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Biodiesel production from lignocellulosic biomass using *Yarrowia lipolytica*

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ABSTRACT

Depletion of hydrocarbons is forcing to find alternative resources to meet the energy demand of the growing population. Microbial biodiesel as a fuel can act as a cheaper and eco-friendly alternative to fossil fuel. Single-cell oil (SCO) consisting of carbon, hydrogen and oxygen grown over the lignocellulosic biomass using oleaginous microorganisms are triacylglycerols which can be converted to biodiesel, with physicochemical properties similar to conventional diesel. However, several cost-effective pretreatment methods are required to utilize lignocellulosic biomasses. The current research study investigates the SCO yield (and biodiesel characteristics) obtained from sugarcane bagasse hydrolysate through various pretreatment techniques. The pretreatment with 4% v/v H_2SO_4 at 25 min of ultra-sonication provided the best depolymerisation results (based on the glucose concentration). *Yarrowia lipolytica* was inoculated into the hydrolysates, allowed to grow at 25 °C, pH of 6.5 and rapid mixing for six days yielded biomass of 16.39 g/l. Biodiesel was extracted from the biomass via in-situ and ex-situ transesterification. In-situ transesterification carried out with the catalyst K_2CO_3 yields 80% biodiesel. In comparison, 63% were achieved with ex-situ transesterification, where lipid extraction was carried out as a first step and transesterified further in the presence of catalyst KOH to obtain biodiesel. The obtained fatty acid methyl esters (FAME) was subjected to FTIR analysis, and the observed physicochemical properties were within the international standards.

Introduction

The economic development (due to the unprecedented utilization of fossil fuels) was attained at the cost of environmental concerns such as global warming, ozone layer depletion, climate change, melting of polar glaciers and defforestation [1]. The present era was confronted with many concerns of population explosion and energy crisis due to depleting fossil fuel reserves resulting in substantial crude oil production. These issues could be partly addressed by exploring non-conventional energy sources [2]. This critical situation warrants the demand for alternative fuels and in the coming decades is expected to have more contribution from biofuels rather than the fossil fuel sector [3,4,5]. Biodiesel is a potential sustainable fuel made from plant, animal, and microbial lipids with oleaginous ability. To meet the high demand of fuel, interest in 3rd generation biodiesel produced from oleaginous microorganisms (OM) has grown significantly [6,7]. Using OMs may also help realise the circular economy and create cost-effective procedures. For example, biodiesel produced from waste product crude glycerol may be used as a carbon source for lipid build-up [8]. These lipids may then be regenerated into fatty acid methyl esters (FAMEs) and glycerol. Adopting to this procedure may result in cost-effective biodiesel synthesis from agro-residues, glycerol, and other waste substrates [9].

It is irrational to depend on the edible feedstock (as raw materials) for biodiesel production because a huge population relies on edible vegetable oil that accounts for a production rate of nearly 30 billion tons per year [10]. The consumption of large amounts of vegetable oils for biodiesel production could result in a shortage of edible oils, thus

Abbreviations: SCO, Single-cell oil; FAME, fatty acid methyl esters; OM, oleaginous microorganisms; LCB, lignocellulosic biomass; SBP, sugarcane bagasse powder; KOH, potassium hydroxide; FTIR, Fourier transform infrared spectroscopy.

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leading to an increase in food prices and triggering food-fuel priority [11,12]. The complications associated with biodiesel production are its high cost, of which raw materials amount to approximately 60–75% of the total production cost [13,14]. Henceforth, unconventional resources should be explored for energy utilization to meet its demand [15,16]. The production of biodiesel should be cheaper and high quality, utilizing non-edible oils and other sources. To mitigate this issue, researchers are exploring the production of microbial oil using various oleaginous organisms and converting them to biodiesel [17,18]. Microorganisms, especially oleaginous species, may accumulate more than 20% of lipid by the mass fraction of biomass [19]. The quantity of lipids that can be recovered from yeasts can vary according to the raw material used.

Oleaginous yeasts have such a wider range of carbon sources and compared to microalgae, they are less affected by seasonal variation or climate change. The most well-known oleaginous yeast strains, such as *Lipomyces*, *Yarrowia*, *Cryptococcus*, and *Rhodosporidium*, may store up to 40% of their cell dry weight in lipids. *Yarrowia lipolytica* was considered as a model yeast for producing lipids as a non-conventional yeast [20]. Since *Yarrowia lipolytica* is completely aerobic, it is well suited to high-cell density modes of cultivation [21]. Moreover, biodiesel from lignocellulosic biomass (LCB) like switchgrass could replace 20% of current United States diesel demand [22]. Since the worldwide production of sugarcane is approximately 1.6 billion tons and the residues generated through the various processes are around 279 million metric tons [23,24], the utilization of sugarcane bagasse as a novel carbon source for the growth of oleaginous organism are still emerging [25,26].

Sugarcane bagasse is a popular waste lignocellulosic biomass having 28–45% of its weight as cellulose and hemicellulose, while lignin constitutes only 15–20% of the total constituent [27]. Lignin being recalcitrant makes the process of utilization of biomass difficult. The limitation with the usage of cellulose in LCB lies with the convertibility to monomeric glucose, which is preferred by microbes for their growth. The cellulose is bound to hemicellulose and lignin, which binds them in an inaccessible and unavailable form. Any conversion technique is being halted by lignin, which cannot be converted unless under implemented under drastic conditions. Hence, pretreatment is necessary for solubilising the cellulose from the recalcitrant matrix.

