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Production and characterization of sophorolipid under yeast-mediated submerged fermentation utilizing Agro-industrial waste

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ABSTRACT

The challenges of pollution and agro-industrial waste management have led to the development of bioconversion techniques to transform these wastes into valuable products. This has increased the focus on the sustainable and cost-efficient production of biosurfactants from agro-industrial waste. Hence, the present study investigates the production of sophorolipid biosurfactants using the yeast strain *Rhodotorula mucilaginosa* IIPL32 under submerged fermentation, employing sugarcane bagasse hydrolysate—a renewable, low-cost agro-industrial waste as the feedstock. By systematically optimizing strain adaptation, medium composition, and scaling up the process from shake flasks to a bioreactor, a maximum sophorolipid yield of 2.6 ± 0.21 g/L was achieved. Extensive characterization was conducted, encompassing emulsification index (54 %), surface tension reduction, and several chemical analyses (anthrone, iodine, saponification, lipid solubility). Advanced structural elucidation techniques such as Fourier-transform infrared (FTIR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy were employed for structural confirmation of the extracted biosurfactant. FTIR spectroscopy identified characteristic functional groups, while LC-MS revealed distinct sophorolipid congeners with varying lipid chain lengths and acetylation. NMR spectroscopy corroborated the presence of disaccharide and fatty acid components, indicating the extracted biosurfactant might be sophorolipid. This study underscores the feasibility of utilizing agro-industrial waste for the eco-friendly production of sophorolipid biosurfactants and provides detailed insights into their structural features, highlighting their potential applications across diverse fields such as pharmaceuticals, cosmetics, and environmental remediation.

1. Introduction

Microorganisms synthesize glycolipid biosurfactants consisting of carbohydrate and lipid components. Glycolipids are classified into rhamnolipids, sophorolipids, and trehalolipids. These biosurfactants are environmentally benign substitutes for synthetic surfactants since they lower surface tension, are biodegradable, and can be utilized in food, cosmetics, and bioremediation (Das and Kumar 2018; Kumar and Das, 2018; Purohit et al., 2024). Sophorolipid biosurfactants, produced primarily by non-pathogenic yeasts, represent a promising alternative to synthetic surfactants due to their biodegradability, low toxicity, and environmental compatibility (I.N.A. Van Bogaert et al., 2007; Ambust et al., 2023; Al-Kashef et al., 2023; Alabi et al., 2023; Amândio et al., 2023). Comprising a sophorose molecule linked to a fatty acid, these glycolipids exist in both lactonic and acidic forms, each with unique properties suitable for diverse applications (Daverey andPakshirajan 2009). The societal importance of sophorolipids is underscored by their environmental benefits, wide-ranging industrial applications, antimicrobial properties, and potential roles in enhanced oil recovery (I.N.A. Van Bogaert et al., 2007). However, the high production costs associated with traditional substrates like glucose and vegetable oils pose a significant challenge alongside sustainability concerns (Makkar and Cameotra, 2002). A sustainable and cost-effective solution lies in the utilization of agro-industrial waste, particularly bagasse (Faria et al., 2018).

The fibrous residue known as bagasse, which is left over after sugarcane juice is extracted, is becoming more and more acknowledged as a cheap and plentiful substrate for microbial production processes, especially in production of valuable biological based microbial products like enzymes, biosurfactants, and biofuels (Pandey et al., 2000). Bagasse has

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a number of benefits over other traditional substrates like glucose or starch. It is a cheap, renewable resource that is frequently discarded as waste, which makes it a desirable substitute for environmentally friendly industrial uses. Cellulolytic microbes may break it down into fermentable sugars because of its high cellulose concentration, which offers them a possible carbon source. Bagasse's value as a substrate is further increased by the fact that it is readily available in areas with sugar industries, which lowers the requirement for expensive raw materials (Pandey et al., 2000).

Bagasse is abundant and inexpensive, offering a viable alternative substrate that can substantially reduce production costs and promote waste valorization (Faria et al., 2018). The process begins with the pre-treatment of bagasse through physical, chemical (using 1.0 % NaOH solution (w/v) prepared with a solid-to-liquid ratio of 1:10) or enzymatic methods(using cellulase enzyme at a dosage of 25 FPU/g) to break down its lignocellulosic structure, followed by fermentation using optimized conditions to maximize product yield (Rocha et al., 2012; Amândio et al., 2023).

