STUDIES ON PRODUCTION OF VALUE-ADDED PRODUCTS FROM ALOE VERA LEAF RIND HYDROLYSATE USING *Rhodosporidium toruloides 3547*

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF REQUIREMENTS

FOR THE AWARD OF THE DEGREE

OF

MASTER OF TECHNOLOGY

IN

[INDUSTRIAL BIOTECHNOLOGY]

Done at

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Submitted by:

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2K21/IBT/22

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CANDIDATE'S DECLARATION

I, **Sukanya Nag**, Roll No. **2K21/IBT/22** of M.Tech (Industrial Biotechnology), hereby declare that the Project Dissertation titled **"Studies on production of value-added products from aloe vera leaf rind hydrolysate using *Rhodosporidium toruloides 3547*"** which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the Degree of Master of Technology, is original and not copied from any source without proper citation. The work has not been previously formed the basis for award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

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This is to certify that **Ms. Sukanya Nag,** a student at **Delhi Technological University** has successfully completed a 6-month Training in Magic Software from **November 15, 2022, to April 30,2023** under the guidance of **Ms. Namrata Popli**.

During the period of her Internship Program with us, she has worked to support the **HMH team**. She worked with minimal supervision, and this proved extremely useful in furthering our company's goals.

We wish her every success in her life and career.

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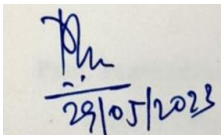
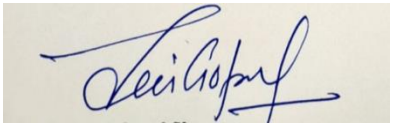
CERTIFICATE

I hereby certify that the Project Dissertation titled **" Studies on production of value-added products from aloe vera leaf rind hydrolysate using *Rhodosporidium toruloides* 3547"** which is submitted by Sukanya Nag, Roll no. 2K21/IBT/22 [Department of Biotechnology], Delhi Technological University, Delhi in partial fulfilment of requirement for the award of Degree of Master of Technology, is a record of the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part of full for any Degree or diploma to this University or elsewhere.





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ABSTRACT

The requirement to construct on the demand for sustainable components in second generation bio-refineries can only be fulfilled through modern and environment friendly technologies based on bioprocess mechanisms such as organosolv treatment, transesterification, and various catalytic procedures to make more composite molecules and matter on which a future durable society can be built. Aloe vera leaf rind (AVLR), a waste biomass has no commercial usage and could provide as an efficient lignocellulosic feedstock for bio-based product manufacturing where the requisite is a viable pretreatment to separate cellulose, hemicellulose and lignin into different streams. The use of unconventional oleaginous yeast *Rhodosporidium toruloides* 3547 to produce value added products using agro-waste like aloe vera leaf rind (a lignocellulosic waste) liquid hydrolysate as the sole carbon source has been hypothesized in this project work which has not been tried so far. Here, AVLR is characterized for compositional analysis and components such as protein (2195.64 mg/l), cellulose (2022.58 mg/l), hemicellulose (993.54 mg/l), total carbohydrates (1693.53 mg/l), and lignin (7.75%) etc, are observed which show a promising probability towards further by-product production. Organosolv method can be utilized to attain pure lignin with less than 1% weight of residual carbohydrate quantity. In this study, various catalyst compositions such as $FeCl_3$ hydrolysate (4 fold increase in reducing sugars), CuCl hydrolysate (3 fold increase in reducing sugars), etc, are analyzed for efficiency and microbial growth profiling as the pretreatment using the organosolv method. "CuCl hydrolysate with yeast basal media" catalyst is found to have the highest sugar consumption rate (94%), thereby showing the highest sugar uptake rate (11.33 mg h^{-1}) and maximum biomass production (95 mg/hour at optimal growth). Qualitative and quantitative analysis of intracellular lipids through techniques such as staining, FTIR, GC-MS and XRD has also provided favorable results in the samples obtained. The qualitative analysis using Sudan Black stain revealed that the" CuCl hydrolysate" catalyst treated sample accumulated maximum lipid while "$FeCl_3$ hydrolysate" catalyst accumulated the least. In quantitative analysis "Cu

Cl hydrolysate" catalyst treated sample is found to have the highest lipid content (48%), due to its higher efficiency in fractionalization of the components. Fourier-Transform Infra-Red (FTIR) spectra released peak at about 1732 cm^{-1}, with the inference that in "CuCl hydrolysate" catalyst treated AVLR, the hemicellulose is extracted fully but not in "$FeCl_3$ hydrolysate" treatment. The presence of peaks near the region 1600 cm^{-1} and 1423 cm^{-1} indicates the presence of lignin in both raw and pre-treated AVLR and these peaks correspond to the phenyl and methyl propane groups present in the lignin. This study found that the organosolv pretreatment of AVLR biomass can efficiently enhance the yield of fermentable sugars and lipid production which can be further utilized for by-product manufacturing such as biofuels, carotenoids, etc.

Keywords: Aloe vera leaf rind, *Rhodosporidium toruloides* 3547, Organosolv pretreatment, Lignocellulosic waste, Lipid, FTIR.

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SUKANYA NAG

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NOMENCLATURE

h^{-1}: Specific growth rate

h : Doubling time

K : kappa number.

p : amount of permanganate actually consumed by the sample.

f : factor for correction to a 50% permanganate consumption.

w : the dry weight of the biomass (g)

CHAPTER -1

INTRODUCTION

Aloe vera (Aloe arbadensis Miller), a flowering plant of succulent nature, is prevalent naturally in multiple tropical and subtropical regions. Aloe vera has been utilized in varied ways in traditional medicine for ages to cure diseases of the skin, as well as for its laxative effect. Currently, it is used worldwide as a prized component for foods and beverages, antioxidant materials, cosmetics and other drugs. The leaves dagger like in shape are the majorly utilized part of the plant, in which dual vital fragments can be differentiated, viz., the inner parenchyma (fillet or pulp) and photosynthetic green cortex (rind) (Ortega *et al*., 2019).