In many cases, a single pretreatment technique could not convert enough cellulose for microbial culturing [28]. Hence, a combination of treatments is necessary for the saccharification of LCB to utilize glucose and xylose. Chandel et al. [29] have reviewed the conversion of hemicellulosic sugars via fermentation into ethanol, xylitol and other value-added products. Wei et al. [30] also reported that the economic feasibility of biofuels from lignocellulosic biomass could be increased via exploration of xylose sugars from hemicellulose (which is the second most abundant sugar). Aguilar et al. [31] reported that under specific conditions of acid hydrolysis, hemicellulose predominates, and cellulose is weekly hydrolysed, thereby contributing significantly to the biofuel yield. However, the utilization of sugars is also dependent on the yeast strain utilized. The study by Yook et al. [32] reported that the metabolism of xylose by *Y. lipolytica* is appreciably low due to its cryptic and conditional catabolism. Thus, utilizing xylose as the sole carbon source for lipid production is not feasible [33]. Rodriguez et al. [34] and Li and Alper [35] have overexpressed enzymes like xylose reductase, xylitol dehydrogenase and oxido-reductase in *Y. lipolytica* to act as a substrate for lipid production. However, the presence of several co-factors during lipid metabolism to provide the reducing power often makes *Y. lipolytica* in-efficient, thereby limiting the utilization of xylose as the only carbon source for obtaining lipids. Further, the yeast strain *Y. lipolytica* is also robust enough to tolerate a wide range of phenolic compounds generated during the pretreatment process as enlisted in the studies by Vatsal et al. [36] and Theerachat et al. [37].

The major objective of the present study was to analyse the effect of different pretreatment conditions, i.e. acid pretreatment, alkaline pretreatment combined with ultrasonication over the glucose yield from sugarcane bagasse hydrolysates. The hydrolysate from sugarcane bagasse obtained was thereafter used as the growth medium for cultivating *Y. lipolytica*. The yeast biomass was then used to evaluate the biodiesel yield under different conditions of in-situ and ex-situ alkali-based transesterification, and further, the properties of biodiesel obtained were qualitatively and quantitatively analysed.

Materials and methods

Yeast strain

Yarrowia lipolytica was obtained from the Institute of Microbial type culture collection and Gene bank (IMTECH, Chandigarh, India) in the form of freeze-dried culture. It was revived from lyophilized stocks stored in ampoules and cultured onto nutrient broth. One colony was inoculated into 1.50 ml nutrient broth. This seed culture was transferred to 100 ml nutrient media, making a 6% inoculum concentration and stirred in an orbital shaker at 28 °C and 150 rpm. The composition of the standard nutrient medium constitutes the following: Malt extract − 3.0 g/L; Yeast extract − 5.0 g/L; Peptone − 5.0 g/L and Glucose −10.0 g/L. The subcultures were then used for inoculation into sugarcane bagasse hydrolysates for later studies.

Sample preparation

Sugarcane bagasse was collected from the regional juice shop and dried in a hot air oven at 65 °C overnight. The dried bagasse was shredded in the shredder and ground into a fine powder using a locally made Willy mill. The fine powder is then subjected to sorting (based on size) using a sieve shaker. The finest particles passing through the 75 μm sieve sizes were taken for further hydrolysis analysis. The particle size of the sugarcane bagasse powder (SBP) was analysed by dissolving a pinch of the sample in ethanol. To obtain successful dispersion of material, the dissolved solution was kept in probe sonicator for about 7 min. Afterwards, the sample was analysed by particle size analyser (Malvern make and Zeta size nano zs90 model). The samples of SBP were subjected to detailed proximate and biochemical composition analysis as per the standard procedures detailed in the study by Praveen et al. [38].

Pretreatment of sugarcane bagasse

Acid pretreatment

1 g of SBP was taken in a 250 ml conical flask. Sulphuric acid (H_2SO_4) (98% purity) was then added at various concentrations such as 1%, 2%, 3%, 4% and 5% v/v of distilled water with a ratio of solid to water ratio of 1:25 w/v. The concentration of sulphuric acid for pretreatment has been chosen based on the previous studies [39–41]. It was kept in an autoclave at 121 °C under 15 psi pressure and allowed to react for a residence time of 20 min. High temperature boils the water above its boiling point, and the high-pressure break the entry of superheated steam into the cellulose molecules. This prolonged hydrolysis facilitates the breakdown of the insoluble bonds of ß-glucose chains. Further, to achieve effective hydrolysis, the solution is mixed at 150 rpm in an orbital shaker for 24 h. All experiments were done in triplicates. The effectiveness of the acid pretreatment was analyzed by determining the glucose concentration after hydrolysis. Afterwards, the acid hydrolysates were neutralized to facilitate the growth and propagation of microbes.

Alkali pretreatment

Similar to the acid pretreatment explained above, alkaline pretreatment was carried out by varying the concentration of potassium hydroxide (KOH) (99% purity) as 1 N, 2 N, 3 N, 4 N and 5 N. A similar procedure of hydrothermal treatment as employed during acid pretreatment described in the previous section was also implemented for alkali based complete degradation of lignin component to expose the cellulose fraction. Glucose concentration was assessed in the

hydrolysates to measure the effectiveness of the pretreatment. All the experiments were done in triplicates, and the deviation observed was less than 5% of the mean values.

Ultrasonication

The molecular breakdown of cellulose and hemicellulose was further enriched by adopting ultrasonication of optimized hydrolysates. The acid and the alkali hydrolysates showing the maximum glucose concentration was thereby combined with ultrasonication carried out for various durations of 5, 10, 15, 20 and 25 min. The effectiveness of co-combined ultrasonication based acid and alkali pretreatment was analysed by determining the glucose concentration of hydrolysates at different time-interval.