Although diverting bagasse from farms to industrial uses has a lot of potential benefits, there may also be disadvantages that should be carefully considered. The effect on agricultural productivity is one issue. When left on the farm, bagasse can be applied as a soil conditioner, improving soil fertility and retaining moisture. Long-term crop yields may be impacted if this resource is diverted to industry, which could result in its depletion (Alabi et al., 2023). Furthermore, there are logistical issues associated with moving bagasse from farms to industrial locations, such as higher fuel usage and greenhouse gas emissions, which may cancel out some of the environmental advantages (Munagala et al., 2021). Furthermore, the energy needed to turn bagasse into bio-based goods may necessitate a large amount of industrial infrastructure, which could lower the economic benefits and increase operating expenses (Kabeyi and Olanrewaju, 2023). Therefore, even though bagasse has industrial potential, its removal from farms needs to be carefully controlled to maintain a balance between its usage in bio-industries and its agricultural utility, thereby fostering both economic viability and environmental sustainability.

Recent studies have demonstrated the feasibility and efficiency of this approach, highlighting similar or superior surfactant properties compared to those produced from conventional substrates (Faria et al., 2018). Innovations in fermentation technology, including advanced bioreactor designs and metabolic engineering of yeast strains, have further improved production efficiency (Daverey and Pakshirajan 2009). The economic benefits of this method are significant, as reduced raw material costs make sophorolipid production more viable and competitive, potentially expanding their market reach and fostering innovation across various industries (Makkar and Cameotra, 2002; Gaur et al., 2022). Environmentally, using agricultural waste enhances resource efficiency, reduces waste disposal issues, and lowers greenhouse gas emissions by replacing petrochemical surfactants (Faria et al., 2018). However, scaling up this process to industrial levels requires further optimization and infrastructure development (Daverey and Pakshirajan 2009). Policy support in the form of incentives for sustainable practices and regulatory approvals will be crucial for the widespread adoption of sophorolipid biosurfactants (I.N.A. Van Bogaert et al., 2007). A sustainable and economical strategy for increasing the production of sophorolipids has been the subject of very few studies. Thus, in order to show that utilizing agro-industrial waste can result in the production of environmentally friendly biosurfactants, this study reports the production of sophorolipid biosurfactants by oleaginous yeast *Rhodotorula mucilaginosa* IIPL32 (MTCC 25056) (hereafter denoted as RMIIPL32) using sugarcane bagasse hydrolysate and confirms its glycolipid nature through FTIR, LC-MS, and NMR. Bagasse-derived sophorolipids thus provide a sustainable production strategy while also being in line with larger environmental and economic objectives, opening the door to a more sustainable and greener future.

2. Material and methods

2.1. Biosurfactant producing yeast strain

The present study utilized an in-house biosurfactant-producing pink oleaginous yeast strain identified as *Rhodotorula musilaginosa. R. mucilaginosa* is known for its ability to accumulate lipids, making it an attractive candidate for biosurfactant production (Khot and Ghosh, 2017). For initial characterization and biosurfactant production studies, RMIIPL32 was cultured in YEPG (Yeast Extract Peptone Glucose) medium with a composition ratio of 1:2:2. This nutrient-rich medium supports robust growth and metabolite production in yeasts (Unrean, 2016). The cultures were incubated at an optimized temperature of 32 °C for 24 h to achieve sufficient biomass for subsequent experiments. Prior to large-scale production, strain adaptation and growth kinetics of RMIIPL32 were thoroughly investigated and optimized at the shake flask level. This step is crucial for understanding the strain's behavior under different cultivation conditions and ensuring reproducible biosurfactant yields (Varjani and Upasani, 2017)(.

The shake flask experiments were carried out in two sets: synthetic xylose and a natural carbon source (sugarcane bagasse hydrolysate, SBH). Initially, 10 mL of YPD medium was prepared in duplicate 100 mL Erlenmeyer flasks, which served as the primary inoculum medium. A small inoculum was then transferred into a 50 mL seed medium of the same YPD composition in 250 mL Erlenmeyer flasks. The medium that supported the highest cell biomass production was selected for further inoculum transfer, always maintaining a 10 % (v/v) inoculum size. The 250 mL flasks containing the mineral salts medium (MSM) were prepared with either synthetic xylose or the natural carbon source (SBH) in 1000 mL Erlenmeyer flasks. These production media were inoculated according to the 10 % (v/v) rule. The pH was maintained between 5.0 and 5.5, while the temperature and agitation speed were kept constant at 32 °C and 180 rpm, respectively. All the media were autoclaved before inoculation.

The screening of biosurfactant production by IIPL32 was carried out using the drop collapse test, which relies on the destabilization of liquid drops by surfactants, lowering the interfacial tension between the hydrophobic surfactant molecules and the liquid. Additionally, foam formation within the reactor vessel during cultivation visually indicated surfactant production. The kinetics of biosurfactant production were monitored by analyzing the cell dry weight (CDW), sugar concentration, and surfactant levels throughout the cultivation period, providing insights into the dynamics of biosurfactant synthesis under the tested conditions.

