0.0214 ± 0.0007 0.0250 ± 0.0003 0.0272 ± 0.0008	(This study)

(Where DCW: Deproteinized cheese whey, UCW: Untreated cheese whey)

4.2.4 Soluble COD removal and lipid generation

Cheese whey is characterized by high COD, which is due to the presence of oxidizable organic matters present in it [Carvalho *et al.*, 2013]. The growth of yeast on cheese whey converts the soluble COD to Volatile Suspended Solids (VSS), thereby treating it and rendering it suitable for discharge. The sCOD was monitored every 24 hours, and the profile is shown in Figure 4.4. The sCOD decreased from 66.3528 ± 1.5159 g/L to 10.0094 ± 0.1323 g/L after 7 days in 100 % DCW at the rate of 8.0490 ± 0.1980 g/L. d. Similarly, sCOD reduced down from 85.5392 ± 1.3232 to 11.2664 ± 0.1750 g/L after 7 days of incubation in 100 % UCW at the rate of 10.6103 ± 0.1656 g/L. d. The total sCOD removal was observed at 84.9125 ± 0.1553 and 86.8287 ± 0.0679 % for 100 % (v/v) DCW and UCW respectively. The initial sCOD removal rate was higher for UCW media than that of DCW media. The remainder of the sCOD might be due to the presence of inert molecules that resist degradation by yeasts. The sCOD removal rate and total removal achieved was higher for this yeast as compared to other yeasts reported in the literature. Different values for the COD removals are compared with the literature in Table 4.2. *D. etchellsii*

showed 58.3 % of COD removal at the rate of 14.05 g/L. d in an expired soft drink medium mixed with cheese whey (1:1) [Arous *et al.,* 2016]. In another study, 94.22 % COD removal at the rate of 0.106 g/L. d was achieved for paper and pulp industry wastewater by *R. kratochvilovae* HIMPA1 [Patel *et al.,* 2017b]. In yet another study, 68 % COD removal at the rate of 6.025 g/L. d was achieved for acetone-butanol-ethanol fermentation wastewater using *T. cutaneum* CH002 [Xiong *et al.,* 2015]. Similarly, *T. cutaneum* ACCC20271 treated cellulosic ethanol fermentation wastewater and removed 55.05 % COD at the rate of 13.054 g/L. d [Wang *et al.,* 2017]. In the present study, the initial sCOD was considerably higher, and the total sCOD removal was also higher than most of the studies involving yeast.

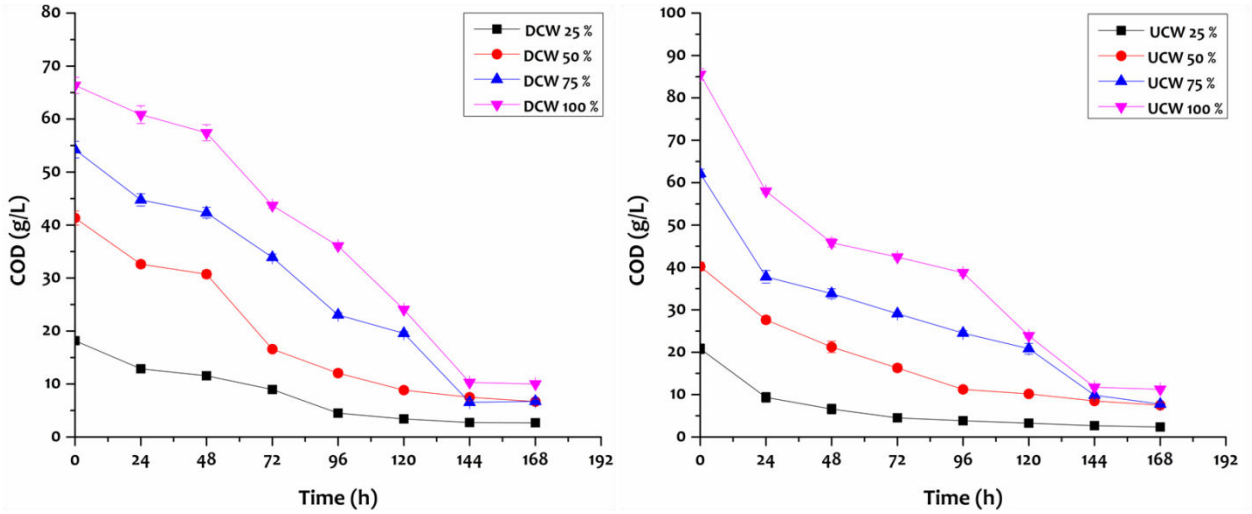


Figure 4.4: Change in sCOD with time for DCW and UCW: Showing soluble COD values for the time course of all DCW and UCW