Aloe vera will turn into a huge industry of 242 million dollars with almost 300 industrial units producing huge amounts of wastes of leaf rinds by 2025 (Global Aloe Vera Extract Market Size Report, 2019). The use of unconventional microorganisms such as the oleaginous yeast Rhodosporidium toruloides to produce value added products using agro waste such as aloe vera rind as the only carbon source has proven to be a crucial field of research in recent times. Rhodosporidium toruloides, an industrial, heterothallic, bipolar yeast has a red colouration and is a natural producer of neutral lipids (collected during nutrient- limiting state as a carbon storage method), carotenoids (cause for the red colouration of cells as well as antioxidant characteristics), and various vital enzymes. All these constituents are commercially revered: carotenoids are utilized as colourants, antioxidants, or vitamin A precursors and applied in the edibles, pharmaceutical and feed departments; lipids and lipid-derived compounds are quality replacements for petroleum in the manufacturing of fuels and synthetics, and upgraded enzymatic activities in this yeast viz. D- amino acid oxidase and L-phenylalanine ammonia-lyase are applicable for the pharmaceutical and synthetic industries. Physiological research has also shown other characteristics as an industrial yeast, such as the ability to grow to high cell density, make use of a broad range of carbon and nitrogen sources, and its potential to be applied to manufacture high-value products from low-cost components (Park *et al*., 2017).
For this purpose, the optimization of the pretreatment method is vital to increase the productivity of the process along with making it environmentally friendly. The hydrolysates are separately treated for detoxification and enzymatic hydrolysis to continue with the fermentation process by *Rhodosporidium toruloides* 3547. The commercial aspect of the byproducts is highly

dependent on the productivity of the entire process whose basis lies on the substrate used, yield per unit, raw material cost and the recovery methods.

Organosolv pretreatment is considered to be propitious, for its inherent benefit viz. fractionation of peak purity cellulose with minute disintegration, isolation of superior lignin, pure and efficient recovery of hemicellulose fractionation in comparison to prevalent treatments and organic solvent recovery. This pretreatment attains a structured fractionation of the cellulose-hemicellulose- lignin matrix into three different streams (Salapa *et al*., 2017). Several methods including organic solvents that allow various conventional and alternative raw constituents to be efficiently delignified have been reported (Rodríguez *et al*., 2008). Organosolv processes are profoundly related to the efficiency of temperature which leads to the separation of the intra and inter polymeric connection between the different biopolymers that compose plant constituents, thus securing lignin dissolution. The amount of delignification attained and the distinctivity of the lignin extraction method is related to the operating state applied, such as the kind of organic solvent, the solvent concentration, extraction temperature and time etc (Jiménez *et al*., 2001).
The goal of the entire research process is to tackle the technical challenges present in the pretreatment methods along with the yield and production optimization to make the byproducts commercially viable by using aloe vera rind as the single carbon source for the oleaginous yeast *Rhodosporidium toruloides* 3547 making the by-products economically viable and energy efficient along with long term sustainability.

CHAPTER -2

REVIEW OF LITERATURE

2.1.1 Rhodosporidium toruloides

Rhodosporidium toruloides come under bipolar yeast and are heterothallic and red. It is a member of the pucciniomycotina which in turn comes under basidiomycota. Its genuine character is to produce carotenoids, neutral, lipids etc. These substances have a wide range of industrial applications. Carotenoids are antioxidants and precursors of vitamin A and hence are major players in the pharmaceutical industry. Lipids are good replacements for conventional fuels and hence are crucial components in biofuel production. Previous research has unveiled that this yeast can grow to higher cell densities and make use of various carbon and nitrogen sources. This gives it the reputation of being able to produce value added products using cheap substrates (Park *et al*., 2017).

Over 60% (of dry cell weight) lipid accumulation was reported in Rhodosporidium species (Li *et al*., 2007). The fatty acids that constitute these lipids are of the long chain type. This is similar to fatty acids found in the usual vegetable oils. Hence, this makes Rhodosporidium a suitable substitute to produce lipids and hence make a more sustainable biofuel industry (Zhao *et al*., 2005). Previous research indicates that Rhodosporidium toruloides can accumulate over 70% lipids and a by titer greater than 100 grams per litre. Further Rhodosporidium toruloides is a powerful strain for producing lipid utilizing hydrolysate from biomass (Hu *et al*., 2009).

R toruloides is capable of converting the mixture of C5 and C6 sugars from lignocellulosic biomass to value added products. The key points to be noted is that the growth and yield were found to be higher in ligno-cellulosic hydrolysate when compared to pure substrates (Yegashi *et al*., 2017).

Lipid extraction from Rhodosporidium toruloides can be done using mild acid- based treatment and recovery. This is followed by hydrogenation and isomerization to give the end product as biodiesel. Results show that approximately 75% of the resulting substance was in the range of T90 and consisted of No.2 diesel fuel (Nogue *et al*., 2018).

2.1.2 Aloe vera leaf rind

Coming under the Liliaceae family, aloe vera is a plant of high medicinal value. More than 500 species of aloe vera exist throughout the globe. The leaf of aloe vera is composed of water making up 95% of the volume. About 75 nutrients, amino acids- 18 in number and minerals-20 in number are found in the leaf (Modi *et al*., 2012).

When aloe vera leaf rind was delignified using laccase and optimized using response surface methodology, the optimum level of delignification was found to be around 77% and lignin retrieved was nearly 4% (w/w). The optimal solid to liquid ratio was found to be 1:3.7. the optimum time and temperature of incubation were respectively found to be 323.15 K and six hours. Enzyme based hydrolysis of the aloe vera rind biomass was done using unpolished cellulase obtained from Aspergillus sp., leading to a 44% saccharification which is the double of level of saccharification obtained in untreated aloe vera leaf rind (Gunasekaran Rajeshwari, 2019).