2.2. Conversion of Agro-industrial waste into fermentable carbon

Agro-industrial waste in the form of sugarcane bagasse (SB) was collected from a local sugar industry. SB was chosen due to its high cellulosic content and potential as a low-cost substrate for *R. mucilaginosa* growth and biosurfactant production (Chandel et al., 2012). The SB was washed three times with distilled water to remove residual sugars and impurities, then dried at 70 °C for 24 h in a hot air oven. The dried SB was ground and passed through a 0.5 mm sieve to obtain a fine powder (Sindhu et al., 2016).

To obtain fermentable sugars from SB, a dilute acid pretreatment was performed. The SB powder was treated with 1 % (v/v) H_2SO_4 at a solid-to-liquid ratio of 1:10 and autoclaved at 121 °C for 1 hour (Martín et al., 2008). After cooling, the pretreated slurry was neutralized to pH 5.5 using $Ca(OH)_2$. The hydrolysate was then separated by filtration and supplemented with an optimized mineral salt solution (MS) containing (per liter): 4 g KH_2PO_4, 13.6 g $(NH_4)_2SO_4$, 0.8 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, and trace elements (Unrean, 2016). The resulting SB hydrolysate was used at various concentrations (25 %, 50 %, and 75 % v/v) diluted with distilled water to develop the fermentation medium in Erlenmeyer flasks for submerged fermentation by IIPL32.

The SBH-based media were evaluated under varying pH, temperature, and agitation rates to determine the optimal conditions for maximum biosurfactant production. The pH of the substrates was adjusted to 4.5, 5.5, and 6.5 using 1 M NaOH or 1 M HCl, encompassing the typical pH range for yeast growth (Khot and Ghosh, 2017). Incubation temperatures were set at 25, 28, 32, and 35 °C to assess the strain's mesophilic preferences. Agitation rates of 120, 150, 180, and 210 rpm were tested to optimize oxygen transfer and mixing, which are critical for lipid-based biosurfactant production in yeasts (Saenge et al., 2011).

The liquid hydrolysate was then analyzed using a High-Performance Liquid Chromatography (HPLC) system with an Aminex HPX-87H column. This analytical technique allowed for the quantification of fermentable sugars, such as glucose and xylose, and the identification and measurement of any inhibitory compounds in the hydrolysate. The results obtained from the HPLC analysis provided insights into the effectiveness of the dilute acid hydrolysis process in converting the lignocellulosic components of sugarcane bagasse into fermentable sugars, as well as the potential challenges posed by the presence of inhibitory compounds (Bandhu et al., 2019).

2.3. Experimental setup for conversion of fermentable carbon into sophorolipid

The production medium consisted of minimal salt medium (MSM) and bagasse hydrolysate, which demonstrated a xylose concentration of approximately 2.3 % and a glucose concentration of around 0.151 %. As a result, bagasse hydrolysate was deemed a renewable carbon substrate instead of a synthetic carbon source. The MSM medium was formulated with a carbon concentration of 20 g/L (xylose obtained from sugarcane bagasse hydrolysate), 1 g/L mycological peptone, 1 g/L yeast extract, 1 g/L malt extract, $NaNO_3$, and 4 mL/L micronutrient solution, with the pH maintained at approximately 5.5 (Das et al., 2024). The experiment was conducted in a batch process within a sterilized stirred tank submerged bioreactor of working volume 5 L (Andel BioSac) equipped with pH, temperature, and aeration probes. Aeration was maintained at 1 v.v.m throughout the bioprocess. Air saturation levels were kept above the critical threshold, with the medium pre-saturated with 100 % oxygen before inoculation. The impeller design heavily influenced the agitation parameters; in this study, the reactor was fitted with open impellers, and agitation was consistently maintained at 400 rpm. Mechanical foam breakers were incorporated into the reactor system. The pH was automatically regulated within the range of 4.5 to 5 by peristaltic pumps. Data acquisition and monitoring were performed using the bioreactor's integrated control system.

2.4. Biosurfactant extraction and purification

The extraction and purification of sophorolipid biosurfactants from the fermentation broth were performed using a well-defined experimental setup (Das and Kumar 2018). Initially, the culture broth was centrifuged at 6000 rpm for 7 min at 4 °C to separate the cells from the spent medium. A 100 mL aliquot of the cell-free supernatant was then acidified to pH 2.0 using 6 N hydrochloric acid (HCl) and left to stand overnight. The acidified broth underwent liquid-liquid extraction with diethyl ether as the organic solvent. This extraction process was conducted in a borosilicate gravimetric separation unit, where the aqueous and organic phases were vigorously mixed, and pressure was released every 30 s to facilitate the partitioning of Sophorolipids into the ether layer. The diethyl ether extract containing the Sophorolipids was collected in a glass container, and the solvent was evaporated to obtain the crude extract. Any residual moisture in the extracted sample was removed by purging it with nitrogen gas. Finally, methanol was added to the purified sophorolipid extract to precipitate and remove any remaining solid particulates. The methanol was evaporated, leaving the concentrated sophorolipid product, which was preserved in an air-tight container for further characterization studies.