Table 4.2: Comparison of COD removal from different types of wastewaters using oleaginous yeasts

Organism name	Type of the wastewater	Cell dry weight X (g/L)	Lipid content (%)	Initial COD (g/L)	Final COD (g/L)	COD removal (%)	Reference
Debaryomyces etchellsii	50 % Expired soft drink + 50 % Cheese whey	7.90	14.90	96.36	40.13	58.35	[Arous *et al.*, 2016]
	62.4 % expired soft drinks + 37.6 % olive mill wastewater	4.6	28.90	113.26	66.48	41.30	
***Rhodosporidium kratochvilovae* HIMPA1**	Paper and pulp Industry wastewater	13.87	61.71	0.675	0.039	94.22	[Patel *et al.* 2017b]

***Trichosporon cutaneum* CH002**	Acetone-Butanol-ethanol (ABE) Fermentation wastewater	4.9	14.7	18.050	6.00	68	[Xiong *et al.*, 2015]
***Trichosporon cutaneum* ACCC20271**	Cellulosic ethanol fermentation wastewater	16.20	13.33	118.58	53.31	55.05	[Wang *et al.*, 2017]
***Cystobasidium oligophagum* JRC1**	Deproteinized cheese whey (100 %)	12.7904 ± 0.0786	44.1210 ± 0.8497	66.3528 ± 1.5159	10.0094 ± 0.1323	84.9125 ±0.1553	(This study)
	Untreated cheese whey (100 %)	20.9809 ±0.3640	21.7997 ± 1.0050	85.5392 ± 1.3232	11.2664 ± 0.1750	86.8287 ±0.0679	(This study)

(Where COD: Chemical oxygen demand)

4.2.5 FT-IR, TLC, and ^{1}H NMR analysis

The FT-IR analysis was done for all the lipid extracts. The peak assignment was done according to previous studies [Tariq *et al.,* 2011]. As shown in Figure 4.5, the broader peak at 3400 cm^{-1} indicates the stretching vibration of -O-H group due to the presence of a water molecule. The lipid extracts from DCW and UCW showed three characteristic peaks in the range of 2800 to 3000 cm^{-1}. These peaks appear due to the presence of symmetric vibrations of -CH_3, -CH_2, and -CH. The peaks in the range of 1450-1370, 1300-1100, and peaks nearby 720 cm^{-1} show the asymmetric vibrations for -CH_3, -CH_2, and -CH, respectively. A sharp peak obtained at nearly 1750 cm^{-1} in all samples confirms the presence of the carbonyl (-C=O) functional group. TLC was performed on a silica gel, and lipid extracts from all the different experiments were analyzed. Triolein standard was used as a standard for the TLC analysis. After incubation at a higher temperature, different spots were identified on the TLC plates. The concentration of the TAG was the highest, followed by DAG and MAG. ^{1}H NMR spectra of the lipid extract were recorded, and the characteristic peaks for triglycerides were assigned according to the previous studies [Sarpal *et al.,* 2014; Tariq *et al.,* 2011]. As shown in Figure 4.6, all the samples showed peaks at 4.0-4.35 ppm indicated the presence of (-OCH_2) functional group. Other peak values such as 2.30, 5.20, 5.05-5.65, 2, 1.26, and 1.30 ppm were observed, which indicated the presence of -CH_2C=O, -OCH, -CH=CH, -CH_2CH=CH, -$(CH_2)_n$ and -CH_3 groups respectively. The sharp peaks in between 3-4 ppm are due to glycol/phospholipids. The triplet at 2.3 ppm represents free fatty acids. The peaks at 2.7-2.8 ppm show the presence of C18: N (N=1-3), which were comparable with the GC results.