The impact of pretreating the aloe vera rind was categorized by analytical methods. An increase to nearly 15% in cellulose crystallinity in pretreated aloe vera leaf rind when compared to the 10.5% in untreated aloe vera leaf rind was revealed by patterns obtained in X-Ray Diffraction (XRD). The level of structural disintegration post pretreatment was significantly shown by Scanning Electron Microscopy (Gunasekaran Rajeshwari, 2019).

A 63% saccharification was found to the maximum under optimal environment and the sugar output was found to be nearly 310 mg/g (Gunasekaran Rajeshwari, 2020).

When assessed for phenolic compounds the rind was shown to contain over
105 mg/g draw out of phenolic compounds. The rind showed the best antioxidant property by offering the top defence against oxidative haemolysis and β-carotene bleaching. The rind showed excellent antimicrobial properties especially against the Salmonella Typhimurium (Ortega *et al*., 2019).

2.1.3 Organosolv

Organosolv fractionation can be employed to attain highly pure lignin with less than 1 wt% of residual carbohydrate quantity. This method can yield first-class cellulose biofuel by eliminating lignin. This procedure characteristically results in the wide-ranging removal of lignin with the least loss of cellulose (less than 2%). This procedure has had its commercial demonstration at a

pilot scale (Alcell procedure established by Repap Enterprises Inc) and yielded nearly 5000t of pulp from numerous hardwood raw materials. The procedure was considered viable with well-known kraft pulping (Mathew *et al*., 2018).

Lewis acids are currently being experimented to be catalyze the pretreatment of wheat hay using organosolv. Fractionation of the lignocellulosic biomass and lignin breakdown has been accomplished in aqueous ethanol using Iron (II) chloride, Copper (II) chloride, Iron (III) chloride, Gallium (III) Triflate, Zirconium oxychloride. The character of the lewis acid also affected the character lignin that was precipitated (Constant *et al*., 2018).

The pre-treatment by ethanol at 453.15 K for nearly two-third of an hour yielded a supreme cellulose conversion of nearly 90% and an ethanol harvest of over 65% of the theoretic harvest. The outcomes specify that organosolv pre- treatment with diethylene glycol at 433.15 K for two-third of an hour is hopeful about the fact that it gives a good ethanol harvest of 65% and a good pulp produce of over 50% (Salapa *et al*., 2017).

Naturally, acids (Hydrochloric acid, sulphuric acid, oxalic, or salicylic) can be used as catalysts if the procedure is carried out at temperatures below 185– 210°C (Nahyun et al., 2010). Also, in the case of using acid catalysts, the rate of delignification is amplified and greater xylose harvests are attained (Zhao *et al*., 2009).

The key fractions obtained post pretreating biomass include: (i) cellulosic fibres; (ii) solid lignin, attained post elimination of volatile solvent; and (iii) liquid solution of hemicellulosic sugars, largely xylose (Pejo *et al*., 2011).
Process environments impacted the functional groups and molecular mass of the mined organosolv ethanol lignins and subsequently impacted the antioxidant action of lignin. Generally, the lignin(s) produced at raised temperature, lengthier reaction period, a higher quantity of catalysts, and diluted ethanol displayed high antioxidant activity. Regression models were made to permit the quantitative prediction of lignin character and antioxidant action grounded on the processing conditions (Pan *et al*., 2006).

2.1.4 Downstream processing

Unfiltered hydrolysate is taken for direct fermentation by Rhodosporidium toruloides, where xylose, glucose, lactate and acetate are completely utilized through fermentation. Additional benefits can be obtained via combining pretreatment, saccharification and fermentation, eliminating the requirement to distinguish ionic liquids from hydrolysates prior to fermentation.

This permits change of saccharified IL pretreated biomass directly to unconventional biofuels without needing further partings or washing, negligible add-ons to affluence fermentation, no loss of performance becauseof IL toxicity, and abridged fuel retrieval through phase separation (Sundstrom *et al*., 2018).

Yeast cell saponification using alcoholic potassium hydroxide followed by recovery using hexane has been identified as a very effective technique to retrieve and separate lipids and carotenoids. This technique when used over yeast cells distorted via bead milling helped extract 75 % lipids and 71% carotenoids. Moreover, 15% of proteins too had been recovered. While saponification using alcoholic potassium hydroxide was conducted on whole wet cells, 86% of lipids and 79% carotenoids were recovered, and no proteins. The purity of the retrieved lipids was similar when complete or distorted cells were used, but then the concentration of the carotenoids obtained was enticingly more when the process was applied on distorted cells (Liu *et al.*, 2020).

To achieve better lipid production by Rhodosporidium toruloides Y4, fermentation was carried out in the presence of illustrative inhibitors. The findings revealed acetate, 5 - hydroxymethyl furfural and syringaldehyde had repressive properties; p-hydroxybenzaldehyde and vanillin were of high toxicity at concentration > 10, and furfural and its derivatives furfuryl alcohol and furoic acid repressed cell growth by 45% at about 1 mM. Moving forward it was verified that inhibition is generally additive, while robust synergistic inhibitions had also been detected (Hu *et al*., 2009).

After cell distortion the complete lipid is recovered either by chloroform and methanol using two different protocols [conferring to Folch (F) and Bligh and Dyer (BD)] and alternatively by ethanol and hexane (EH). BD extraction was executed for S. podzolica. For S. podzolica, complete lipid harvest and cell distortion efficiency were found to be allied. Reliably, retrival effectiveness was constantly greater with EH, trailed by BD and F for all cell distortion methods. The best method for complete cell lipid recovery of S. podzolica is subsequently the blend of HPH and EH which is 2.7 times higher than with the least desired blend. The most effective blend was BM and BD with 20.0 ± 3.2%. While highest complete lipid harvests too were obtained from the more effective cell distortion method (BM), learnings do not give a clear depiction as clear as for S. podzolica and detected variances are not statistically remarkable (Gorte *et* al., 2020).