2.5. Characterization of extracted biosurfactant

2.5.1. Emulsification index (E24)

The emulsification index (E24) of the extracted biosurfactant was determined using the method described by Cooper and Goldenberg (1987). In this method, equal volumes of the biosurfactant solution and motor oil were mixed and allowed to stand for 24 h. The height of the emulsified layer was measured and expressed as a percentage of the total height of the mixture.

2.5.2. Oil displacement test

The oil displacement test was conducted according to the method described by Ohno et al. (1993). A Petri dish was filled with distilled water, and a thin layer of motor oil was added to the surface. A drop of the biosurfactant solution was then placed on the oil layer, and the diameter of the resulting clear zone was measured.

2.5.3. Surface tension measurement

Samples were drawn from the fermentation broth at initial inoculation, log phase, and stationary phase to confirm the production of Sophorolipids. The surface tension of the biosurfactant solution was determined on Kruss DSA30 surface angle goniometer. The measurements were conducted at room temperature, and the results were expressed in mN/m.

2.5.4. Chemical analysis of the compound

The chemical analysis of the extracted compound involved several tests. Anthrone and iodine tests were conducted for preliminary identification. Saponification and lipid solubility analyses were performed following the methods of Sawhney and Singh (2000) and Mahesh et al. (2006) Glycolipid production was assessed by following the method of Dubois et al. (1956).

2.6. Structural characterization of extracted biosurfactant through analytical techniques

2.6.1. Attenuated total reflection (ATR) FTIR spectroscopy

The purified biosurfactant sample was characterized using attenuated total reflection (ATR) Fourier-transform infrared (FTIR) spectroscopy. A small sample was placed on the diamond crystal surface of the ATR accessory, and the spectrum was recorded using a Thermo Scientific FTIR spectrometer. The measurements were conducted at room temperature with a resolution of 4 cm^{-1} and 32 scans per sample. Prior to sample measurement, the background spectrum was collected using the clean ZnSe crystal at 45° The spectra were recorded over the wavenumber range of 4000–400 cm^{-1}.

2.6.2. Liquid chromatography and mass spectrometry (LC-MS) analysis

The sophorolipid samples were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) using a C18 column. The eluted compounds were then analyzed via electrospray ionization mass spectrometry (ESI-MS), operating in both positive and negative ion detection modes. This combined LC-MS technique enabled the identification and structural elucidation of the various sophorolipid congeners in the samples. Key parameters assessed included the degree of lactonic ring formation, the length and saturation level of the fatty acid chain, and the presence and extent of acetylation. For the LC-MS analysis, 50 μg of the extracted biosurfactant was dissolved in 5000 μL of HPLC-grade methanol and passed through a 0.22 μm syringe filter. 1000 μL of the filtered solution was then analyzed using LC-ESI-MS.

2.6.3. NMR characterization

The structural features of the sophorolipid biosurfactants produced by RMIIPL32 were further investigated using nuclear magnetic

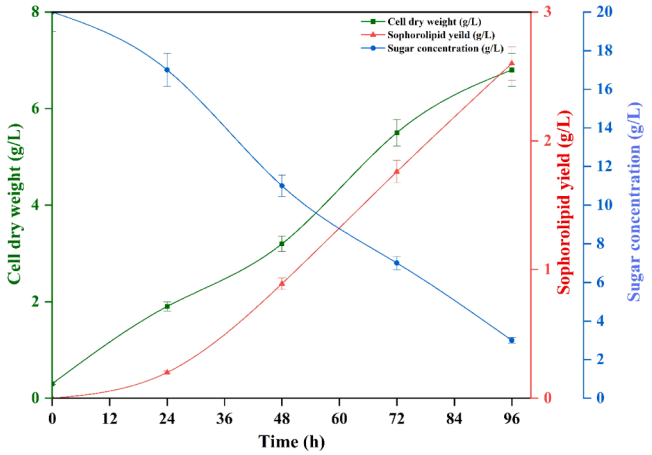

Fig. 1. Batch Kinetic of bioreactor for biosurfactant production utilizing renewable substrate.

