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Cystobasidium oligophagum JRC1 Tolerance Assessment on Inhibitors Released on Lignocellulosic Biomass Hydrolysis and Growth on Acid Hydrolyzed Agro-industrial Wastes

Lignocellulosic biomass consists of cellulose (40-53 %), hemicelluloses (11-35 %) and lignin (15-28 %), and it is a most abundant organic material in the world [Chandel *et al*., 2013; Osorio-Gonzalez *et al*., 2019; Zhao *et al*., 2012]. The cellulose and hemicellulose are hydrolyzed to simple sugars before being used as a growth substrate [Leiva-Candia *et al*., 2014]. Pre-treatment methods include acid/alkali, mechanical, thermal, oxidative, steam explosion, liquid hot water extraction, microwave, biological, ammonia fiber explosion, and enzymatic treatment. The pretreatment liberates pentoses (C_5), mainly xylose and hexose (C_6) such as glucose, mannose, rhamnose, arabinose, and galactose [Matsakas *et al*., 2017; Patel *et al*., 2016]. Dilute acid treatment is one of the rapid, cheaper, and widely used techniques for the purpose [Zhao *et al*., 2012]. However, acid hydrolysis also releases certain toxic compounds that inhibit microbial growth [Palmqvist and Hahn-Hagerdal, 2000]. The inhibitors include furan derivatives [furfural, 5-hydroxymethyl furfural (5-HMF)], organic acids (acetic acid, levulinic acid, formic acid, etc.,) [Osorio-Gonzalez *et al*., 2019; Palmqvist and Hahn-Hagerdal, 2000; Sitepu *et al*., 2014b]. These inhibitors can have a sufficient inhibitory effect on growth and lipid accumulation by yeast [Zhao *et al*., 2012]. Therefore, pre-treatments are often followed by inhibitor removal [Patel *et al*., 2016]. Yeast capable of growing and accumulating lipids in the presence of the inhibitors is desirable for cost-effective oil production

Different species exhibit differential tolerance to inhibitors [Chen *et al*., 2009; Martin and Jonsson, 2003]. It is necessary to obtain the inhibitor tolerance profile of oil [Almeida *et al*., 2007]. The literature presents several reports mentioning the effect of lignocellulosic inhibitors on the growth of yeast and ethanol production [Sakai *et al*., 2007; Taherzadeh *et al*., 2000], as well as lipid accumulation [Osorio-Gonzalez *et al*., 2019; Patel *et al*., 2016].

In this study, the effect of the three most common lignocellulosic inhibitors [Sitepu *et al*., 2014b] furfural, 5-HMF, and acetic acid on growth and lipid accumulation by *C. oligophagum* JRC1 was studied. The objective of the study was to assess the suitability of isolate to grow on undetoxified lignocellulosic acid hydrolysates. Further, the isolate was cultivated on undetoxified & detoxified acid hydrolysate of sugarcane bagasse and corn cob (containing xylose) [Figure 5.1].