2.1.5 Importance of industrial production of intracellular lipid

Current commercial manufacture of bio-diesel is chiefly based upon vegetable oils, which can lead to a lack of comestible oils in the food market and raise their costs. Lipids from microorganisms, which are formed by oil-containing microorganisms, are gaining a lot of consideration currently as a basis of acid, such as polyunsaturated and alternative raw materials to manufacture biodiesel. Yet, the manufacture of microbial oils is associated with many obstacles related to the cost of lipid withdrawal, the sources of carbon and the operating costs for culturing of microorganisms in typical stirred tank bioreactors that make manufacture not possible economically. Inedible raw materials, lignocellulosic biomass, and various lignocellulosic wastes are inexpensive renewable sources of carbon that could meaningfully lessen the expense of lipid production from microorganisms (Santek *et al*., 2018).

CHAPTER -3

MATERIALS AND METHODS

3.1 Substrate

Aloe vera leaf rind biomass was obtained as a by-product from a neighbourhood juice shop. The rinds were dried, powdered and kept in a zip lock plastic bag for later use.

3.2 Substrate Protein estimation

The protein extraction from aloe vera rind biomass was carried out where 500 mg of aloe vera leaf rind extract was mixed into 30 ml of phosphate buffer and crushed using a mortar and pestle followed by centrifugation for 15 minutes at 5000 rpm. The supernatant was collected and Lowry's estimation for protein analysis was carried out where reagent 1 and 2 were added followed by measuring OD at 660nm (Lowry *et al*., 1951).

3.3 Aloe vera leaf rind Biomass Biochemical Compositional Analysis

Standard protocols were used to determine the total reducing sugars and volatile organic solids present in aloe vera leaf rind biomass (Miller *et al*., 1959).

Cellulose estimation was carried out in accordance with the protocol (Sun *et al*., 2004) where 5 g of aloe vera leaf rind biomass was used followed by acid treatment for 20 minutes with a mixture of (10 mL of 70% v/v nitric acid and 100 mL of 80% v/v acetic acid) in boiling water. The residues obtained following pre-treatment were alternately washed with distilled water and the samples were dried overnight at 60°C. The cellulose content of aloe vera leaf rind biomass was determined and calculated using Equation (1).

Cellulose content (%) = (Final biomass dry weight (g) / Initial biomass dry weight (g)) × 100 (1)

Dried aloe vera leaf rind biomass was used to estimate hemicellulose where it was treated using (1 percent, v/v) of sulphuric acid for 4 hours at 100°C, followed by drying overnight, the hemicellulose content of AVR biomass was calculated using the variability between the total dissolved carbohydrate concentration prior to and following pre-treatment (Marlett *et al.*, 2006) (Updegraff *et al.*, 1969). The amount of lignin present in aloe vera leaf rind biomass was determined in accordance with the protocol using the titration method in which 0.05 g of dried aloe vera leaf rind biomass was used and treated with a solution of (0.1 N) of 7.5 mL potassium permanganate and 7.5 mL sulphuric acid (Hussain *et al.*, 2002). In order to carry out aloe vera leaf rind lignin oxidation, 4 N of the sulphuric acid was used. The reaction was carried out at 25°C for 10 minutes before adding (1 N, 1.5 mL) of potassium iodide and titration was done using (0.1 N) sodium thiosulfate solution with starch as an indicator. Similarly, blank titration was performed using the same procedure. An intuitive method was used to determine the Klason lignin present in aloe vera leaf rind biomass using Equations 2 and 3,

$$\textit{Kappa number} = (P \times f) / w \qquad (2)$$

where P is the millilitres of (0.1 N) potassium permanganate decinormal solution of biomass consumption and w is the dry weight of the biomass (g); f is the correction factor for 50% permanganate consumption (f=1).

$$\textbf{Lignin content (\%)} = (\textbf{Kappa number} \times 0.155) \times 100 \qquad (3)$$

Where, the slope of the linear graph of kappa number versus klason lignin is 0.155.

3.4 Organosolv based Hydrolysate and Lignin Extraction

The procedure is a standard organosolv process. Here, 4 g of aloe vera leaf rind biomass was combined with aqueous ethanol of 1.25 L (65% v/v) in an autoclave of 2L (Autoclave France®) with 8 mmol/L catalyst ($FeCl_3$, Cu Cl). Stir the reaction mixture for 2 hours at a temperature of 160°C. Once the reaction was over, reactor temperature was brought to room temperature. A Rotary vacuum evaporator was used to extract the

liquor from the reaction mixture (pulp and black liquor). Wash the pulp at a temperature of 60°C with an aqueous ethanol solution 300ml (65% v/v), and the washes were mixed with the black liquor. To precipitate the lignin, add 3 volumes of water to the liquor. The obtained solution was centrifuged at 20°C for 20 minutes at 4950 rpm and filtered using Whatman filter paper. Wash the residue with water and dry for 72 hours at 50°C. The residue was the lignin fraction, and the filtrate is hydrolysate where extractable compounds were dissolved (Sandra *et al*., 2005).

3.5 Fermentation using Rhodosporidium Toruloides 3547

Here,2 ml of inoculum was collected and transferred to 5 separate tubes with 18ml of $FeCl_3$ hydrolysate, 18ml CuCl hydrolysate, 18ml of yeast basal media, 18ml of $FeCl_3$ hydrolysate and 1% yeast basal media, 18ml of CuCl hydrolysate and 1% yeast basal media. The procedure was carried out under sterilised conditions, and the culture was taken after 24 hours.