resonance (NMR) spectroscopy, focusing on ^{13}C NMR analysis. The purified sophorolipid samples were dissolved in deuterated methanol (CD_3OD), and the ^{13}C NMR spectra were recorded. The ^{13}C NMR data provided valuable insights into the carbon skeleton and degree of saturation within the sophorolipid structures. Signals corresponding to the carbonyl, olefinic, and aliphatic carbons along the fatty acid chains were carefully analyzed and assigned. Furthermore, the ^{13}C NMR spectra facilitated the identification of the sugar head group carbons and the carbons involved in the lactonic or acidic functionalities present in the sophorolipid molecules. By interpreting the chemical shifts and signal patterns observed in the ^{13}C NMR data obtained in deuterated methanol, this comprehensive NMR characterization was essential for elucidating the diverse molecular structures of the sophorolipid biosurfactants produced by RMIIPL32.

3. Results and discussion

3.1. Screening of process kinetics and biosurfactant production

Agro-industrial waste biomass, such as lignocellulosic materials from sources like sugarcane bagasse and agricultural residues, presents an abundant and renewable source for sophorolipid production. These wastes are rich in carbohydrates like xylose and glucose, which can be utilized by yeasts for biosurfactant synthesis (Kosaric et al., 2021; Wang et al., 2018). IIPL32 produced biosurfactants when grown on a minimal salt medium (MSM), as evidenced by foam formation in the reactor vessel. The drop collapse test confirmed surfactant production, with liquid drops spreading and collapsing, indicating the presence of surface-active compounds. This test works by surfactants lowering the interfacial tension between hydrophobic molecules and the liquid, causing drop destabilization. This initial confirmation of biosurfactant production warrants further chemical and structural analysis for detailed characterization. Cell dry weight, sugar concentration, and surfactant levels were tracked throughout the cultivation period to study the kinetics of biosurfactant production. As illustrated in Fig. 1, cell dry weight increased steadily while sugar concentration in the medium decreased, showing that the carbon source was being utilized for growth. Notably, surfactant production also increased over time, peaking at the end of the harvest period. This kinetic analysis provided valuable insights into the dynamics of biosurfactant synthesis by RMIIPL32 under the tested conditions. The data showed the dynamics of carbon consumption, cell growth (CDW), and biosurfactant yield throughout the fermentation. The carbon concentration decreased from 20 g/L to 3 g/L with the decrease in cell dry weight by the end of fermentation, indicating efficient substrate utilization by the yeast Fig. 1. The biosurfactant yield steadily increased, reaching a maximum of 2.6 ± 0.21 g/L at the end of the fermentation period. These results highlighted the capacity of *Rhodotorula mucilaginosa* to effectively convert carbohydrate-rich agro-industrial wastes into value-added biosurfactant products. The production of Sophorolipids typically involves an optimization study that includes optimizing several factors such as pH, temperature, agitation rate, and carbon source concentrations (Vasileva-Tonkova et al., 2015; Makkar and Cameotra, 2002). Hence, the present study demonstrates that pH between 4.5, agitation rates of 180 rpm, and a temperature around 30 °C to 32 °C support optimal growth along with biosurfactant production. Similar results were reported in previous studies that indicate maintaining a pH between 4.5 and 6.5 and a temperature around 28–32 °C supports optimal yeast growth and biosurfactant production. Agitation rates and aeration levels also influence fermentation efficiency by ensuring adequate oxygen transfer and nutrient availability for yeast metabolism (Wang et al., 2019; Cameotra and Makkar, 2010).

3.2. Characterization of extracted biosurfactant

The extracted sophorolipid biosurfactant displayed a 54 % emulsification index and reduced surface tension to 42 mN/m, underscoring its significant potential in various industrial applications. Sophorolipids, known for their environmentally friendly properties and biodegradability, are effective in stabilizing emulsions, crucial for applications in bioremediation, pharmaceuticals, and cosmetics (I.N.A. Van Bogaert et al., 2007). The emulsification index reflects the biosurfactant's capability to stabilize oil-water mixtures, a critical property for enhanced oil recovery and pollutant degradation (Mulligan, 2005). Furthermore, the reduction of surface tension to 42 mN/m indicates a strong surface-active property, essential for lowering the energy required for spreading and wetting, which is advantageous in detergency and dispersion of hydrophobic substances (Guerra-Santos et al., 1986). These properties, combined with the eco-friendly nature of Sophorolipids, make them a promising alternative to synthetic surfactants in various sectors (Daniel et al., 1999).