Glucose analysis of sugarcane bagasse hydrolysates

After pretreatment, the sample was filtered using Whatman filter paper (#1) to acquire hydrolysates rich in fermentable sugars. Then the hydrolysates were adjusted to the pH value between 6 and 7 since *Yarrowia lipolytica* shows high growth at such range. The effectiveness of the pretreatment technique relies on the amount of glucose liberated into the solution from the treatment. The finest technique should give a high value of glucose through which it can be concluded that the cellulose and hemicellulose present in the bagasse were converted into fermentable carbohydrates. In this study, the phenol–sulphuric acid method of glucose analysis was adopted.

For standard glucose measurement, a 1000 ppm solution of glucose was prepared by dissolving 100 mg of dextrose (LR) into 100 ml of distilled water. For subsequent solutions, a serial dilution of stock solution was performed to yield 200, 400, 600, 800, 1000 ppm. 1 ml of standard glucose solution was added to 1 ml of 5% phenol. 5 ml of conc. H_2SO_4 was added to the above solution and kept for 1 h to attain room temperature. Then 3 ml of the final solution was used for glucose measurement in a double beam ultra-violet (UV) visible spectrophotometer (PerkinElmer make and Lambda 35 model). The sample absorbance was read at the wavelength of 490 nm, and the values were obtained. The standard curve was drawn by plotting absorbance along Y-axis and the concentration along X-axis. For glucose estimation of hydrolysates, 1 ml of 0.1% pretreated samples were taken, and the same procedure was followed. The obtained absorbance values were compared to the standard curve, and the corresponding glucose concentration was identified. The experiment was repeated in triplicate for concordant values of glucose.

Growth of Yarrowia lipolytica in the fermenter

The growth of the oleaginous organism (*Yarrowia lipolytica*) was studied on a dry weight basis to observe the growth profile and to ensure suitable environmental conditions for rapid reproduction. The dynamics of the microbial growth can be charted employing a population growth curve through plotting the biomass concentration versus time of incubation. 20 ml of sample from the pretreated hydrolysates solution were taken and centrifuged at 7000 rpm for 5 min. The supernatant was discarded, and the leftover biomass was washed with deionized water a couple of times. Then it was transferred to the crucible, weighed and kept in an oven at 65 °C until it was completely dehydrated. By using the growth curve, the cultivation period for the organism was determined.

Bio-age autoclavable fermenter (Bio-Spin 05A, Bio-Age, India) with the capacity of 5 L was used for mass cultivation of biomass along with bioprocess monitoring of pH, DO, and mixing. A condenser arrangement is provided to maintain the temperature. Aerobic condition is maintained employing an artificial air pump, which makes the oleaginous yeast grow well. After sterilising the fermenter in an autoclave, pretreated hydrolysates and oleaginous yeast were transferred into the fermenter. Pre-determined growth conditions such as pH of 6.5, the temperature of 25 °C and a stirring rate of 120 rpm were maintained in the fermenter. Acid, base, antifoaming solutions were provided to adjust the pH if it exceeds the pre-set limit and avoid foaming.

After sufficient biomass growth, the broth was centrifuged to separate the biomass and was weighted. The cell wall removal of biomass was performed using 0.1 N of Hydrochloric (HCl) acid followed by homogenization with mortar and pestle for 5 min. Then the solution was dried under a hot plate to remove excess HCl, and the dried biomass was stored until further use. The breakdown of cell wall is essential to release the fermentable sugars from the residue as well as the excessive HCl will be released due to the mild heating.

Biodiesel production

Biodiesel production was carried out via the transesterification process, and it is the chemical conversion of triglycerides into fatty acid alkyl ester groups and glycerol through a reaction with ethanol/methanol in the presence of a catalyst. In this research study, transesterification was performed via two methods, i.e. in-situ transesterification (single step production of biodiesel from yeast biomass using an alkali catalyst) and ex-situ single step alkali catalysed transesterification methods.

In-situ transesterification of biomass

In-situ direct transesterification, the dried crude biomass was treated with chemical processing without any lipid extraction. Biomass was taken in the range of 2.5 % w/v of methanol in a round bottom flask, and 3 g of K_2CO_3 was added to the sample. The sample filled flask was kept in a water bath along with the reflux set up. The water bath is heated to a temperature of 55 °C for two hours. At this temperature, some amount of methanol gets vaporized and escapes upward into the condenser column. This vapour is condensed and returns to the solution. The reaction was allowed to take place for about 90 min, and then the solution was allowed to cool down to room temperature.

Hexane was added in the ratio of (5:3) into the solution, and then it is rapidly mixed with the help of a magnetic stirrer at 700 rpm for about 30 min. The mixture was transferred into the separating funnel and kept untouched for about two hours. The layer separation was clearly observed, the top layer contains hexane along with biodiesel, and the bottom layer contains methanol, catalyst and biomass. The top layer containing hexane and biodiesel was separated employing heating above the boiling point of hexane using a thermo-mixer. Then the water-soluble contents were removed by reacting with an equal volume of 0.9 N sodium sulphate solution to separate the biodiesel.

Ex-situ alkali catalysed transesterification of extracted lipids from biomass

In the ex-situ transesterification process, lipid was extracted (first step), and subsequent lipid transesterification (second step) was carried out. Lipids were extracted from the biomass by adopting Hara and Radin method with minor modification. Hara and Radin [42] utilized ratio of hexane: isopropanol at 3:2, whereas, in this study, it was modified to a 5:3 ratio to homogenize the mixture and produce a higher yield of lipid extraction. The carbohydrates and proteins were separated from lipids using hydrophilic and hydrophobic solvents. 1 g of dried biomass mixed with 20 ml of hexane: isopropanol solution (5:3) was then poured into 50 ml of the conical flask. The biomass was later subjected to bead milling with the glass beads for 150 min in a magnetic stirrer at 200 rpm and subsequently speeding up to 700 rpm for a period of 30 min.