3.6 Biomass measurement

For biomass measurement, 2 ml of sample was collected and transferred to separate Eppendorf. OD value was measured for all 5 samples at 600 nm followed by centrifugation for 10 min at 3000 rpm. Collected supernatant was estimated using Total carbohydrate by anthrone method (Hedge *et al*., 1962) and Total reducing sugar by Dinitrosalicylic acid method (Miller *et al*., 1959). Then the biomass was dried and the weight of biomass was noted.

3.7 Lipid Extraction

The fermentation broth (6ml) was centrifuged at 6000 rpm ,10 minutes at a temperature of 4°C. The supernatant was discarded and the pellet was washed twice with distilled water. The dried cell weight was determined by letting the pellet dry. Add 12ml of chloroform: methanol (2:1) using glass beads and then vortex the pellet. For 45 minutes sonication was done, centrifuged at 10,000 rpm, 10 minutes at a temperature of 4°C. Once 12 mL of Chloroform: Methanol (2:1) was added to the supernatant, vortexing was done followed by adding 3ml NaCl. For separation at phase, vortexing is done

followed by incubating for 10 minutes. The top layer was removed, and the bottom layer (lipid) was stored (Bligh *et al*., 1959). Lipid yield calculated using the following Equation 4.

Lipid Yield (mg/ml) = Lipid Dry Weight(mg)/ Volume of Sample(L) (4)

For each of the five samples, 100 μL of fermented broth was collected in an Eppendorf tube. 10 μL of Sudan Black dye (0.3% dye in 70% EtOH) was added to all samples. A 10 μL sample was loaded onto a slide for Fluorescence Microscopy.

3.8 XRD Analysis of Raw and Pre-treated AVR Biomass Characterization

Before doing organosolv treatment and after doing organosolv treatment, the crystallinity of the AVR biomass powder formed was monitored using XRD (Malvern Analytical, AERIS -High resolution benchtop XRD, UK) this was done and radiation CuKα (40 kV). The speed for scanning was kept as 3 degrees /minute and the grade was kept at 10 to 75°(2θ). For native form of cellulose an analytical form of approach was used, the crystallinity index was calculated using Segal 's method (Segal *et al*., 1959).

3.9 FTIR Analysis

To determine the changes in the functional groups, present in the aloe leaf rind biomass formed after organsolv treatment (480W) FTIR was used. (Binod *et al*., 2012) reported that, the AVR sample (raw and treated) was dried (60 ℃) and then mount thin sections with potassium bromide, this is done in order to note the spectrum from 4000 to 400 / cm at spectral resolution (0.5 per cm) using an FTIR spectrometer (Agilent Technologies, Cary 600 Series, USA).

3.10 SEM Morphological Analysis

Raw and pretreated AVR biomass was observed using a Scanning Electron Microscopy (FEI, QUANTA 200, USA) at 30 kV. This was used to study the morphological changes after organosolv pretreatment.

CHAPTER -4

RESULTS AND DISCUSSION:

4.1 Characterization of AVLR

The cellulose, hemicellulose, lignin and total volatile solids content in the AVLR used in this research is comparatively less to the results obtained in the research carried out by (Gunasekaran Rajeshwari, 2019). This may be attributed to the fact that the AVLR used in this research were that of very young leaves of growing shoots.

Table 1: Characterization of biomass

S.no	Characterization of AVLR	Concentration values (mg/l)
1	Protein	2195.64 ± 100.69
2	Total carbohydrate	1693.53 ± 132.65
3	Cellulose	2022.58 ± 35.70
4	Hemicellulose	993.54 ± 126.05

Table 2: Amount of lignin and total volatile solids

S.no	Characterization of AVLR	Concentration Values (%)
1.	Lignin	7.75 ± 2.24
2.	Total volatile solids	84.48 ± 0.37

4.2 Cell growth determination

"Cu Cl hydrolysate and Yeast basal media" catalyst is seen to provide the optimal growth condition for microbial growth amongst the different samples as it has the highest specific growth rate and least doubling time amongst all the samples with different substrate sources analyzed. In the presence of different catalysts, the maximum biomass was obtained for "Cu Cl hydrolysate and Yeast basal media" catalyst at 96th hour.

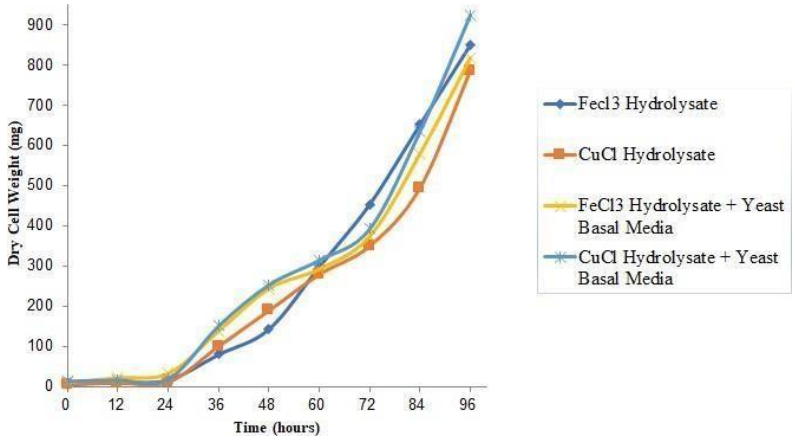


Fig 1: Growth curve for *Rhodosporidium toruloides 3547*

Table 3: Specific growth rate (h-1) and doubling time (h) of different hydrolysates

Substrate	Specific growth rate (h^{-1})	Doubling time (h)
FeCl3 hydrolysate	0.61	1.14
Cucl hydrolysate	0.55	1.26
FeCl3 hydrolysate + Yeast basal media	0.58	1.20
Cucl hydrolysate + Yeast basal media	0.68	1.02

Significant growth can be seen for all the samples around the 36th hour, where it attains the log phase. The possible reason for the high biomass accumulation in "Cu Cl hydrolysate and Yeast basal media" catalyst could attribute to a balanced C/N ratio. A balanced C/N ratio indicates nearly similar amounts of carbon and nitrogen. When the C/N ratio is high, biomass accumulation is lowered. This is due to the fact that an osmotic shock caused by carbon if present in high quantity in the media. This occurs due to a reduction in Krebs cycle activity, decreased energy produce, and an intracellular upsurge of citric acid (Saini *et al*, 2020). The yeast basal media in "Cu Cl hydrolysate and yeast basal media" sample provides the required amount of nitrogen to balance the C/N ratio and hence this particular sample combination is able to generate high biomass.