3.3. Chemical analysis of the extracted biosurfactant

The extracted biosurfactant demonstrated a positive anthrone test for carbohydrates, evidenced by a bluish-green color change, indicating the presence of carbohydrates. A red precipitate in Barfoed's test and the absence of a blue or reddish-brown complex in the iodine test confirmed the presence of monosaccharides and the absence of polysaccharides. In Bial's test, the appearance of a greenish-blue complex suggested the presence of pentose sugars. The biosurfactant was insoluble in water but dissolved in alcohol and chloroform. The saponification test with NaOH confirmed the presence of lipids in the biosurfactant. Moreover, the phenol-sulfuric acid method was light orange, indicating that the biosurfactant is a glycolipid (Das and Kumar 2018). Thus, the chemical analysis results revealed that the extracted biosurfactant likely belongs to the glycolipid group.

3.4. Structural characterization of extracted biosurfactant through analytical techniques

The structural characterization of the extracted biosurfactant was accomplished using various analytical techniques. Fourier Transform Infrared Spectroscopy (FTIR) revealed characteristic peaks indicating the presence of functional groups such as hydroxyl, ester, and carboxyl, which are typical of glycolipids. Nuclear Magnetic Resonance (NMR) spectroscopy provided detailed information on the molecular structure, confirming the presence of carbohydrate moieties and fatty acid chains. Mass spectrometry (MS) further validated the molecular weight and composition of the biosurfactant, aligning with glycolipid structures. These combined analytical techniques confirmed that the extracted

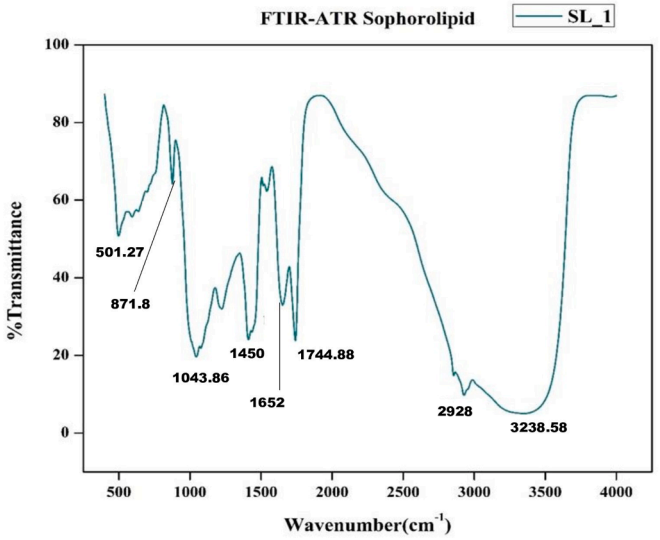

Fig. 2. FTIR spectroscopy of the liquid biosurfactant fraction.

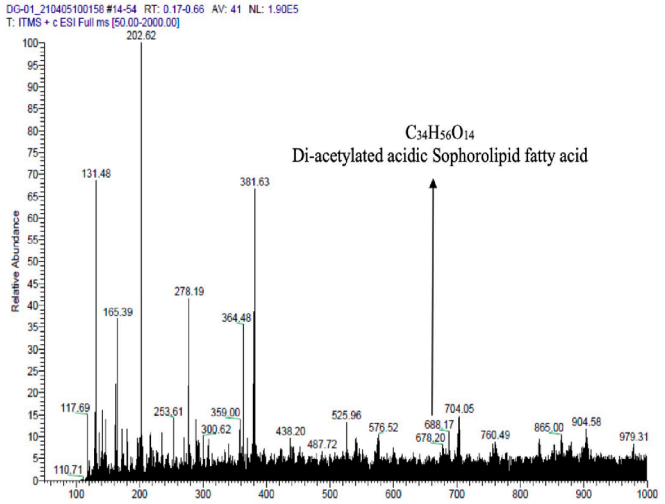

Fig. 3. MS spectrum for the purified biosurfactant fraction and congeners based on m/z value.

biosurfactant might belong to the glycolipid group and sophorolipid class.

3.4.1. FTIR/ATR analysis

The attenuated total reflection (ATR) spectroscopic analysis of the biosurfactant compound revealed the presence of characteristic functional groups and structural features consistent with a sophorolipid molecule Fig. 2. The observed wavenumbers and their corresponding functional groups were as follows: 1043.86 cm^{-1}, indicating the CO-O-CO stretching, which corresponds to the glycosidic linkage in the disaccharide structure of sophorolipid; 1450 cm^{-1}, corresponding to the C-H bending vibration of the methylene group in the lipid chain of sophorolipid; 1744.88 cm^{-1}, associated with the C=O stretching vibration of the ester linkage in the sophorolipid structure; and 3238.58 cm^{-1}, reflecting the OH stretch in-plane bending of carboxylic acid, typical of acidic sophorolipids. These results were consistent with findings from previous studies by Ankulkar and Chavan (2019), Kumari et al. (2021), and Pontes et al. (2016).