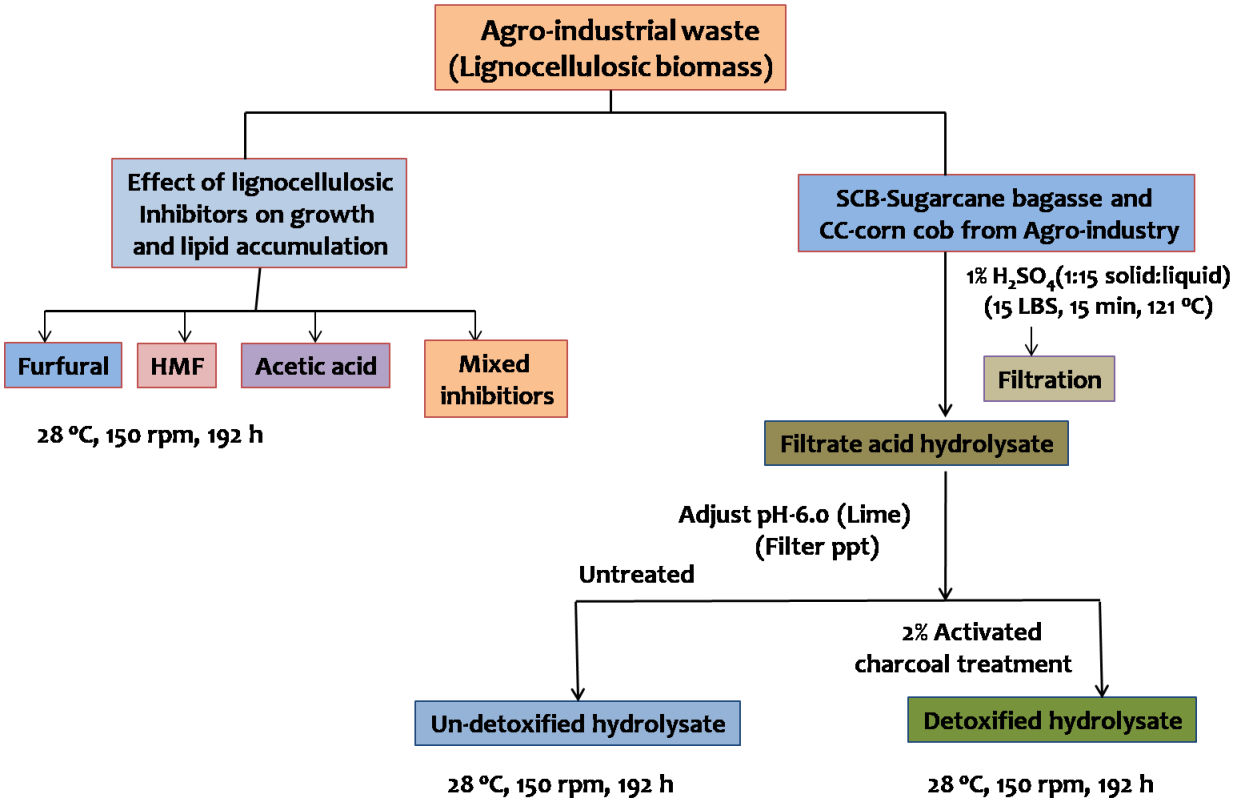

Figure 5.1: Summary of the work

5.1 EFFECT OF LIGNOCELLULOSIC INHIBITORS AND ACID HYDROLYSATES

5.1.1 Dilute acid treatment of sugarcane bagasse and corn-cobs

The Sugarcane bagasse (SCB) was obtained from the sugar factory based in Bardoli, Gujarat (India). The Corn-cobs (CC) were obtained from the nearby food-processing industry unit. The substrates were dried and grounded to achieve 2-10 mm particle size followed by treatment with 1 % H_2SO_4 (Dilute acid) at a solid to liquid ratio of 1:15 (w/v). The digestion was carried out at 121 ^{0}C and 15 psig for 30 minutes (in an autoclave) [Brar *et al*., 2017]. The digested biomass was filtered using a muslin cloth, and the filtrate was labelled as acid hydrolysate. The pH of the acid hydrolysate was adjusted to 6.0 using $Ca(OH)_2$ (lime) followed by precipitate removal by filtration using Whatman filter paper. The solution so obtained was labeled as undetoxified hydrolysate. When the filtrate was treated with 2 % activated charcoal for 2 h at 40 ^{0}C [Brar *et al*., 2017], it was labeled as detoxified hydrolysate.

5.1.2 Organism and the culture conditions

C. oligophagum JRC1 was cultivated on the lipid production medium containing (g/L): Glucose, 40; KH_2PO_4, 7; NaH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4$. $7H_2O$, 1.5; Vitamins (mg/L): D-biotin, 0.002; calcium pantothenate, 0.4; folic acid, 0.002; inositol, 2; niacin, 0.4; para-aminobenzoic acid (PABA), 0.2; pyridoxine HCl, 0.4; riboflavin, 0.2; thiamine, 0.4, 100 mL. The three different inhibitors (5-HMF, Furfural and acetic acid) were added at the concentration of 0.5, 1.0, 1.5 and 2.0 g/L in three separate flasks containing the lipid production medium. In another experiment, the three inhibitors were mixed at equal ratio of (1:1:1) and added at (g/L) for four separate flasks: for 0.1 g/L of mixed inhibitors (0.1 g/L, 5-HMF + 0.1 g/L, Furfural + 0.1 g/L, Acetic acid), for 0.25 g/L of mixed inhibitors (0.25 g/L, 5-HMF + 0.25 g/L, Furfural +

0.25 g/L, Acetic acid),for 0.5 g/L of mixed inhibitors (0.5 g/L, 5-HMF + 0.5 g/L, Furfural + 0.5 g/L, Acetic acid), and for 1.0 g/L of mixed inhibitors (1.0 g/L, 5-HMF + 1.0 g/L, Furfural + 1.0 g/L, Acetic acid), into NLM medium. The lipid production medium without any inhibitor served as control. The undetoxified (SCBUT and CCUT) and detoxified acid hydrolysate (SCBT and CCT) of SCB and CC, along with the Yeast nitrogen base (YNB) was used to cultivate the isolate in separate experiments.

The inoculum was prepared by cultivating the isolate in 100 mL YPD medium (yeast extract, 10; Peptone, 20; and glucose, 10 g/L) at 28 °C and 150 rpm. A 5 mL of the log-phase culture (approximately 1.74×10^8 cells/mL) was aseptically centrifuged at 8000 rpm for 15 min separating the cells and supernatant. The supernatant was discarded, and the cells were re-suspended in sterile saline (0.85% NaCl) and centrifuged again. This step was repeated twice. The washed cells were finally re-suspended in 5 mL sterile saline and introduced into the all sterile media aseptically. All the flasks, along with control, were incubated at 28 ^{0}C, 150 rpm.

5.2 RESULTS AND DISCUSSION

5.2.1 Effect of lignocellulosic inhibitors on the growth and lipid accumulation by *C. Oligophagum* JRC1

The three most common inhibitors found in the lignocellulosic hydrolysates are Furfural, 5-Hydroxymethyl Furfural (5-HMF), and Acetic acid. The concentrations vary according to the type of feedstock, conditions, and the pre-treatment method [Almeida *et al.*, 2007; Sitepu *et al.*, 2014b]. In general, concentrations of inhibitors range from 0.2-2.8, 0-3.4, and 0.2-7.8, g/L for Furfural, 5-HMF, and acetic acid, respectively [Sitepu *et al.*, 2014b]. For example, acid-treated sugarcane bagasse contains 0.41, 0.07, 2.40 g/L for Furfural, 5-HMF, and acetic acid, respectively. On the other hand, acid pretreated corn stover hydrolysate contains 0.10, 0.51, and 2.30 g/L for Furfural, 5-HMF, and acetic acid, respectively [Zha *et al.*, 2012]. The growth and lipid accumulation by *C. oligophagum* JRC1 was studied at different concentrations of these inhibitors. Generally, the inhibitor profile and their concentrations change with the change in the feedstock and treatment method.