Finally, the lipid components were separated by centrifugation at 8000 rpm for 10 min. The separation of distinct layers was observed in the centrifuge tube. The bottom and top layers contain protein and lipid, respectively. The top layer containing lipids was separated by pipette and dried in a hot air oven at 105 °C for 12 h. An ethanol emulsion test is conducted to confirm the presence of lipids in the given sample. Ethanol interacts with any lipid suspended in water and forms a cloudy appearance, whereas, absence of lipids leads to a clear solution. The lipid samples were tested with this conventional method as a

preliminary assessment before being subjected to the analysis of the characteristic functional groups via attenuated total reflectance via Fourier transform infrared spectroscopy (FTIR) scan from 4000 cm^{-1} to 700 cm^{-1}.

For transesterification of lipids, 0.5 g of KOH catalyst was dissolved into 20 ml of methanol. Then this solution was mixed with the lipid in the ratio of 2:4 and heated in a thermo-mixer for 10 min at 55 °C to obtain fatty acid methyl esters (FAME). The methyl groups corresponding to the FAMEs in the transesterified biodiesel samples were quantified via FTIR analysis as outlined by the standard protocols by ASTM D7371-14 and EN-14078. The physicochemical properties like the density, viscosity, flash point were also quantified as per the standard protocols detailed by Behera et al. [43] and Supraja et al. [14].

Results and discussion

Physicochemical characterization of sugarcane bagasse

Sugarcane bagasse is a pulpy fibrous residue obtained after the extraction of sugar molecules, and it is abundantly available at throw-away cost or free. However, the utilization of sugarcane bagasse for the desired fermentation process demands a series of pretreatment steps. Particle size reduction is the key step in pretreatment as it could offer a higher surface area to volume ratio, which further helps in the efficient breakdown of cellulose. The shredded and powdered sugarcane bagasse was subjected to sieve analysis, and the particles passing through 75 μ sieves was utilized in this study. Particle size analysis was carried out to determine the exact distribution of particles. It was performed at the temperature of 25 °C, count rate of 342.5 for the duration of 50 s, measurement position of 4.65 mm with 11 attenuation shows Z- Average as 572 nm and an intercept value of 0.978. The smaller and uniform particle size of sugarcane bagasse ensures better pretreatment efficiency. Smaller particle size often helps in achieving better hydrolysis and cellulose conversion rates [44]. Fig. 1 shows the size distribution of powdered sugarcane bagasse by intensity curve. The proximal composition of the powdered sugarcane bagasse is listed in Table 1. Biochemical composition analysis showed the presence of 50% carbohydrates, 1.8% proteins and 0.6% fats in the sugarcane bagasse sample. The presence of 2.1% ash with 50% carbohydrates makes sugarcane bagasse an ideal substrate for facilitating the growth of *Y. lipolytica*. Similar proximate compositions for sugarcane bagasse were also presented in the study by Daniyanto et al. [45] and Shukla and Kumar [46].

Table 1
Proximate and biochemical composition of sugarcane bagasse.

Properties	Values (%)
Biochemical Composition	
Protein	1.8
Carbohydrates	50.0
Fat	0.6
Proximate Composition	
Ash	2.1
Crude fibre	30.4
Moisture	45.0

Pretreatment of sugarcane bagasse

Low-cost carbon substrates as a growth medium to sustain yeasts with are more appealing today in laboratory-scale research and industrial-scale applications to achieve a high biomass concentration and lipid content [20]. Bagasse from sugarcane is one of the most prevalent agricultural wastes [7]. It is lignocellulosic biomass that releases sugars through pretreatment and enzyme hydrolysis [47]. Its structure comprises 37–46% cellulose and 21–27% hemicellulose [48,49], yielding 40–51% glucose and 26–33% xylose following full hydrolysis.

Acid pretreatment of sugarcane bagasse

The principal monomeric compound released during the pretreatment process is glucose, which acts as a major carbon source for increasing biomass growth and, in turn, is responsible for the lipid content in the oleaginous organism. The pretreatment technique should be chosen based on the maximum amount of glucose concentration after process completion. In the case of the acid pretreatment technique, the concentration of acid was increased from 1 to 5% v/v for the breakdown of complex macromolecules. It was observed that the glucose concentration increased from 810 mg/l to 1190 mg/l with an increase in acid concentration from 1% v/v to 4% v/v (Fig. 2). However, in 5% v/v acid pretreatment, the concentration of glucose dropped down to 790 mg/l, inferring that the optimal acid concentration for obtaining the maximal glucose concentration from sugarcane bagasse is 4% v/v. Sritrakul et al. [50] projected 5.9 g (glucose)/100 g bagasse treated with 4% v/v of dilute sulphuric acid after 90 min treatment time. Similar results were also reported by Roslan and Salini [51], where an increase in acid concentration during pretreatment increased the yield of glucose until a particular concentration beyond which it declined.