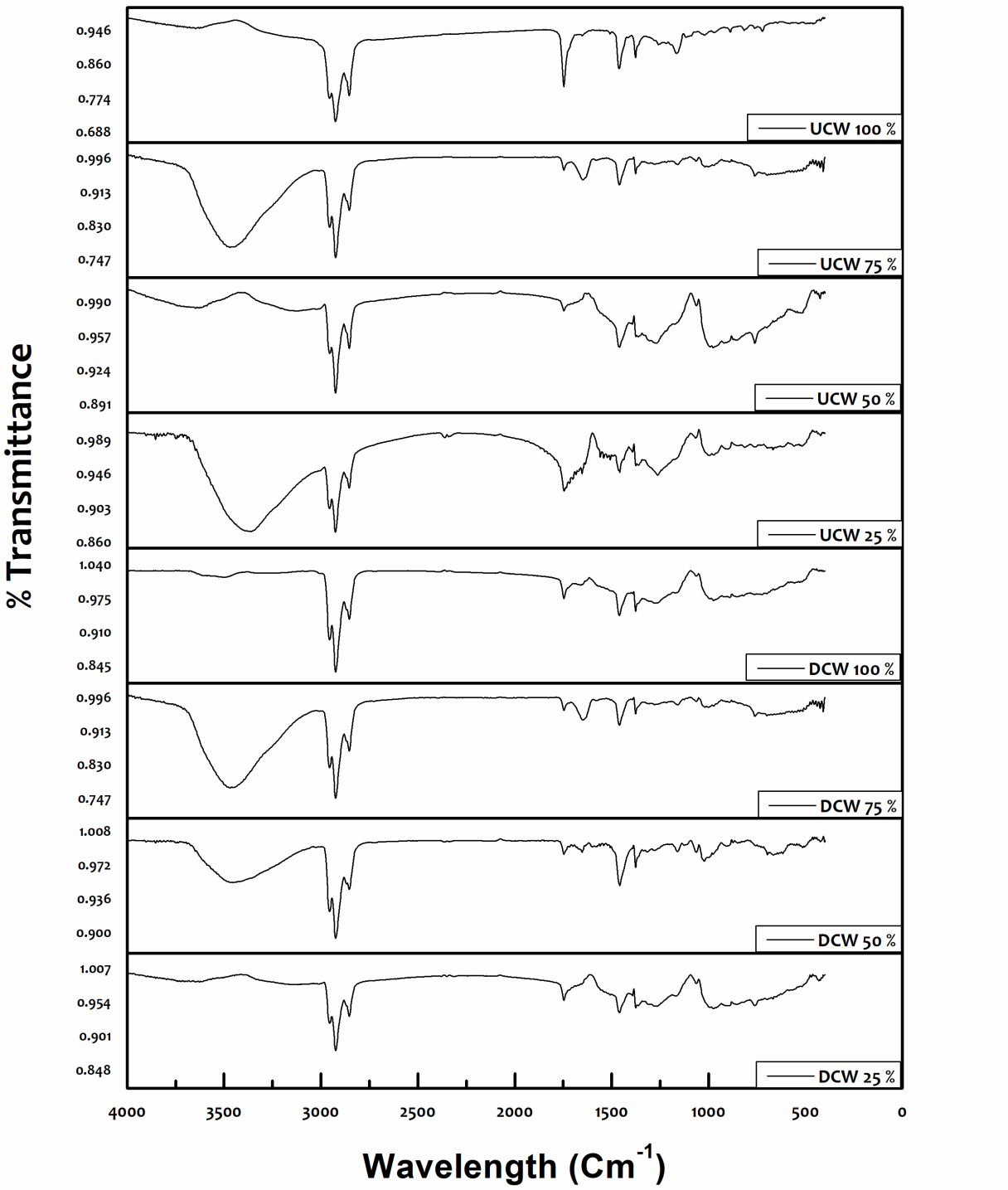


Figure 4.5: FT-IR spectra recorded for the lipid extracts obtained from yeast cultivated in DCW and UCW