5.2.1.1 Effect of 5-Hydroxymethyl Furfural (5-HMF)

For example, high temperature and pressure during pre-treatment degrade hexoses into furan derivatives such as 5-HMF and furfural [Patel *et al*., 2016]. Inhibitor tolerance also varies from species to species [Martin and Jonsson, 2003]. The species having high resistance is an obvious choice for a bioprocess. Figure 5.2 shows the reducing sugar consumption, as well as the growth profile of isolate, recorded every 24 h in the presence of 5-HMF. The sugar consumption rate was rapid in the first 72 h at all 5-HMF concentrations. The sugar uptake rate declined by 33.00 % at 0.5 g/L of 5-HMF, 55.78 % at 2.0 g/L of 5-HMF when compared to control. The cellular growth also dropped proportionally with increase 5-HMF. The CDW at the end of the experiment (192 h) was 11.0460 ± 0.6025, 10.2570 ± 0.4686, 9.2234 ± 0.5913, 8.3318 ± 0.4686 and 8.2292 ± 0.4574 g/L at 0, 0.5, 1.0, 1.5, and 2.0 g/L of 5-HMF respectively. The 5-HMF was least toxic amongst all the inhibitors, and the same has been observed earlier [Hu *et al*., 2009; Taherzadeh *et al*., 2000]. The 5-HMF is believed to have an inhibitory effect on the hexokinase & glyceraldehydes-3-phosphate dehydrogenase, thus inhibiting glycolysis and Tricarboxylic acid (TCA) pathway [Horvath *et al*., 2003].

The lipid accumulation and lipid content can be seen in Table 5.1. The lipid dry weight was 4.5105 ± 0.5649, 3.4695 ± 0.3005, 2.8465 ± 0.0884, 2.2991 ± 0.2689, and 2.1799 ± 0.0202 g/L (40.7554 ± 2.8910, 33.7940 ± 1.3858, 30.9566 ± 2.9439, 27.5475 ± 1.6778 and 26.5374 ± 1.7210 % of CDW) at 0, 0.5, 1.0, 1.5 and 2.0 g/L 5-HMF. Furans often inhibit the synthesis of the precursor

needed for lipid synthesis [Beopoulos *et al*., 2011; Osorio-Gonzalez *et al*., 2019]. It can be seen from the results that the isolate could accumulate lipids even at 2 g/L of 5-HMF. Similar observations were made earlier on *T. cutaneum* ACCC 20271, which grew at 5.0 g/L of 5-HMF attaining 35 .3 % of cell dry weight and 24.7 % (of CDW) of lipid content [Wang *et al*., 2016].

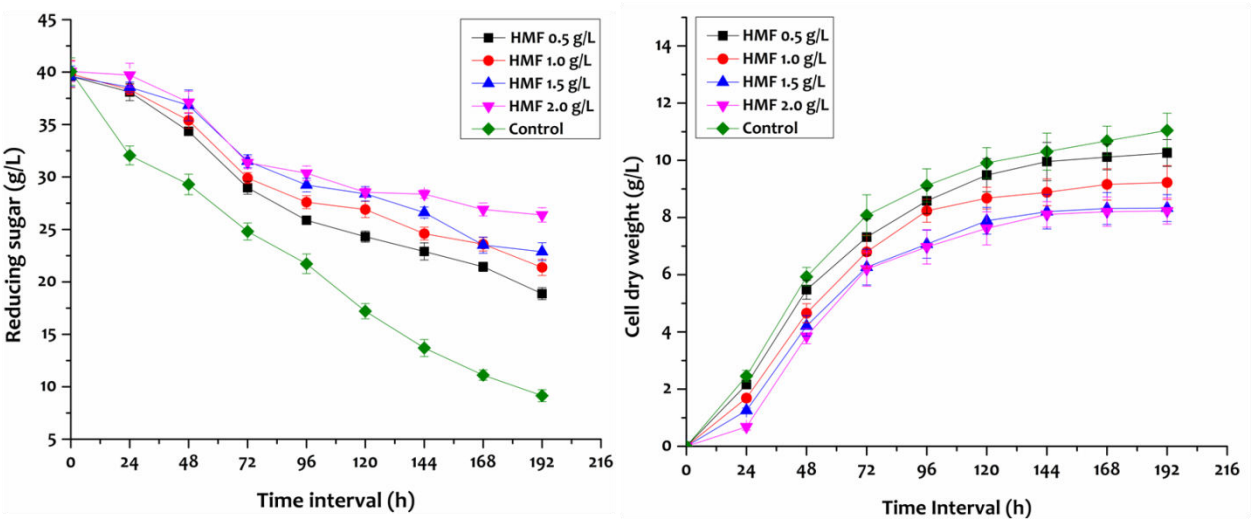

Figure 5.2: Effect of 5-HMF on reducing sugar uptake and cellular growth

Table 5.1 Lipid accumulation

	Concentration (g/L)	Cell dry weight (g/L)	Lipid production (g/L)	Lipid content (% w/w)
Control	**0.0**	11.0460 ± 0.6025	4.5105 ± 0.5649	40.7554 ± 2.8910
5-HMF	**0.5**	10.2570 ± 0.4686	3.4695 ± 0.3005	33.7940 ± 1.3858
	1.0	9.2234 ± 0.5913	2.8465 ± 0.0884	30.9566 ± 2.9439
	1.5	8.3318 ± 0.4686	2.2991 ± 0.2689	27.5475 ± 1.6780
	2.0	8.2292 ± 0.4574	2.1799 ± 0.0202	26.5374 ± 1.7210
Furfural	**0.5**	1.5622 ± 0.4240	0.4038 ± 0.0954	25.9765 ± 0.9412
	1.0	1.0730 ± 0.3570	0.2210 ± 0.0452	21.0599 ±2.7903
	1.5	No growth	N.A.	N.A.
	2.0	No growth	N.A.	N.A.
Acetic acid	**0.5**	8.9946 ± 0.6471	3.1100 ± 0.3648	34.5197 ± 1.5727
	1.0	6.3909 ± 0.6471	1.7625 ± 0.3199	27.4663 ± 2.2252
	1.5	No growth	N.A.	N.A.

	2.0	No growth	N.A.	N.A.
Mixed inhibitors	**0.1**	2.8246 ± 0.4463	0.9221 ± 0.2163	32.4449 ± 2.5336
	0.25	1.4359 ± 0.6025	0.4367 ± 0.1641	30.7214 ± 1.4616
	0.5	No growth	N.A.	N.A.
	1.0	No growth	N.A.	N.A.