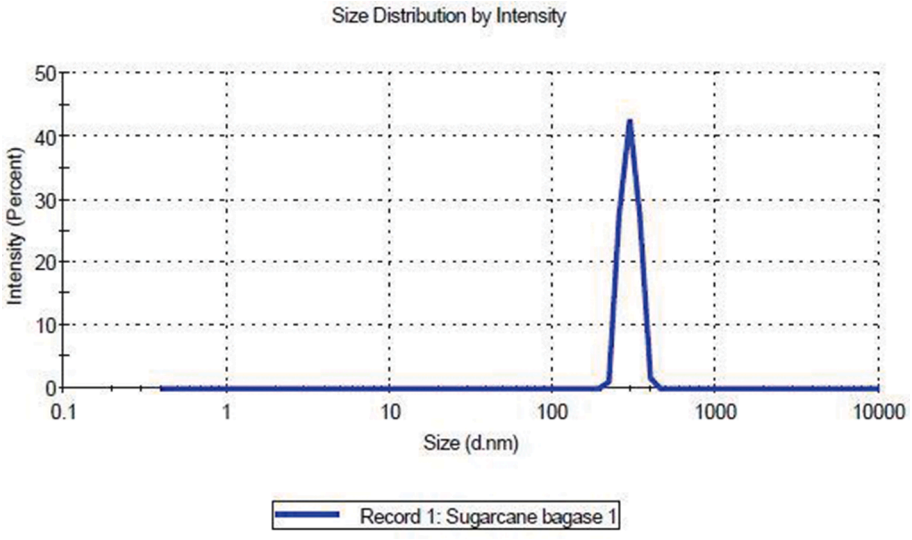

Fig. 1. Particle size distribution of powdered sugarcane bagasse.

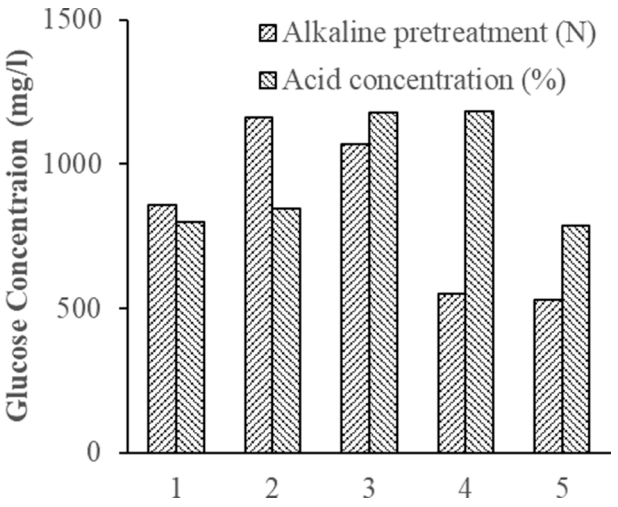

Fig. 2. Effect of alkaline pretreatment and acid pretreatment of sugarcane bagasse on the glucose yield.

Alkaline pretreatment of sugarcane bagasse

In the alkaline pretreatment of sugarcane bagasse, the alkalinity concentration was varied from 1 N to 5 N solutions (see Fig. 2). The yielded sugarcane bagasse hydrolysates were observed for glucose concentration to check the efficacy of the pretreatment. It was found that up to 2 N alkaline solutions, the sugarcane bagasse could yield higher concentrations of glucose up to 1170 mg/l. Afterwards, the glucose concentration dropped down from 1080 mg/l to 530 mg/l while varying the alkaline concentration from 3 N to 5 N. However, comparing both acid and alkaline pretreatments has been done so far with the sugarcane bagasse; 4% v/v of acid pretreatment yielded the maximum glucose concentration (see Fig. 2). Similar to the present study, Zhu et al. [52] also reported higher glucose yield during sulphuric acid pretreatment compared to the alkaline pretreatment. Contrary to the present study, Yoon et al. [53] reported that alkali treatment causes better delignification compared to acid-based pretreatments. Nevertheless, the efficiency of the pretreatment methods depends on the hydrolysing potential of the concentrated acid/alkali and the biomass utilized.

Combined acid pretreatment followed by sonication of sugarcane bagasse

The combination of two pretreatment techniques, i.e., physical and chemical, was attempted in the present study to evaluate whether higher glucose yields from sugarcane bagasse could be attained. Hence, a novel sonic pulse wave generated from the ultrasonicator was utilized sequentially with the acid pretreated sugarcane bagasse to check for its impact on glucose yield. The ultrasonication was done with varying durations (5, 10, 15, 20, 25 min). It was observed that the glucose concentration at 25 min treatment of sugarcane bagasse was at a maximum corresponding to a value of 1380 mg/l. However, the sugarcane bagasse subjected to 4% v/v acid pretreatment followed with 25 min sonication yields a 15% higher glucose concentration than that of 4% v/v acid pretreatment alone. Wu et al. [54] reported that ultrasonication provided the requisite heating and increased the porosity of the biomass, thereby increasing the efficacy of chemical solvents during pretreatment.

Growth of Yarrowia lipolytica

The obtained sugarcane bagasse hydrolysates were further subjected to the fermentation process by *Yarrowia lipolytica* yeast strain under optimal environmental conditions. The growth of *Y. lipolytica* under the same environmental conditions with the standard nutrient medium was considered. On the first day of the fermentation process, both biomass increase and lipid accumulation were not significantly seen, as it can be due to the fact that the cells were adapting to the new environmental conditions. After a few hours, the *Y. lipolytica* could able to grow steadily and multiply exponentially, as evident from the 2.43 g/l of biomass concentration in the standard medium at the end of the second day (Fig. 3). Also, the biomass concentration in sugarcane bagasse hydrolysate was at 1.83 g/l at the end of the 2nd day of fermentation. Similar trends were observed till the end of the sixth day, where biomass concentration on standard medium and sugarcane bagasse hydrolysates were 21.38 g/l and 16.39 g/l, respectively. Beyond that sixth day, not much significant increase in biomass concentration was observed in both the mediums. Likewise, Fontanille [55] observed, under initial glucose concentrations of 40 g/L, *Y. lipolytica* produced 31 g/L biomass and 40% lipid content. Sarris et al. [56] studied the growth of *Y. lipolytica* in glucose enriched olive mill effluent resulting in 12.7 g/l biomass concentration. Tsigie et al. [57] reported 11.42 g/l biomass for *Y. lipolytica* with peptone as the nitrogen source, thereby accumulating 58.5% of lipids. The review by Jin et al. [58] has discussed similar biomass concentrations of different oleaginous yeasts in alternative low-cost carbon substrates. Obviously, the concentration of carbon sources is also important. Carbon source concentrations that are either too low or too high have a detrimental impact on yeast growth and lipid formation [59]. Our findings underscored the critical role of an adequate carbon source concentration in maximising substrate utilisation and lipid and biomass synthesis.

