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Economical Di-Rhamnolipids Biosynthesis by Non-Pathogenic *Burkholderia thailandensis* E264 Using Post-Consumption Food Waste in a Biorefinery Approach

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Abstract: Rhamnolipids (RLs) are one of the most promising eco-friendly green alternatives to commercially viable fossil fuel-based surfactants. However, the current bioprocess practices cannot meet the required affordability, quantity, and biocompatibility within an industrially relevant framework. To circumvent these issues, our study aims to develop a sustainable biorefinery approach using post-consumption food waste as a second-generation feedstock. In-depth substrate screening revealed that food waste hydrolysate (FWH) was rich in readily assimilable carbohydrates, volatile fatty acids, and amino acids. The fermentative valorization of FWH as a sole carbon and energy source with *Burkholderis thailandensis* E264 in a bioreactor showed active RLs biosynthesis of up to 0.6–0.8 g/L (34–40 mg/g FWH) in a short duration (72 h). In terms of the kinetic parameters, the FWH-RLs outperformed other supplemented pure/waste streams. Interestingly, the recovered RLs had a long chain length, with Rha-Rha-C_{12}-C_{14} being the predominant isoform and exhibiting a strong emulsification ability ($E_{24,}$ 54.6%). To the best of our knowledge, this study is the first to prove bioreactor-level RLs production and their abundance in food waste. Moreover, the feasibility of this developed process could propel next-generation biosurfactants, lower waste burdens, and increase the industrial applicability of RLs, thereby significantly contributing to the development of a circular bioeconomy.

Keywords: substrate screening; *Burkholderia thailandensis*; second-generation feedstock; bioreactor; di-rhamnolipids; emulsification

Citation: Kumar, R.; Johnravindar, D.; Wong, J.W.C.; Patria, R.D.; Kaur, G. Economical Di-Rhamnolipids Biosynthesis by Non-Pathogenic *Burkholderia thailandensis* E264 Using Post-Consumption Food Waste in a Biorefinery Approach. *Sustainability* **2023**, *15*, 59. https://doi.org/10.3390/su15010059

Academic Editor: Giovanni Esposito

Received: 4 December 2022
Revised: 15 December 2022
Accepted: 19 December 2022
Published: 21 December 2022

1. Introduction

Biosurfactants are a class of surface-active agents often viewed as an alternative to synthetic petrochemistry-based surfactants. Synthetic surfactants are highly damaging to the environment. Therefore, it has become crucial to have sustainable products on the market [1], biosurfactants being one of them. The global biosurfactant market is predicted to reach USD 1.9 billion with a CARG of ~11.2% by the end of 2027 [2]. Due to this huge expected demand, researchers are focusing on various aspects of microbial surfactant synthesis and optimization.

Biosurfactants such as glycolipids are given particular attention as they possess an excellent ecological framework, the lowest biotoxicity, structural diversity, and resistance to high temperature, pH, and salinity [3]. Due to their biochemical nature and structural diversity, rhamnolipids (RLs) are one of the most studied representatives of glycolipid biosurfactants. They comprise a hydrophilic rhamnose sugar moiety attached to one or

two hydrophobic acyl-hydroxy fatty acid chains [1,3]. The chemical synthesis of these RLs biomolecules has been reported using environmentally friendly materials. Nevertheless, the cost of chemical production is relatively higher than biosynthetic routes [4]. The scalability of this chemical synthesis is another key aspect that needs to be considered for any technology derived from a chemical or biological process. While chemical methods are touted as "green", the scalability potential needs to be established and/or demonstrated. In this aspect, bioprocessing has demonstrated scalability and has already been adopted on an industrial scale. One such example is Evonik, which recently constructed a new commercial-scale plant for bio-based rhamnolipids [5].

In recent times, the main biological producer species for RLs has been *Pseudomonas aeruginosa*. However, their potential to secrete toxins and their opportunistic pathogenicity in humans hinder their use for large-scale industrial purposes [6,7]. An alternate to this is a newly identified and environmentally safe species of bacteria, i.e., *Burkholderia thailandensis* [8,9]. The present work focuses on this recently identified non-pathogenic strain for RLs catalysis with product titers different from conventional *Pseudomonas* species. Generally, RLs can be produced from a variety of hydrophilic (glucose, glycerol) [8] and hydrophobic substrates such as fatty acids (heptadecanoic acid, oleic acid) [10] and oils (canola oil) [8]. Residual substrates like waste cooking oil (WCO) and agricultural residues have also been proposed as alternatives for RLs biosynthesis [11–13].

Potential valorization of waste streams using *B. thailandensis* as cell factories can prove to be highly beneficial. The biggest challenge for making use of such valuable commodities is the requirement of expensive nutrient supplementation and the absence of definitive process development [14]. In addition, the separation of the abovementioned hydrophobic waste substrates from the produced RLs is relatively expensive and demands additional downstream processing [15]. To circumvent this, we hereby develop the use of post-consumption food waste hydrolysate (FWH) for RL production. According to estimates, 1.3 billion tons of food are wasted annually, accounting for 8–10% of greenhouse gas emissions that contribute to global emissions [16]. Thereby, the valorization of food waste into value-added products as RLs not only reduces the cost of fermentation but also provides an effective means of dealing with the burgeoning waste load.