3.4.2. LC-MS analysis

The LC-MS analysis of the sophorolipid sample revealed distinct peaks across the m/z spectrum. Notably, peaks at m/z 202 and 318 correspond to the disaccharide and fatty acid residues, respectively, indicating key structural features of the sophorolipid. Additionally, the analysis identified several congeners within the sophorolipid complex, as evidenced by prominent peaks at m/z 525, 688, and 904. These molecular weight distributions are attributed to variations in lipid chain length and degree of acetylation, affecting the overall molecular weight of the sophorolipid molecules. The diverse range of congeners highlights the structural complexity of the sophorolipid mixture, influenced by factors such as microbial strain, cultivation conditions, and downstream processing steps (Hirata et al., 2009; Daverey and Pakshirajan, 2010). The observed m/z values and their corresponding assignments are summarized in Fig. 3 & Table 1.

Table 1
The MS spectrum of the purified biosurfactant fraction, detailing the identification of congeners based on their m/z values.

m/z	Molecules	References
202	Disaccharide residue	Ashby et al. (2006)
318	Fatty acid residue	Ashby et al. (2006)
525	Mono acetylated Lactonic Sophorolipid	Dekoster et al. (1995)
688	Diacetylated Lactonic Sophorolipid	Thaniyavarn et al. (2008)
725	Diacetylated Acidic Sophorolipid	Ingham et al. (2024)

3.4.3. NMR analysis

The NMR analysis revealed signals in the 100–90 ppm range, indicating the anomeric carbon atoms (C-1) of the sugar moieties in the biosurfactant structure Fig. 4. This range is typically associated with the resonance of anomeric carbon atoms in glycosidic compounds (de Koster et al., 1995). These signals suggest the presence of disaccharide or oligosaccharide units, which are common structural features of glycolipid-based biosurfactants such as sophorolipids. Additionally, the 80–70 ppm range signals are attributed to the sugar moieties' remaining ring carbon atoms (C-2 to C-6). This range is characteristic of the chemical shifts associated with the carbon atoms in the pyranose ring structure of carbohydrates (de Koster et al., 1995; Rau et al., 1999).

Based on the comprehensive experimental data and analyses presented, several significant conclusions can be drawn about the production and characterization of sophorolipid biosurfactants by IIPL32 using agro-industrial waste as a substrate. Firstly, the successful use of sugarcane bagasse hydrolysate as a fermentable carbon source demonstrates the potential for valorizing low-cost, renewable feedstocks in biosurfactant production. The kinetic study showed efficient substrate consumption, cell growth, and biosurfactant synthesis, achieving a maximum yield of 2.6 ± 0.21 g/L. This finding highlights the ability of IIPL32 to convert lignocellulosic biomass into value-added Sophorolipids, contributing to sustainable bioprocessing strategies. The multifaceted characterization of the biosurfactant product provided strong evidence for the production of Sophorolipids. Chemical tests indicated the presence of carbohydrates and the absence of polysaccharides, consistent with the glycolipid nature of Sophorolipids. FTIR spectroscopy identified characteristic functional groups, including glycosidic linkages, ester bonds, and carboxyl groups are hallmarks of sophorolipid structures. Furthermore, LC-MS analysis identified a range of sophorolipid congeners with varying molecular weights, highlighting the structural diversity within the biosurfactant mixture. The detection of distinct m/z values corresponding to disaccharide (202) moieties, along with larger molecular ions (525, 688, and 904), indicates differences in lipid chain length and acetylation patterns. This structural heterogeneity can influence the physicochemical properties and functionality of the biosurfactant product. The ^{13}C NMR spectroscopic data provided additional insights into the sophorolipid architecture, with signals in the 100–90 ppm, 80–70 ppm, and ~60 ppm regions corresponding to anomeric carbons, pyranose ring carbons, and hydroxyl-bearing carbons, respectively. These spectral features align with the sophorose