Growth on glucose can be considered as a basis (for comparison), as this substrate has been broadly used. The growth of *Y. lipolytica* on the sugarcane bagasse hydrolysates was accompanied by the production of various organic acids, which resulted in the decline of pH of the culture medium. However, the growth of oleaginous yeasts exhibits three phases of growth under nutrient limitation conditions. During the exponential growth phase, the yeast cells of *Y. lipolytica* were undergone rapid proliferation. The oleaginous yeast cells show minimum growth due to nutrient limitation in the lipid accumulation phase (or late log phase). In the stationary phase (or late accumulation phase), the accumulation of lipid occurs at maximum pace along with a little catabolic breakdown of lipids. The shift of catalytic growth of oleaginous microbes to accumulate lipids generally occurs when excessive carbon in the medium is associated with a nutrient limitation that impacts biomass production [60,55].

Lipid accumulation in Yarrowia lipolytica

From the obtained biomass, the lipid accumulation efficiency of *Y. lipolytica* was evaluated by following the methodology outlined in Hara and Radin method. The lipid yield in sugarcane bagasse hydrolysate was observed as 0.587 g/g of the dry weight of the biomass, suggesting the potential of this yeast strain for biodiesel production. Since

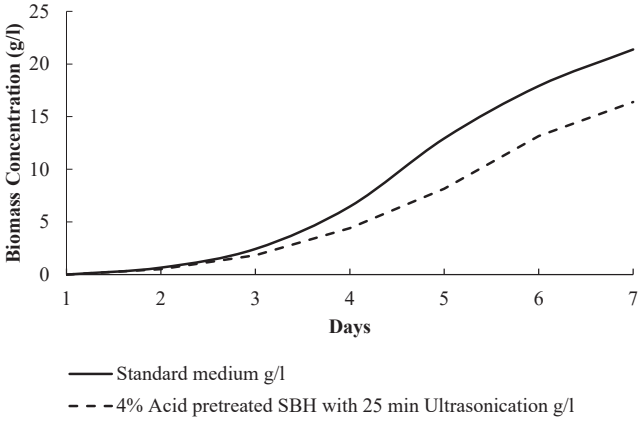

Fig. 3. Growth profile of Yarrowia lipolytica on the sugarcane bagasse hydrolysates.

the utilized yeast strain *Y. lipolytica* belongs to oleaginous microbes, the capability of transforming organic acids into Acetyl Coenzyme A (ACA) is quite high. The ACA is the key intermediate on the biosynthesis of lipids in these microorganisms [55]. However, the accumulation of lipids by the microbial strains primarily depends on the microbial physiology, nutrient limitation and other environmental factors such as pH and temperature of the nutrient medium [60]. Besides *Y. lipolytica*, the other predominant oleaginous microorganisms are from the genus *Candida, Cryptococcus, Rhizopus,* and *Trichosporon*. Jin et al. [58] have summarized the lipid accumulation capacity of several oleaginous yeasts grown in different alternative growth mediums. Sarris et al. [56] reported 0.28 g/g lipids during the growth of *Y. lipolytica* in glucose enriched olive mill effluent. Tsigie et al. [57] reported that *Y. lipolytica* grown in synthetic medium with peptone as the nitrogen source could accumulate 58.5% of lipids. *Y. lipolytica* has grown in acid hydrolysed wheat straw accumulated 0.4 g/L lipids [61], while in sulphuric acid-treated defatted rice bran hydrolysate, *Y. lipolytica* could accumulate 5.2 g/L of lipids [62]. However, the lipid accumulation capacity varies among the species, as on average, these yeasts, under normal conditions, could accumulate lipids up to 40% of their biomass. Under nutrient limiting conditions, the lipid accumulation could reach more than 70% of their biomass [60,63]. The characteristic functional groups of lipid formed during the growth of *Y. lipolytica* in sugarcane bagasse hydrolysate was verified by FTIR analysis, and the results are shown in Fig. 4. The peak at 3475.29 cm^{-1} represents the presence of hydroxyl group, peaks at 2954.51 to 2853.17 cm^{-1} indicates the existence of C–C and C = C groups. The peak at 1745.51 cm^{-1} corresponds to the ester groups of lipids, and the 721.83 cm^{-1} peak shows the presence of long-chain ($>CH_2$) groups in the molecular chain. From these peaks, the presence of long-chain unsaturated fatty acids in the sample was confirmed. Similar spectral peaks for lipids extracted from oleaginous yeast - Cryptococcus vishniaccii with the potential to be converted into biodiesel was also reported in the study by Deeba et al. [64]. The library search performed with FLUKA yielded the same, with several fatty acids such as linoleic acid (C18), oleic acid (C16) and erucic (C24) acids. A similar spectrum of lipid extracts was also reported for oleaginous yeasts by Shapaval et al. [65].