[Where 5-HMF: 5-Hydroxymethyl furfural; N.A.: Not attempted]

5.2.1.2 Effect of Furfural

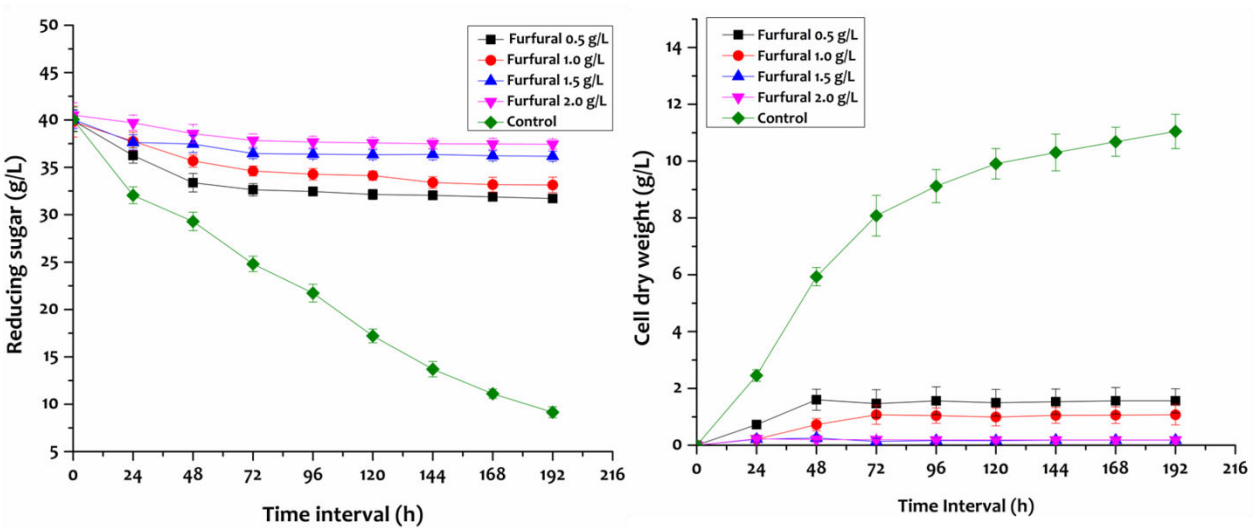

Figure 5.3: Effect of Furfural on reducing sugar uptake and cellular growth

Furfural is found at a lower concentration in the hydrolysates when compared to HMF, yet it is a very toxic [Taherzadeh *et al*., 2000; Chandel *et al*., 2013]. The studies in the literature show that most of the isolates cannot grow beyond 1.0 g/L furfural [Palmqvist and Hahn-Hagerdal, 2000]. Low concentrations also reduce cell growth by 45-50 % [Almeida *et al*., 2007]. In our study, four different concentrations were chosen (0.5-2.0 g/L), and results were alike literature. The time course of sugar uptake and cellular growth is shown in figure 5.3. The sugar uptake dramatically decreased by up to 73 % at 0.5 g/L to up to 99.62 % at 2.0 g/L. At 0.5 and 1.0 g/L, the growth reduced up to 85.52, and 90.64 % and the CDW achieved was 1.5622 ± 0.4240 and 1.0730 ± 0.3570 g/L, respectively. Similar results were observed for *R. toruloides,* which showed 80 % of inhibition in the cellular growth at 0.5 g/L of furfural and could not grow at 2.0 g/L concentration [Hu *et al*., 2009]. However, in another report, *R. toruloides* could grow at 2.0 g/L of furfural with 67 % of inhibition [Zhao *et al*., 2012; Chen *et al*., 2013; Sitepu *et al*., 2014b]. With few exceptions, such as *P. stipitis,* which grow uninhibited at 0.5 g/L furfural, most of the yeast species are sensitive to furfural [Roberto *et al*., 1991]. Furfural is believed to generate reactive oxygen species, which damages the chromatin, mitochondrial membrane, and other cell components severely affecting the growth [Almeida *et al*., 2007].

The lipid accumulation with furfural was 0.4038 ± 0.0954 and 0.2210 ± 0.0452 g/L (25.9765 ± 0.9412 and 21.0599 ± 2.7903 of their % CDW) at 0.5, and 1.0 g/L respectively. The overall lipid production was inhibited by 91.2 and 95.2% at 0.5 and 1.0 g/L, respectively. Furfural was also reported to inhibit the malic enzyme, redirecting the pool of NADPH and intracellular ATP, inhibiting fatty acid synthesis [Huang *et al*., 2011].