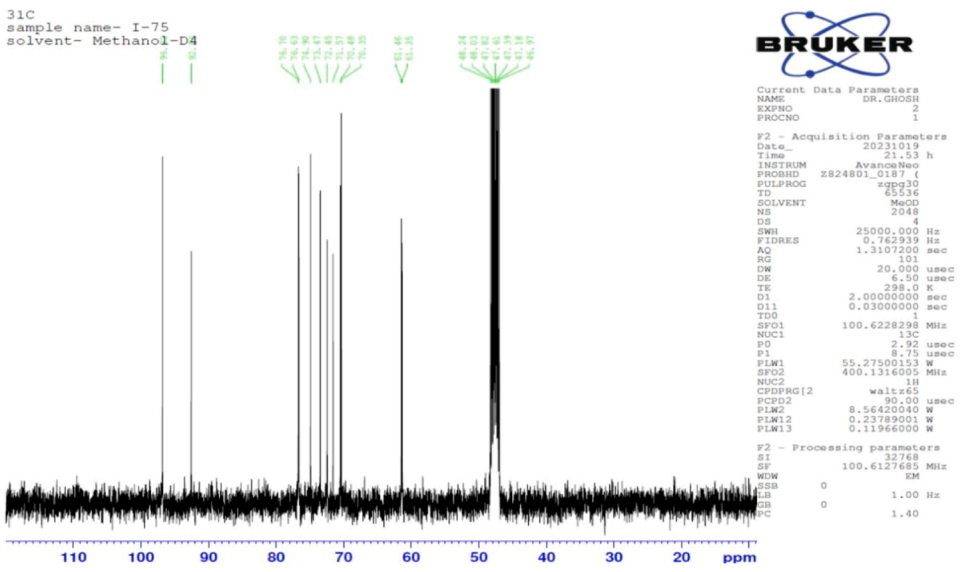

Fig. 4. NMR ^{13}C spectrum of the purified biosurfactant.

Table 2
Comparative studies with recent research on scaling up sophorolipid production from renewable substrate.

Type of Sophorolipids	Scale of Production	Renewable Substrate Used	Microorganism Used	References
Lactonic Sophorolipids	Large-scale (Submerged fermentation)	Wheat feed, potato scraps, sugar-beet pulps	*Starmerella bombicola*	To et al., 2023
Lactonic Sophorolipids	Large-scale(Submerged fermentation)	Food Waste (FW)	*Starmerella bombicola*	To et al., 2023
Diacetylated Lactonic C18:1	Lab-scale (Solid-State Fermentation)	Winterization oil cake, industrial sludges, sweet candy wastewater	*Starmerella bombicola*	Eras-Muñoz et al., 2024
Acetic and Lactonic Sophorolipids	Lab-scale (Submerged fermentation)	Palm oil, glucose	*Starmerella riodocensis, Starmerella bombicola*	Intasit and Soontorngun, 2023
Lactonic Sophorolipids	Pilot-scale Solid state fermentation	Molasses, winterization oil cake	*Starmerella bombicola*	Oiza et al., 2024
Acetylated Sophorolipids (Acidic and Lactonic)	Lab-scale (Submerged fermentation)	Palm fatty acid distillates, soy fatty acid distillates, sunflower oil	*Starmerella bombicola* MTCC 1910	Pal et al., 2024
Monoacetylated and Diacetylated Sophorolipids	Lab-scale (Submerged fermentation)	Glucose, cellobiose, sodium carboxymethyl cellulose	*Pseudohyphozyma bogoriensis*	Msanne et al., 2024
Acidic and Lactonic Sophorolipids	**Lab Scale 5 L (Submerged fermentation)**	**Sugarcane bagasse (Hydrolysate)**	***Rhodotorula mucilaginosa* IIPL32**	**Present Study**

disaccharide unit typical of sophorolipids.

The findings of the present study were compared with earlier research to illustrate the efficiency of the work. Table 2 included various studies that showcased the conversion of agro-industrial waste into sophorolipids. The parameters compared encompassed the renewable substrates utilized, the strains employed, the types of fermentation processes applied, and the production scales involved.

4. Conclusion

The study successfully demonstrated the potential of using sugarcane bagasse hydrolysate, an abundant and low-cost agro-industrial waste, as a substrate for the eco-friendly production of sophorolipid biosurfactants by *Rhodotorula mucilaginosa* IIPL32. Through meticulous optimization of strain adaptation, medium composition, and process scale-up from shake flasks to bioreactor systems, a significant sophorolipid yield of 2.6 ± 0.21 g/L was achieved. This outcome not only underscores the feasibility of utilizing renewable feedstocks for biosurfactant production but also mitigates environmental impact by repurposing agricultural waste. This approach promotes sustainability in biotechnological processes, offering a green alternative to traditional chemical surfactants. The findings of this study provide a robust framework for scaling up sophorolipid production using renewable resources, paving the way for sustainable industrial applications in diverse fields such as pharmaceuticals, cosmetics, and environmental remediation. Overall, this research highlights the potential of integrating waste management and biosurfactant production to foster eco-friendly and cost-effective industrial solutions.

E-supplementary data for this work can be found in the e-version of this paper online

CRediT authorship contribution statement

Aishwary Purohit: Methodology, Formal analysis, Investigation, Writing – original draft. **Amar Jyoti Das:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision. **Debashish Ghosh:** Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

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

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Data availability

No data was used for the research described in the article.

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