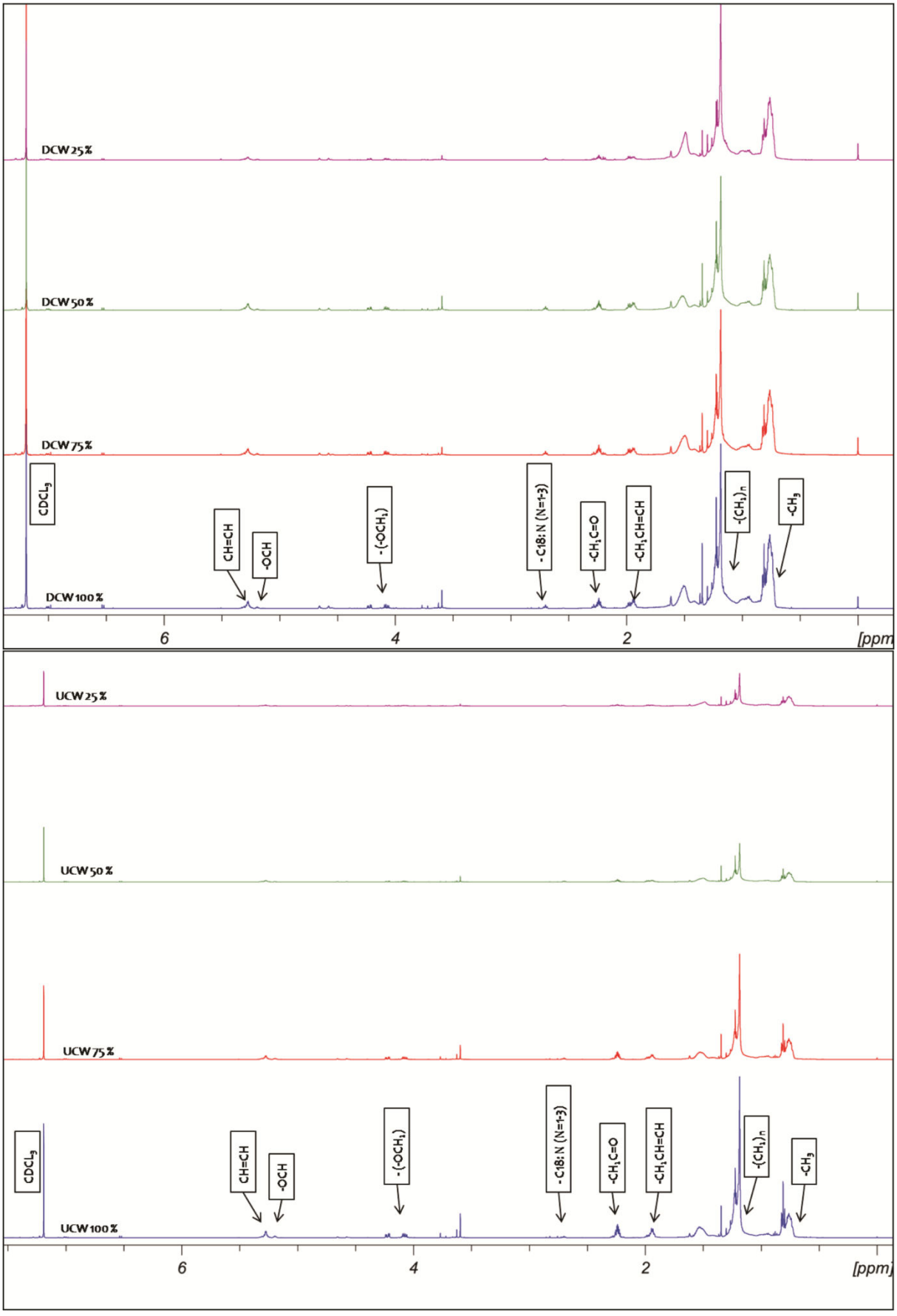
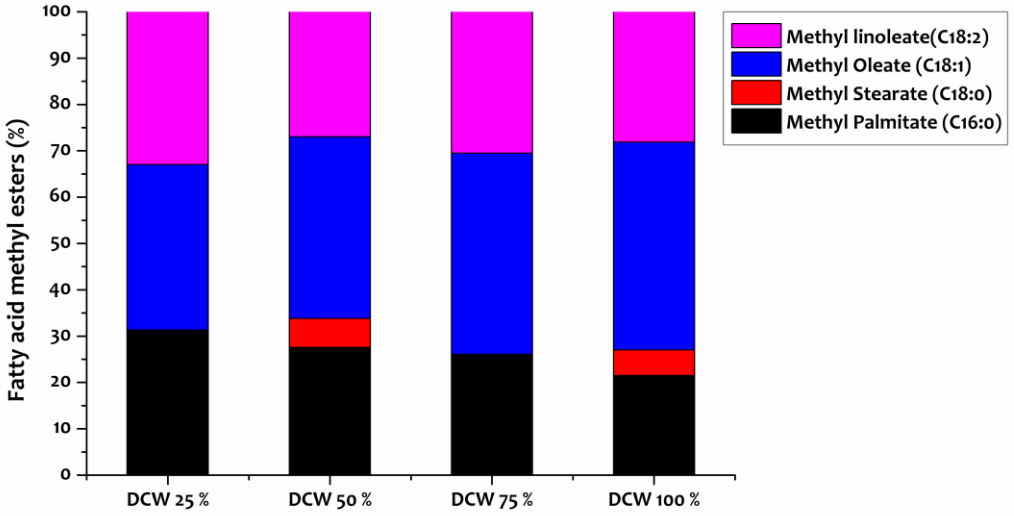