5.2.1.3 Effect of Acetic acid

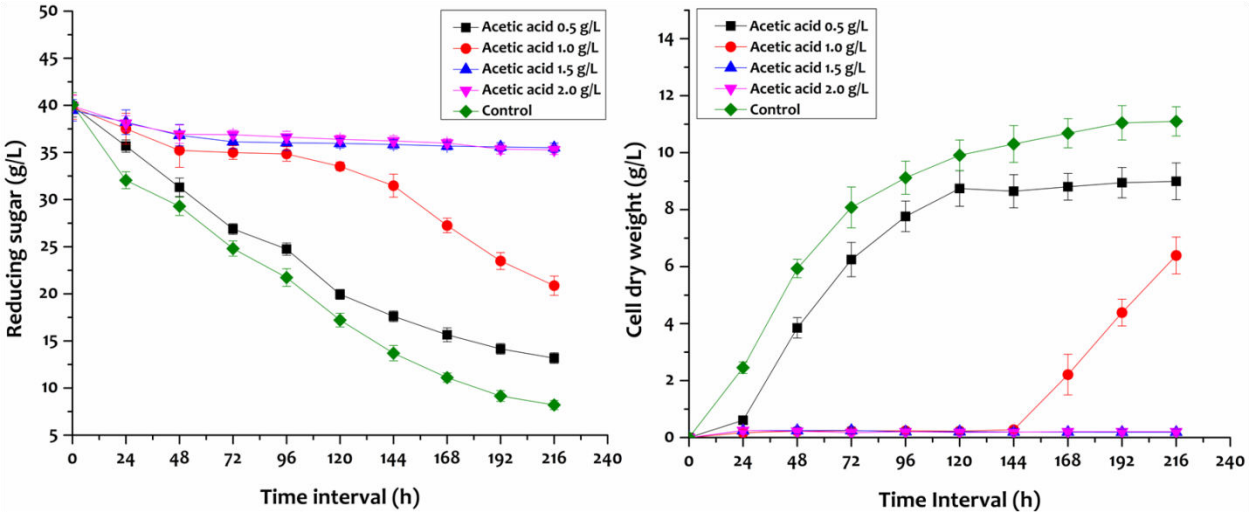

Figure 5.4: Effect of Acetic acid on the reducing sugar uptake and cellular growth

Acetic acid is produced during the de-acetylation of hemicelluloses. The acetic acid in its undissociated form tends to cross the cell membranes and enters the cytosol decreasing the cytosolic pH [Palmqvist and Hahn-Hagerdal, 2000]. Therefore maintenance of intracellular pH becomes necessary for cell survival, and it happens at an energy cost. Thus, the maintenance energy increases and the energy available for cell synthesis and growth reduces [Palmqvist and Hahn-Hagerdal, 2000]. The time course for sugar uptake and cellular was recorded in the presence of acetic acid, as shown in Figure 5.4. The sugar uptake was reduced by 15.15 % at 0.5 g/L acetic acid. Culture exhibited a lag phase of 144 h at 1.0 g/L of acetic acid. It caused a significant delay in growth and sugar consumption. The final CDW obtained was 8.9946 ± 0.6471 and 6.3909 ± 0.6471 at 0.5 and 1.0 g/L acetic acid, respectively, after 216 h of fermentation. Concentrations at 1.5 and 2.0 g/L did not support growth. The (pKa) value for acetic acid is 4.75; at this pH, the dissociated and undissociated forms are equal. Thus, the pH of the broth plays an important role [Liu *et al*., 2015; Palmqvist and Hahn-Hagerdal, 2000]. At the pH value less than the pKa, the undissociated form of acetic acid pre-dominates leading to longer lag phase and delayed cellular growth. This was observed during the cultivation of *T. fermentans* [Liu *et al*., 2015]. While, pH value higher than pKa will support the dissociated form of acetic acid allowing yeast such as *R. toruloides* Y4 to utilize it as the carbon source for the lipid synthesis directly [Hu *et al*., 2009]

The lipid accumulation was 3.1100 ± 0.3648 and 1.7625 ± 0.3199 g/L (34.5197 ± 1.5727 and 27.4663 ± 2.2252 % of CDW) for 0.5 and 1.0 g/L of acetic acid respectively. Literature studies indicate very high acetic acid tolerance in some of the species, such as *T. cutaneum*

ACCC 20271, which could survive at 25.0 g/L of acetic acid with only 49.6 and 34.0 % reduction in CDW and lipid content respectively [Wang *et al*., 2016].

5.2.1.4 Effect of a mixture of inhibitors

The hydrolysate is expected to contain all the inhibitors at different concentrations [Hu *et al*., 2009]. Therefore, the effect of all three inhibitors on sugar consumption and growth was studied. The results can be seen in Figure 5.5.

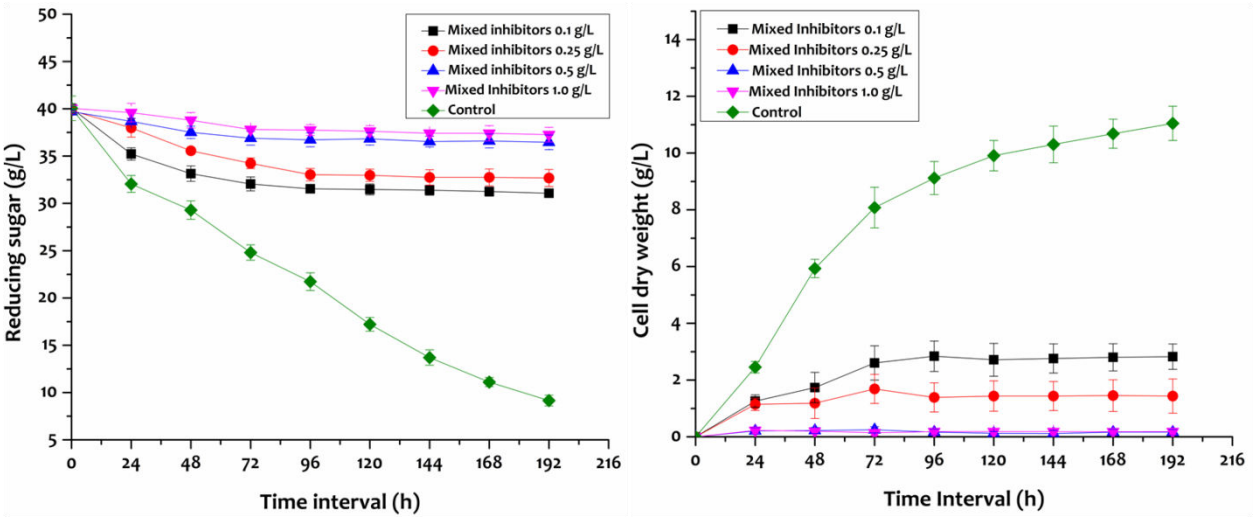

Figure 5.5: effect of mixed inhibitors on the reducing sugar uptake and cellular growth

The mixture exhibited higher inhibition of cell growth compared to individual inhibitors. Also, the mixed inhibitors at their minimum concentration (0.1 g/L) showed substantially reduced cell growth. The sugar uptake dropped by 69.5 % at 0.1 g/L to 99.06 % for 1.0 g/L of each inhibitor. The cell growth was reduced by 74.43 % at 0.1 g/L of each inhibitor. The lipid accumulation was 0.9221 ± 0.2163 and 0.4367 ± 0.1641 g/L (32.4449 ± 02.5336 and 30.7214 ± 1.4616 % of CDW) at 0.1 and 0.25 g/L. The mixture of inhibitors is known to have a pronounced effect on cell growth of baker's yeast than each of the inhibitors alone [Palmqvist *et al*., 1999]. Similar observations were made with *P. stipitis* [Nigam, 2001]. Furfural is expected to have the highest inhibitory effect, and it can be specifically removed after biomass pre-treatment [Fonseca *et al*., 2011].