4.3 Biomass estimation

The relation between Dry cell weight and OD at 600 nm was found. The maximum biomass was obtained for "CuCl hydrolysate and Yeast basal media" catalyst. The

graph can be further utilized as a correlation graph to calculate biomass weight values for different ODs obtained at variable parameters.

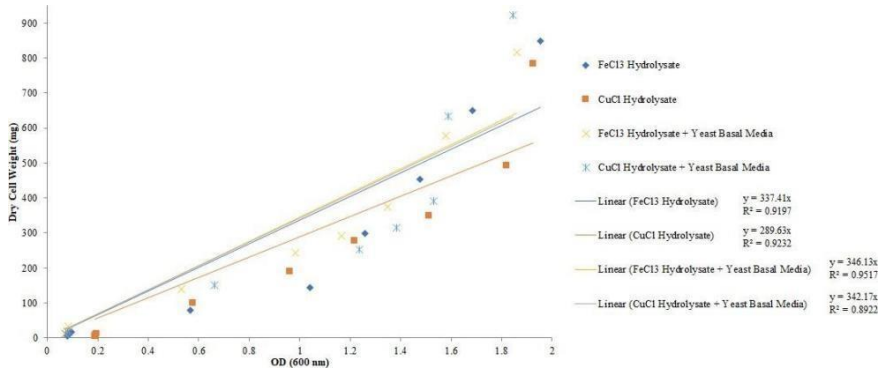


Fig 2: Correlation curve for *Rhodosporidium toruloides* 3547

4.4 Quantification of Total reducing sugars

Quantity of reducing sugars is observed to decrease in all samples with time, showing utilization of sugars by the microorganism for growth. "CuCl hydrolysate and Yeast basal media" catalyst has the highest amount of sugar uptake hence also showing the maximum biomass production with time. The reason for such high sugar uptake is due the catalyst being more efficient in delignification leading to easier uptake of total sugars by *R.toruloides* 3547. From figure 3 its evident that the yeast was in exponential phase till 48 th hour for all the catalysts post which the stationary phase was observed, indicated by decreased concentration of total reducing sugars right after 48th hour. Significant levels of lipid accumulation occurred during the late exponential phase with the plummeting reducing sugar concentration (Tiukova *et al.*, 2019).

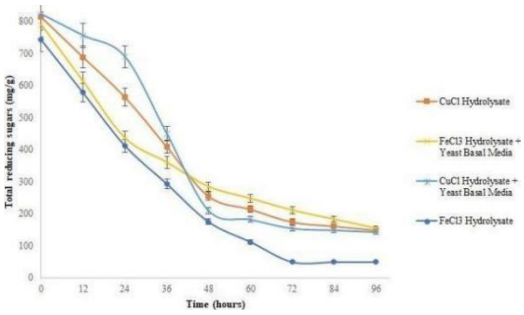


Fig 3: Sugar uptake rate of total reducing sugars (DNS estimation)

Table 4: Reducing sugar uptake rate with different hydrolysates

Substrate	Sugar uptake rate ($mg\ h^{-1}$)
$FeCl_3$ hydrolysate	11.84
CuCl hydrolysate	11.65
$FeCl_3$ hydrolysate + Yeast basal media	14.73
CuCl hydrolysate + Yeast basal media	19.99

4.5 Quantification of Total sugars

Quantity of total sugars are observed to decrease in all samples with time, showing utilization of sugars by the microorganism for growth. "$FeCl_3$ hydrolysate and Yeast basal media" catalyst has the highest amount of total sugar uptake whereas the uptake by the "$FeCl_3$ hydrolysate" catalyst is seen to be the least. The sugar consumption percentage was found to be nearly 80% in case of "CuCl hydrolysate and Yeast basal media" sample and 73% in case of "$FeCl_3$ hydrolysate + Yeast basal media" catalyst.

These outcomes are found to be similar to the outcomes obtained by (Yaegashi *et al.*, 2017) where the sugar consumption rate was nearly 76%. Further the samples $FeCl_3$ hydrolysate and Cu Cl hydrolysate (without yeast basal media) showed nearly 94% sugar consumption, which is very close to the 95% sugar consumption in the work done by (Saini *et al.*, 2020).

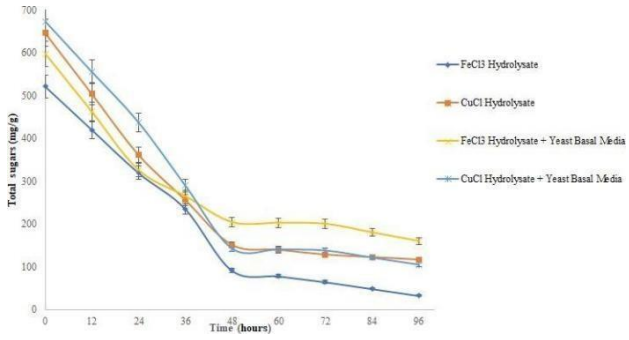


Fig 4: Sugar uptake rate of total sugars (Anthrone estimation)

Table 5: Total sugar uptake rate with different hydrolysates

Substrate	Sugar uptake rate (mg h^{-1})
FeCl3 hydrolysate	7.98
Cu Cl hydrolysate	10.96
FeCl3 hydrolysate + Yeast basal media	11.33
Cu Cl hydrolysate + Yeast basal media	10.56