Figure 4.6: Proton NMR spectra recorded for the lipid extracts obtained from yeast cultivated in DCW and UCW

4.2.6 Lipid profile and GC analysis of the samples

The FAME profile of *C. oligophagum* JRC1 cultivated on different concentrations of DCW and UCW measured at 168 h of the fermentations are shown in Figure 4.7. Of the total FAME, 93.7- 99.9 % were a mixture of C16 and C18 saturated fatty acids (SFA) as well as unsaturated fatty acids. The FAME profiles for DCW and UCW were having slight differences in the concentrations of SFA. The FAME profiles for both DCW as well as UCW were comparable to the FAME composition obtained on glucose medium, as previously reported (chapter 3). The lipid samples contained 68- 72 and 56-71 % of unsaturated fatty acid methyl ester for DCW and UCW, respectively. The linoleic acid methyl esters (C18:2) were in the range of 28-32 and 13-31, while oleic acid methyl esters (C18: 1) were in the range of 35-44 and 29-43 % for the DCW and UCW respectively. SFA, such as palmitic acid methyl ester (C16:0) was in the range of 21-31 and 22-29, while stearic acid methyl ester (C18:0) was in the range of 5-6 and 5-9 % for DCW and UCW media respectively.

The values were comparable with other oleaginous yeasts. *W. anomalus* grown on DCW medium showed similar composition containing palmitic acid (C16:0), and stearic acid (C18:0) methyl ester while oleic acid (C18:1) methyl esters and linoleic acid methyl ester (C18:2) was lower than in the present studies [Arous *et al*., 2017]. The higher amounts of oleic acid (C18:1) methyl esters indicated better biodiesel properties [Arous *et al*., 2017; Sitepu *et al*., 2014a]. When compared with other oleaginous yeast cultivated on cheese whey, the lipid profiles were nearly the same [Arous *et al*., 2016]. The differences in the lipid composition are inevitable, considering the heterogeneity of substrates used and the yeast species employed [Sitepu *et al*., 2014a]. In general, longer incubation period supports a better fatty acid profile for biodiesel generation [Sitepu *et al*., 2013]. Yet, the overall FAME composition was of high quality and comparable with different plant and vegetable oils [Ramos *et al*., 2009].



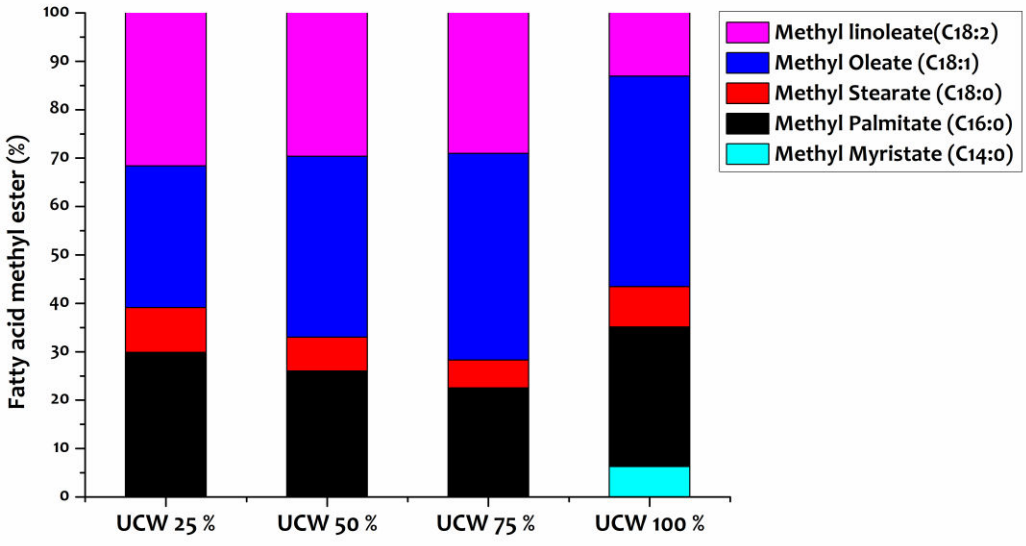


Figure 4.7: FAME compositions obtained by GC-FID analysis: Showing comparison of FAME compositions for the lipid extracts from DCW and UCW medium