5.2.2 Cellular growth and lipid accumulation on lignocellulosic biomass

The utilization of pretreated lignocellulosic biomass fractions is needed for second-generation biofuels. Therefore, it was important to test the growth of the isolate on such substrates. For this purpose, dilute acid-treated sugarcane bagasse and corn cob were used. The acid hydrolysate and the insoluble residues were tested as growth substrates.

The acid hydrolysate was used as undetoxified (SCBUT) and detoxified (SCBT). The initial reducing sugars concentration was 20.0278 ± 0.9766 and 18.4164 ± 0.6511 g/L for SCBUT and SCBT, respectively. The yeast exhibited growth in both conditions. Table 5.2 summarizes the results of the experiment. The CDW was 5.3777 ± 0.5649 and 6.4865 ± 0.8421 for SCBUT and SCBT, respectively. The lipid accumulation was 1.4787 ± 0.2967 and 1.9435± 0.3294 g/L (27.3589

± 2.6442 and 29.6706 ± 0.8970 % of CDW) for SCBUT and SCBT respectively. The lipid yield was 0.0817 and 0.114 g lipid/g of reducing sugar for SCBUT and SCBT, respectively.

Similar experiments were conducted with undetoxified (CCUT) and detoxified (CCT) corn cob acid hydrolysate. Both the conditions supported yeast growth. The initial sugar concentration in the corn-cob hydrolysates was 27.6244 ± 0.3255 and 24.8620 ± 0.9766 g/L for CCUT and CCT, respectively. The CDW were 4.3240 ± 0.4101 and 5.3440 ± 0.6972 for CCUT and CCT, respectively. The lipid accumulation in the yeast after 168 h of fermentation was 1.0161 ± 0.2373 and 1.3760 ± 0.3209 g/L (23.3448 ± 3.2755 and 25.5753 ± 2.6692 % of CDW) for CCUT and CCT respectively. The lipid yield was 0.0485 and 0.0678 g lipid/g of reducing sugar utilized for SCBUT and SCBT medium, respectively. The yeast could grow and accumulate lipid on undetoxified hydrolysates with sugarcane bagasse supporting more growth.

There are various reports on the pre-treated detoxified lignocellulosic biomass hydrolysates in the literature. Few reports discuss the utilization of undetoxified hydrolysates for lipid accumulation by oleaginous yeast, as mentioned in Table 5.2. One study conducted the experiment on five yeasts, with all of them except *R. toruloides* could grow on the undetoxified hydrolysate obtained from wheat straw and accumulate lipids in the range of 4.60-31.20 % of CDW [Yu *et al*., 2011]. In another study, *Y. lipolytica* P01g could grow up to 5.88 g/L of CDW on undetoxified sugarcane bagasse. Detoxification was found to improve the growth and lipid accumulation (11.42 g/L CDW; lipid content 58.50 % CDW) [Tsigie *et al*., 2011]. Similarly, *C. Curvatus* accumulated 40 % lipids of CDW (3.04 g/L) after 6 days of fermentation on undetoxified sweet sorghum bagasse hydrolysates [Liang *et al*., 2014]. The corn stover hydrolysate was used without biodetoxification, followed by cellulase treatment in the 3-L fermenter (with 20 % w/w solids). *T. cutaneum* ACCC20271 could accumulate lipids up to 5.78 g/L after 144 h [Wang *et al*., 2016]. *L. starkeyi* was able to grow and accumulate lipids up to 2.5 g/L (26.9 % of CDW) directly on undetoxified hemicelluloses hydrolysate of sugarcane bagasse [Xavier *et al*., 2017]. In another study, *R. toruloides* accumulated 1.2 ± 0.0 g/L (16.05 ± 0.50 % of CDW) on undetoxified acid hydrolysate of sugarcane bagasse [Bonturi *et al*., 2017]. Results from our study and that of the literature suggest that yeast can grow on undetoxified biomass hydrolysates, and the organism can be engineered or culture-adapted to improve growth.

Table 5.2 Comparison of lipid production by oleaginous yeasts cultivated on different undetoxified lignocellulosic acid hydrolysates**.**

Organism name	Feedstock	Pretreatment method	Mode of culture	Incubation period (h)	Cell dry weight (g/L)	Lipid production (g/L)	Lipid content (% w/w)	Reference
Yarrowia lipolytica Po1g	Sugarcane bagasse hydrolysate	Undetoxified	Shake flask	96	5.88	N.M.	N.M.	[Tsigie *et al*., 2011]
Yarrowia lipolytica Po1g	Sugarcane bagasse hydrolysate	Overliming and vaccum filteration	Shake flask	96	11.42	6.68	58.50	[Tsigie *et al*., 2011]
Rhodotorula glutinis	Wheat straw acid hydrolysat	undetoxified	Shake flask	144	13.8 ± 0.3	3.5 ± 0.0	25.0 ± 0.6	[Yu *et al*., 2011]