4.6 Sugar syrup concentration using Rotary Vacuum Evaporator (RVE)

The sugar syrup concentration was increased through organosolv pretreatment for Reducing sugars. For CuCl catalyst after RVE the sugar concentration was found to be 3130.41 mg/l and before RVE was 1548.61 mg/l. For $FeCl_3$ catalyst the sugar concentration after RVE was 3026.38 mg/l and before RVE was 1459.72 mg/l. In both the catalysts the sugar syrup concentration was found to be two folds after RVE and concentration of reducing sugars for CuCl hydrolysate was found to be higher than $FeCl_3$ hydrolysate for both before and after RVE step. Similarly, the sugar syrup

concentration increased after undergoing organosolv pretreatment for Total Sugars. For CuCl catalyst, before RVE the sugar concentration was found to be 81.92 mg/l and after RVE was found to be 203.17 mg/l which is a three-fold concentration compared to concentration of sugar before RVE. For $FeCl_3$ catalyst the sugar concentration before RVE was 39.32 mg/l and after RVE was 134.36 mg/l which is a fourfold syrup concentration compared to sugar concentration before RVE concentration of total sugars for CuCl hydrolysate was found to be higher than $FeCl_3$ hydrolysate for both before and after RVE step.

The effectiveness of the catalyst used in organosolv process in this research can be compared to the results seen in the work done by Constant *et al.*, (2018) where $FeCl_3$ catalyst proved to be more effective in delignification of the wheat straw substrate compared to $CuCl_2$. When these two catalysts were used with AVLR in this research, the effectiveness was reversed and CuCl showed to be more efficient than the $FeCl_3$ catalyst.

4.7 Qualitative analysis of lipid

In this research the qualitative analysis has revealed that the Cu Cl hydrolysate sample was found to have accumulated maximum lipid while $FeCl_3$ the minimum. Microscopic examination reveals that lipids are accumulated in the form of oil globules within the inclusion bodies of R.toruloides.

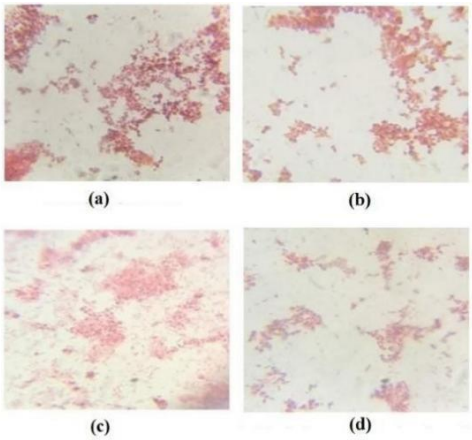


Fig 5: fluorescence microscopic view of lipid- a) Cu Cl hydrolysate; b) Cucl hydrolysate + Yeast basal media; c) $FeCl_3$ hydrolysate; d) $FeCl_3$ hydrolysate + Yeast basal media

The sudan black B stain is absorbed by the inclusion bodies bearing the lipid bodies are stained blue. The safranin which is the counterstain is absorbed by the extracellular bodies which are devoid of lipid, and are stained pink in the cytoplasmic regions which acts as a light background for the lipids which appear as dark spots (Thancharoen *et al.*, 2017, Thakur *et al.*, 1988).

4.8 Quantification of lipid

R.toruloides 3547 has the ability to accumulate lipid upto 70% during its log and stationary phase by consuming both C5 and C6 as carbon sources present in AVLR and converts them as lipid (Osorio-González *et al.*, 2019). Such lipid accumulation varies with different catalysts. In "CuCl hydrolysate" catalyst it can be seen that the lipid content was maximum with 48 % which is similar to that of the lipid content provided in (Shakeri *et al.*, 2021).

Second highest lipid content is obtained in "CuCl hydrolysate and Yeast basal media" catalyst with 45.1 % which was also closely associated with (Shakeri *et al.*, 2021). Next in decreasing order of lipid content is the "FeCl3 hydrolysate and Yeast basal media" catalyst with lipid content of 39.49% which aligned with (Saini *et al.*, 2020). The least lipid content is obtained in "$FeCl_3$ hydrolysate" catalyst with a lipid content of 36.36% which can be related with (Zhou *et al.*, 2012). One of the important parameters in efficient lipid extraction is with pretreatment using organosolv which is more efficient than acid hydrolysis as discussed in (Delgado *et al.*, 2017).

Table 6: Lipid yield, pellet yield and lipid% in all hydrolysates

Samples	Lipid yield (g/l)	Pellet yield (g/l)	Lipid Content (%)
Cu Cl hydrolysate	4 ±1.11	8.33 ±2.07	48 ±5.28
FeCl3 hydrolysate	1.33 ±1.11	3.67 ±2.07	36.36 ± 5.28

Cu Cl hydrolysate +Yeast basal media	2.33 ±1.11	5.17 ±2.07	45.10 ± 5.28
FeCl3 hydrolysate +Yeast basal media	2.83±1.11	7.17 ±2.07	39.49 ± 5.28