Biodiesel from transesterified lipids of Yarrowia lipolytica

Transesterification is being used to generate microbial biodiesel from microbial lipids. The present study considered the transesterification process of accumulated lipids from *Y. lipolytica* by direct (in-situ) and ex-situ single-step alkali-based transesterification. FAME produced from K_2CO_3 under the direct transesterification yielded 80% conversion efficiency of lipids. However, the biodiesel yield in the ex-situ single-step alkali-based transesterification process with KOH catalysis resulted in 63% biodiesel yield. In general, alkali-catalyzed transesterification is the most preferred route due to milder reaction conditions and minimal reaction time and costs; as compared to the other processes [66]. It might be due to the fact that the derived lipid accumulation in *Y. lipolytica* grown in sugarcane bagasse has less free fatty acid content, which might contribute to the higher yield of biodiesel in the single-step transesterification process. Hazmi et al. [67] also reported slightly higher microbial biodiesel yield through in-situ alkali catalyzed transesterification from oleaginous yeasts compared to the ex-situ transesterification following Soxhlet and Bligh and Dyer based lipid extraction. Chopra et al. [68] projected 92% FAMEs yield in the form of microbial biodiesel via integrated in-situ transesterification well comparable to that of the ex-situ process.

The appropriateness of fatty acid methyl esters is primarily evaluated by the physicochemical characteristics of the generated FAME, which must be within the permitted limits of applicable standards (ASTM D-3751, EN14214), as well as petrodiesel comparison studies [69]. Additionally, the characteristics of FAME are influenced by the fatty acid composition of the oleaginous oils used. The spectral analysis was carried out for FAME derived from the transesterified lipids obtained from the lipid accumulation of *Y. lipolytica* grown on sugarcane bagasse pretreated with 4% v/v acid followed by 25 min ultrasonication, through FTIR analysis as laid down by the EN-14078 and ASTM D7371-14. The spectral profile for FAME is similar to that of lipids with the exceptions. The FAME is almost devoid of free hydroxyl groups, and hence the peaks corresponding to the OH groups are absent in their infrared spectra. As seen in Fig. 5, the peaks at 3500–3200 cm^{-1} are reduced by 99%, implicating the replacement of ester bonding during the transesterification process.

The library search from FLUKA databases yielded the profile with various long-chain unsaturated fatty acid esters like methyl linoleate, ethyl linoleate and methyl oleate, which together constitute more than 95% of the spectral match. The various groups of the fingerprint region correspond to many bonds that are present in a typical long-chain molecule. The depth of the peaks is a measure of the purity of the extracted samples. Similar to the present study, Ami et al. [70] reported that FTIR combined with multivariate analysis that can act as an essential spectroscopic technique to detect fatty acid (FA) accumulation in oleaginous yeasts. Several other researchers, Akthar et al. [71] and

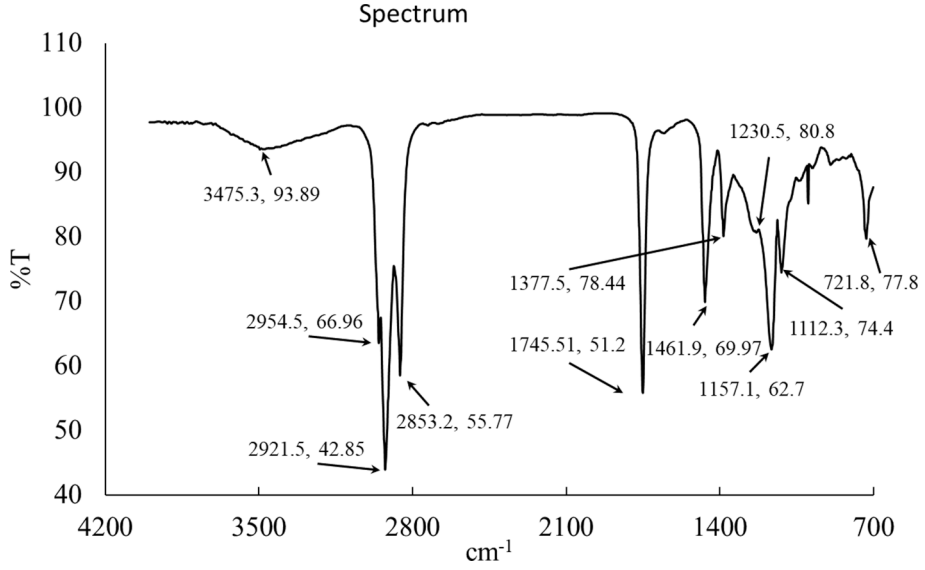

Fig. 4. FTIR spectrum of lipids derived from oleaginous yeast Yarrowia lipolytica on the sugarcane bagasse hydrolysates.