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Yarrowia lipolytica	Wheat straw acid hydrolysate	undetoxified	Shake flask	144	7.8 ± 0.7	0.4 ± 0.0	4.6 ± 0.0	[Yu *et al.*, 2011]
Lipomyces starkeyi	Wheat straw acid hydrolysate	undetoxified	Shake flask	144	14.7 ± 0.5	4.6 ± 0.0	31.2 ± 1.3	[Yu *et al.*, 2011]
Cryptococcus curvatus	Wheat straw acid hydrolysate	undetoxified	Shake flask	144	17.2 ± 0.4	5.8 ± 0.1	33.5 ± 0.1	[Yu *et al.*, 2011]
Trichosporon cutaneum ACCC 20271	Corn stover Acid hydrolysates	Freshly pretreated undetoxified + enzymatic treatment (20 % solid content)	3-L-Bioreactoer	144	N.M.	5.78	N.M.	[Wang *et al.*, 2016]
Cryptococcus curvatus	Sweet sorghum bagasse acid hydrolysate	Undetoxified	Shake flask	144	10.8	3.04	40	[Liang *et al.*, 2014]
Lipomyces starkeyi DSM 70296	Sugarcane bagasse acid hydrolyste	Undetoxified (culture adaptation method)	Shake flask	72	9.3	2.50	26.90	[Xavier *et al.*, 2017]
Rhodosporidium toruloides CCT 0783	Sugarcane bagasse hydrolysate	Undetoxified (culture adaptation method)	Shake flask	72	7.6 ± 0.1	1.2 ± 0.0	16.0 ±0.5	[Bonturi *et al.*, 2017]
Cystobasidium oligophagum JRC1	Sugarcane bagasse acid hydrolysate	Undetoxified	Shake flask	168	5.3777 ± 0.5649	1.4787 ± 0.2967	27.3589 ± 2.6442	[This study]
Cystobasidium oligophagum	Sugarcane bagasse acid	Detoxified with 2% activated	Shake flask	168	6.4865 ± 0.8421	1.9435 ± 0.3294	29.6706 ± 0.897	[This study]

JRC1	hydrolysate	charcoal					0	
Cystobasidium oligophagum JRC1	Corn cob acid hydrolysate	Undetoxified	Shake flask	168	4.3240 ±0.4101	1.0161 ± 0.2373	23.3448 ± 3.2755	[This study]
Cystobasidium oligophagum JRC1	Corn cob acid hydrolysate	Detoxified with 2% activated charcoal	Shake flask	168	5.3440 ± 0.6972	1.6060 ± 0.3209	25.5753 ± 2.6692	[This study]

(Where N.M.: Not mentioned)

5.2.3 Qualitative analysis of the lipid extracts

5.2.3.1 Thin layer chromatography analysis

The lipid extracts obtained from were subjected to TLC using glycerol trioleate (triolein) as a standard. As shown in Figure 5.6, the composition of lipids was quite comparable to the control. Triacylglycerol (TAGs) were observed in most of the conditions except with furfural.

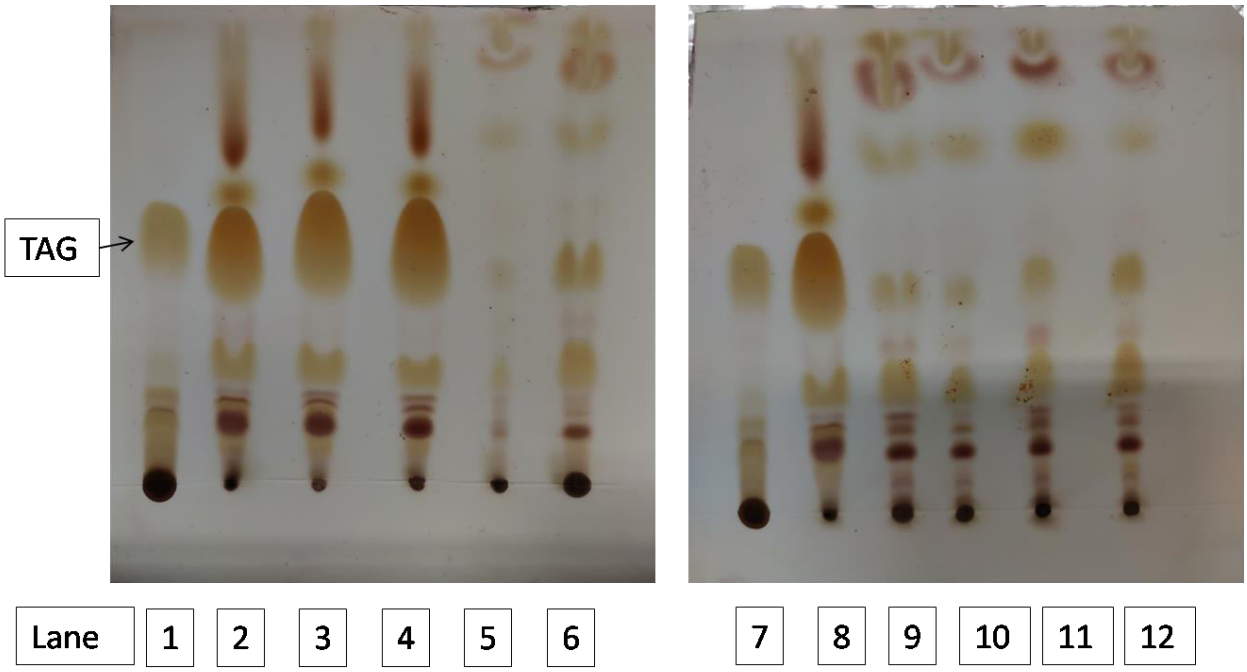

Figure 5.6: TLC analysis of the lipid extract; Lane 1: Triolein standard; 2: Lipid extract (LE) Control ; 3: LE of HMF 2.0 g/L; 4: LE of Acetic acid 1.0 g/L; 5: LE of furfural 1.0 g/L; 6: LE of mixed inhibitors 0.1; 7: Triolein standard; 8: Lipid extract (LE) Control; 9: LE of SCB-T; 10: LE of SCB-UT; 11: LE of CC-T; LE of CC-UT.