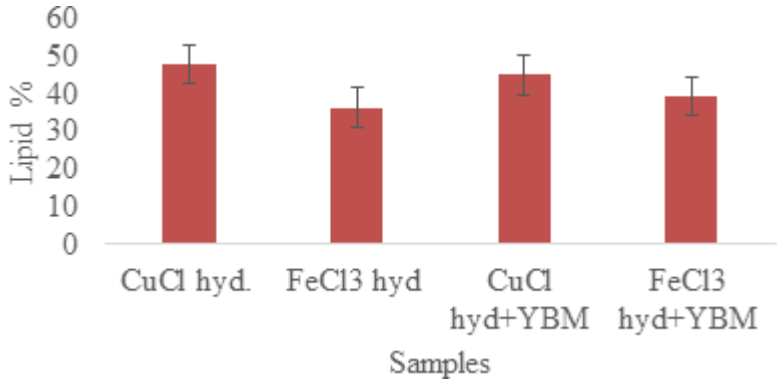


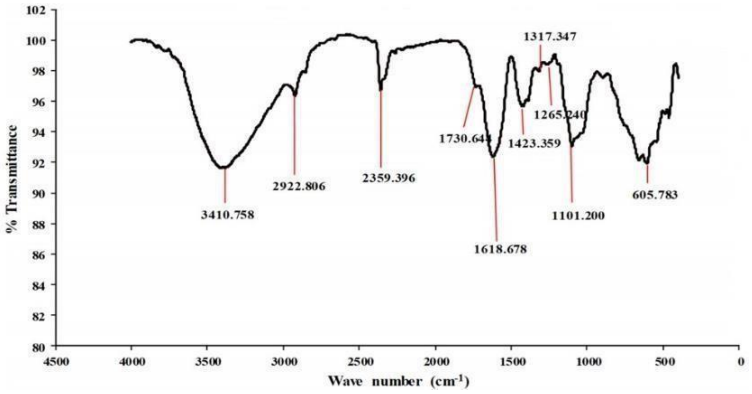
Fig 6: Lipid accumulation by R. toruloides 3547 in different treatment derived hydrolysates

In comparison from the aforementioned paper the lipid content of CuCl treated AVLR was similar to organosolv treated lipid content and $FeCl_3$ treated AVLR had similarity with acid hydrolysed lipid content. This indicates that CuCl treated AVLR is superior in efficient lipid extraction compared to $FeCl_3$ treated AVLR. Hence it can be inferred that *R. toruloides* can be used for microbial lipid production using AVLR as a lignocellulosic biomass and the lipid obtained has promising industrial interests (Osorio-González et al., 2019).

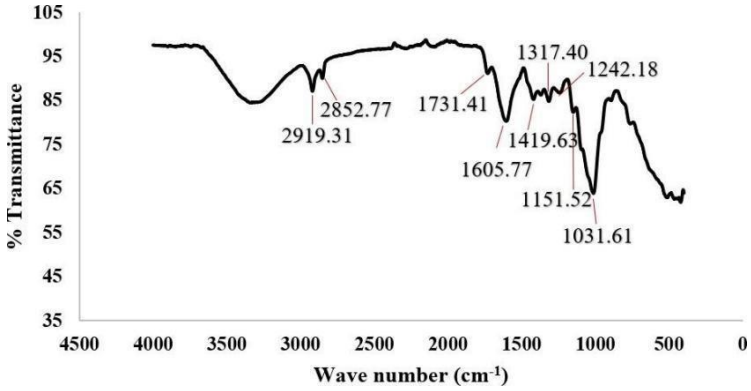
4.9 FTIR Analysis

The FTIR analysis shows the modifications of the AVLR functional groups for both before and after organosolv pretreatment. The existence of peak near the region 1600 cm-^{1} and 1423 cm-^{1} indicates the presence of lignin in both raw and pre-treated AVLR and these peaks corresponds to the phenyl and methyl presence of groups of propane in the lignin.

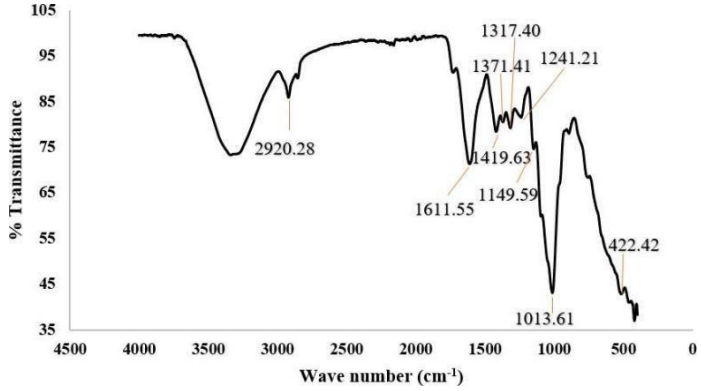
The peak about 1732 cm^{-1} signifies the band for hemicellulose containing saturated alkyl esters and this peak is present in raw and FeCl3 treated AVLR but absent in CuCl treated. This shows that in CuCl treated AVLR, the hemicellulose is extracted fully but not in $FeCl_3$ treatment (Corredor *et al.*, 2008).



(a)



(b)



(c)

Fig 7: FTIR analysis of a) Untreated/raw AVLR; b) $FeCl_3$ treated AVLR: c) CuCl treated AV

The peaks near 1245 cm^{-1} in CuCl treated AVLR and 1732 cm^{-1} in FeCl3 treated AVLR represents the pectins and phenolics solubilization. Modifications near 1245 cm^{-1} parallels to C–O stretch and C–O–H distortion of phenolics (Sene *et al*., 1994).

The peak at 1318 cm^{-1} and 2922 cm^{-1} attributes to C1–O vibrations and C–H deformation of lignin especially in the syringyl group. The peak near 3413 cm^{-1} represents the OH stretching and this peak is absent in both CuCl and $FeCl_3$ treated AVLR indicates the disruption of hydrogen bonds in cellulose meaning the effective degradation of lignin (Gunasekaran Rajeshwari, 2019).

CHAPTER -5

Conclusion

CuCl catalyst in comparison to $FeCl_3$ catalyst in the organosolv pretreatment method is found to be more efficient. Given the high values of sugar consumed post fermentation by *R.toruloides* 3547 in the different substrates, the organosolv method can be considered as a convenient procedure for pre- treatment. *R.toruloides* 3547 is found to utilize the "CuCl hydrolysate and Yeast Basal media" catalyst treated substrate with maximum efficiency as can be seen through the amount of biomass produced and growth analyzed with time. Maximum lipid content can be observed in AVLR treated with "CuCl hydrolysate" and FTIR results also confirmed that "CuCl hydrolysate" is efficient in delignification and increased sugar consumption by *R.toruloides* 3547.

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