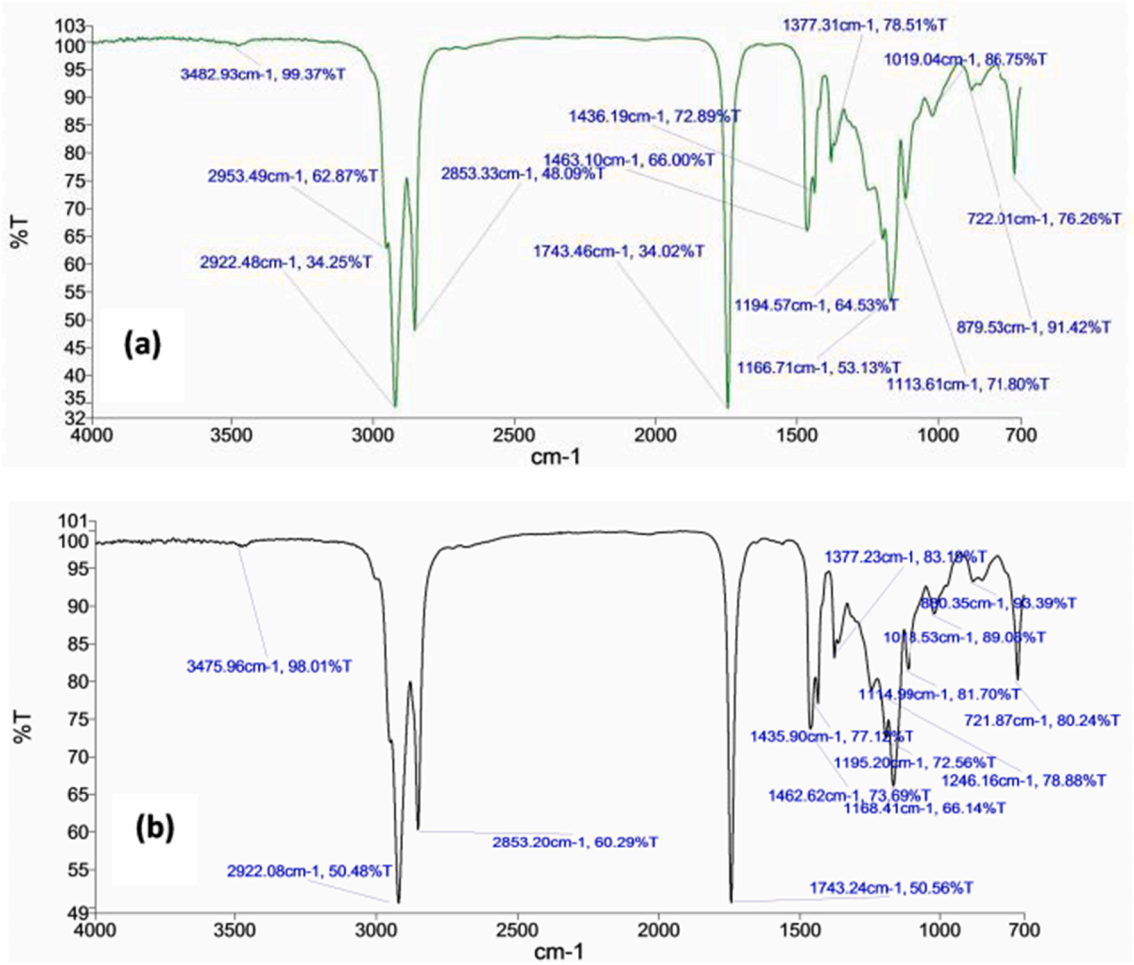

Fig. 5. FTIR spectrum of biodiesel obtained from Yarrowia lipolytica lipids by a) in-situ and b) ex-situ single step alkali catalysed transesterification.

Purandaradas et al. [72] have analysed the functional groups corresponding to methyl groups in biodiesel samples for corroborating the presence of FAMEs.

Physico-chemical properties of biodiesel were examined in order to ensure that the obtained biodiesel meet the specific standards values. Density, kinematic viscosity, flash point, fire point and specific gravity were tested based on the standard codes of ISO 3675, ISO 3104, ISO 3679, and IS 1448, respectively. The quality of biodiesel produced from *Yarrowia lipolytica* grown on sugarcane bagasse hydrolysates. The estimated physical characteristics of *Yarrowia lipolytica* were compared to the ASTM 6751–08 biodiesel standard. As demonstrated in Table 2, the characteristics of *Y. lipolytica* derived biodiesel are similar to those specified by ASTM 6751–08 and IS-15607 for biodiesel. The results of obtained biodiesel properties are compared with the various standards as listed in Table 2. The density and the kinematic viscosity of the obtained biodiesel were comparable with the various international biodiesel standards. Similar physicochemical properties for biodiesel were also reported by Behera et al. [43] and Supraja et al. [14].

Table 2
Physicochemical properties of biodiesel obtained from transesterification of lipids from Yarrowia lipolytica grown on sugarcane bagasse hydrolysates

Properties	Unit	Values	ASTM standards	European standards	IS standards
Density	g/cc	0.893	0.86–0.90	0.86–0.90	0.86-0.090
Kinematic Viscosity	mm^2/s	3.2	1.9–6.0	3.5–5.0	3.5–5.0
Flash point	°C	118	93	101	101
Fire point	°C	127			
Specific gravity	–	0.87			

Conclusion

The present study demonstrated the potential of sugarcane bagasse as a low-cost substrate for biodiesel production through the growth of oleaginous yeast *Yarrowia lipolytica.* Acid pretreatment with 4% v/v H_2SO_4 and ultrasonication (25 min) favour the efficient hydrolysates (85%) of monomeric glucose from the cellulose of sugarcane bagasse. Around 1.3 g/l of glucose were obtained from 10 g/l of sugarcane bagasse, and the subsequent growth of *Yarrowia lipolytica* yielded 58% DCW lipids. The transesterification by in-situ and ex-situ alkali catalysed transesterification process with K_2CO_3 and KOH as catalysts respectively yielded 80% and 63% biodiesel, which were comparable with the international standards. The outcome of the study could sensitize the waste to wealth and energy approach for the efficient utilization of discarded sugarcane bagasse. Finally, the total lipid yield evaluation showed that a critical component for increasing commercial viability of bioconversion for lignocellulose-based microbial lipid production is maximum sugar recovery from raw biomass and strain development for improved fermentation efficiency.

Declarations

Ethics approval and consent to participate
NA.

Consent for publication
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Availability of data and material
Not applicable.

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Authors' contributions
Madhu (Experimental study, data analysis and manuscript drafting); Murugan & Gobinath (Concept and Supervision); Balasubramanian, Gayathri & Karri (Compilation and Manuscript editing).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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