5.2.3.2 ^{1}H NMR analysis

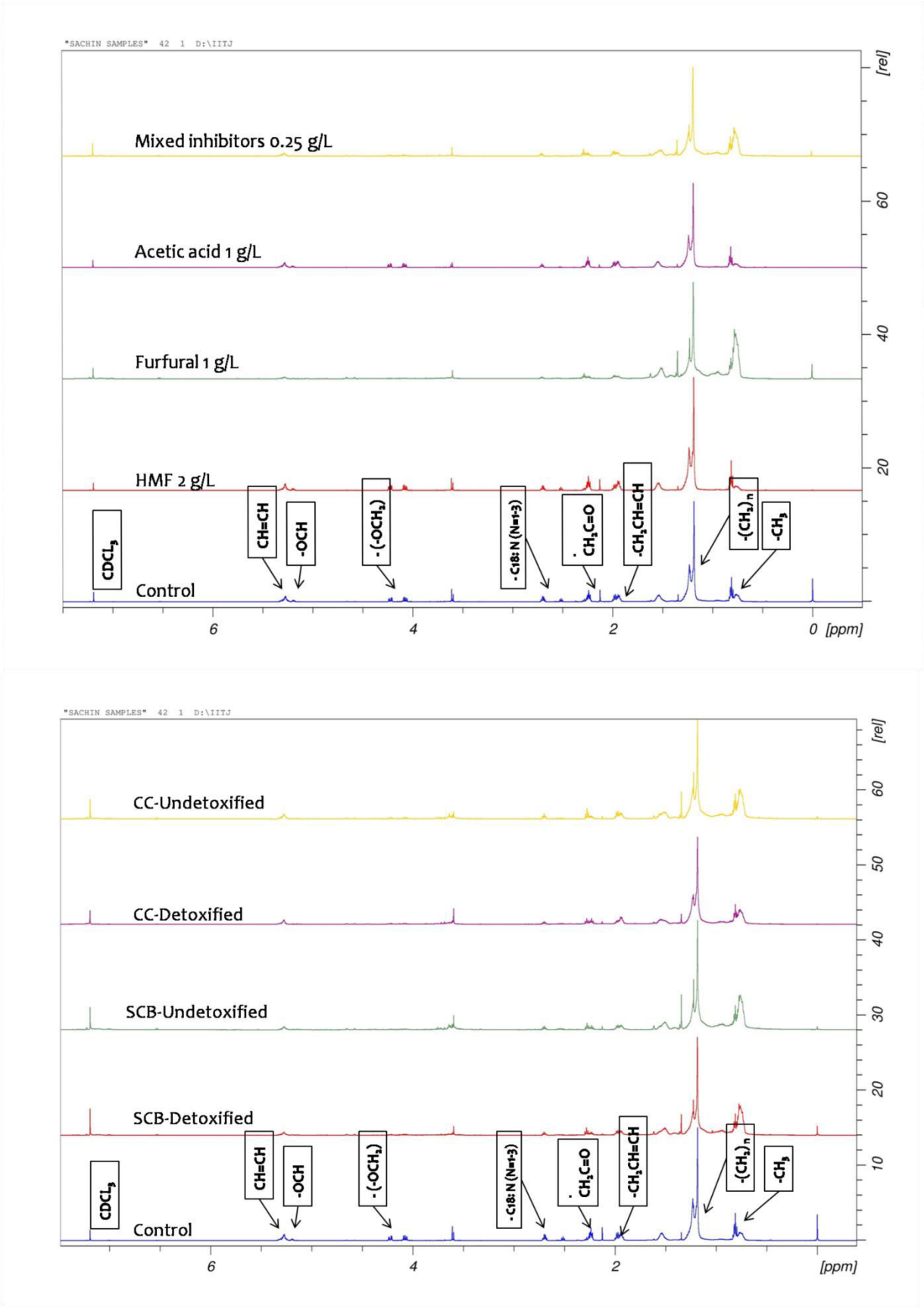

Figure 5.6: A: ^{1}H NMR of lipid extracts from the inhibitors growth conditions B: ^{1}H NMR of lipid extracts of yeast in lignocellulosic hydrolysates (CC: corn cob; SCB: sugarcane bagasse).

Lipid analysis was done using proton- ^{1}H NMR. The spectra were recorded, and the characteristic peaks for lipids were assigned based on the previous studies [Sarpal *et al*., 2014; Tariq *et al*., 2011]. Figure 5.6 shows the NMR spectra of the lipid extracts. All the samples except furfural showed peaks at 4.0–4.35 ppm, indicating the presence of (–OCH_2) functional group. Other peak values such as 2.30, 5.20, 5.05–5.65, 2, 1.26, and 0.85 ppm were observed, which indicated the presence of –CH_2C=O, –OCH, –CH=CH, –CH_2CH=CH, –$(CH_2)_n$ and terminal –CH_3 groups, respectively. The sharp peaks between 3 and 4 ppm were 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 C_{18}: N (N = 1–3) (Polyunsaturated fatty acid). The NMR study indicated that the lipid composition changes with the presence of furfural in the medium.

Nevertheless, the isolate appeared to have exhibited the traits desirable for biofuel production.

5.3 CONCLUSIONS

The study revealed a moderate resistance of *C. Oligophagum* JRC1 towards lignocellulosic inhibitors and very high sensitivity on the mixture of them. Nevertheless, the growth of lignocellulosic biomass hydrolysate was supported. Particularly, the yeast could grow and accumulate lipids on the undetoxified lignocellulosic hydrolysates. The lipid profile as obtained through NMR experiments was found suitable for biodiesel and other applications

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