4.2.7 Biodiesel potential of FAME

Based on the data available from the GC experiments, some predictions on biodiesel properties can be made using the formulas (Annexure A) [Deeba *et al*., 2018; Patel *et al.,* 2017a]. The calculated values were compared with ASTM D6751 (US biodiesel specification) and EN14214 (European biodiesel specification) standards, as shown in Table 4.3. These properties are used to assess the ability of feedstock to be used as automotive fuel, and the values must be within the prescribed ranges. The higher cetane number (CN), longer oxidative stability, and low cold filter plug point are the criteria for proper engine performance [Deeba *et al*., 2018]. CN is a measurement of fuel's autoignition quality [Knothe, 2014]. As shown in Table 4.3, the CN was in the range of the standard values of 49-60. The CN value above 50 reduces the formation of white smoke [Balat and Balat, 2010]. Oxidative stability is which determines the shelf-life of the fuel [Patel *et al.,* 2017a] was higher for UCW lipids (11.62 h) as compared to DCW media (6h). However, both of the values fall in the required range. Kinematic viscosity (KV) determines the ability of the fuel to flow. The values were in the standard range of 1.9-6.0 mm^2/second. Iodine value (IV) is a measurement of unsaturation with all the samples exhibiting moderate unsaturation. Cold filter plug points (CFPP) determines the freezing point of the fuel, and as per the standard ASTM or EN, the CFPP values were low and better than that of vegetable oils and jatropha oils [Ramos *et al*., 2009].

Table 4.3: Biodiesel properties predicted for DCW and UCW from the GC data

Quality parameters	DCW 25 %	DCW 50 %	DCW 75 %	DCW 100 %	UCW 25 %	UCW 50 %	UCW 75 %	UCW 100 %	ASTM standard	EN standard
Saponification value (SV) (mg KOH)	205.02	203.33	203.95	202.90	204.57	203.81	203.08	203.63	N.D.	N.D.
Iodine value (IV) (g I2/100 g)	91.84	84.12	94.29	91.29	83.56	87.26	90.95	50.93	N.D.	120 (max.)
Cetane number (CN) (min)	49.51	51.69	49.02	49.92	51.68	51.25	49.97	60.11	47	51
Degree of Unsaturation (DU) (% w)	101.67	93.16	104.42	101.12	92.48	96.61	100.72	69.60	N.D.	N.D.
Long chain saturation factor (LCSF) (% w)	3.132	5.887	2.608	4.912	7.621	6.095	5.145	7.052	N.D.	<5/<-20
Cold filter plug points (CFPP) (^oC)	-5.77	3.63	-7.566	0.314	9.56	4.34	1.1	7.619	N.D.	N.D.
High Heating value (HHV) (MJ/kg)	39.640	39.833	39.655	39.740	39.796	39.764	39.739	40.317	N.D.	N.D.
Kinematic viscosity (KV) (mm^2/sec)	3.7073	4.4827	3.7073	4.4827	4.4827	4.4827	4.4827	4.9726	1.9-6.0	3.5-5.0

Density (D) (g/cm^3)	0.887	0.886	0.887	0.886	0.886	0.886	0.886	0.885	N.D.	0.86-0.90
Oxidative stability (OS) (h)	6.16	6.96	6.45	6.78	6.32	6.57	6.653	11.62	N.D.	≥ 6

(Where N.D.: Not Determined, DCW: Deproteinized cheese whey, PCW: Proteinized cheese whey, ASTM: American Society for Testing and Materials, EN: European Norms)

4.3 CONCLUSIONS

The oleaginous yeast *C. oligophagum* JRC1 was successfully grown on cheese whey. The deproteinized cheese whey supported higher lipid content, while untreated cheese whey supported high cellular biomass. Higher sCOD removal was obtained in both of the conditions. The lipid productivities were considerably higher. The lipid profile was assessed, and the composition was suitable for biodiesel feedstock. The predicted biodiesel properties were also in accordance with ASTM and EN standards. The study shows the potential of the given yeast for the utilization of cheese whey. Further, process optimization can increase cellular yield and lipid yield.

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