In contrast to other waste streams, food waste as a feedstock releases trapped water-soluble and highly rich nutrients, which increases their bioavailability and bioconversion to RLs. No additional supplements are required during the fermentation process, and overall batch fermentation time is significantly lowered. The results of the research prove that the macronutrient heterogeneity in FWH acts to delimit the synthesis of newer congener profiles in *B. thailandensis* E264 with enhanced applicability.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

The *Burkholderia thailandensis* E264 was obtained from the American Type Culture Collection (ATCC 700388^{TM}) and cryopreserved at −80 °C by supplementing with 25% *v/v* glycerol (master stocks). For inoculum preparation, loopful bacteria were streaked onto solid nutrient agar plates with a composition of peptone (5 g/L), beef extract (3 g/L), sodium chloride (8 g/L), and agar (12 g/L).

2.2. Processing of Food Waste Hydrolysate

The food waste was collected from the Harmony cafeteria of Hong Kong Baptist University, Hong Kong, and mainly consisted of boiled rice, vegetables, meat, noodles, and eggshells. The unwanted materials, such as bones, chopsticks, plastic, and tissue papers were, manually removed. Following proper mixing, a fixed amount of samples were loaded into pre-weighed crucibles and dried overnight in a 105 °C oven before being transferred to a muffle furnace and heated to 550 °C. The residues were weighed again after each step and used for total solids (TS) and volatile solids (VS) estimation, respectively [17]. Following this, food waste was hydrolyzed in a leach bed reactor (LBR) as per the detailed

protocol provided elsewhere [18]. In brief, a two-phase anaerobic digestion system was established by operating LBR as acidogenic reactors under mesophilic conditions. The LBR was loaded with food waste as substrate, which had a TS and VS/TS of 28.26% and 98.87%, respectively. The anaerobically digested sludge (ADS) collected from the Yuen Long sewage treatment plant in Hong Kong was used as inoculum with a TS of 1.66% and VS/TS of 99.23%. The working volume of the LBR was 2 L, loaded with 2 kg (*w/w*) food waste, 400 gm inoculum (20%) and 200 gm glass cubes as bulking agent. Following completion, the hydrolyzed leachate was adjusted to 6.8 pH and autoclaved at 121 °C for 20 min before use in fermentation.

2.3. Screening of Substrates for Rhamnolipid Production

To carry out the batch fermentation screening with pure substrates, small-scale studies at the shake flask level were performed. The seed culture was prepared by transferring a single isolated colony of *B. thailandensis* E264 in nutrient broth and grown for 24 h at 30 °C and 200 rpm. Further re-inoculation was conducted with a transfer of 10% seed culture in an Erlenmeyer flask containing nutrient broth (gelatin peptone 5 g/L and beef extract 3 g/L) supplemented with 4% (*w/v*) of pure substrate (glycerol, glucose, fructose, sucrose, oleic acid, canola oil) or low-cost WCO and maintained for 72 h. The experiments were carried out in triplicate at 30 °C with an initial pH 6.8 and 200 rpm.

During the utilization of FWH, the bacterial cells were acclimatized through an additional re-inoculation with 10% seed culture in an Erlenmeyer flask containing FWH for 24 h at 30 °C and 200 rpm. This acclimatized culture was then used for the production of RLs by inoculating Erlenmeyer flasks containing FWH and growing it for 72 h at 30 °C and 200 rpm.

2.4. Batch Rhamnolipid Fermentation

The controlled fermentation was carried out with *B. thailandensis* E264 in a 1 L fermenter with a working volume of 0.6 L. Growth and biosynthesis were carried out using a dissolved oxygen (DO_2) level of 20% with 0.5 L/min airflow and cascade stirring control between 50–550 rpm. The temperature was maintained at 30 °C and the pH was maintained at 6.8 via the addition of 2N NaOH/HCl throughout the fermentation process. The bioreactor fermentation was performed for FWH and glycerol supplemented medium. The experiments were performed in duplicate, and the average mean values with standard deviation are reported.

2.5. Sampling and Analysis

The samples were collected every 3 h, and cell growth was quantified by measuring optical density (OD) at 600 nm with a microplate reader (Synergy HTX multi-mode reader). This was used to determine cell dry weight (CDW) through a previously established linear correlation of CDW = 0.3082(OD) with R^2 = 0.9864.

2.6. Rhamnolipid Quantification and Characterization

2.6.1. Extraction and Quantification of Rhamnolipids

For the separation of RLs, the solvent extraction method was used as previously described with slight modifications [7]. In addition, the culture broth was centrifuged (10,000 rpm, 15 min) to obtain a cell-free suspension. This cell-free suspension was then filtered through a filter paper (Advantec Grade No. 5C Ashless). The filtered supernatant was mixed with an equal volume of n-hexane and subjected to extraction twice. The emulsion layer was collected, and the residual solvent was evaporated under a constant stream of nitrogen. The crude RLs were then freeze-dried and determined gravimetrically.

2.6.2. Structural Characterization of Produced Rhamnolipids by FTIR

The Fourier transform infrared (FT-IR) was employed for the determination of functional and chemical bonds using an FT-IR spectrometer (Perkin Elmer FT-IR Spectrum 2).

The isolated RL sample (0.5–0.8 mg) was directly loaded onto the diamond high-refractive-index prism for attenuated total reflectance within a spectral range of 4000–400 cm^{-1} [19].

2.6.3. Chemical Characterization of Rhamnolipids by LC-MS-MS

The RL extracts were dissolved in an appropriate volume of a 3:1 *v/v* acetonitrile-water solution. Mass spectra (MS) were measured for RLs identification with an API 4000 QTRAP Triple Quadrapole mass spectrometer (AB Sciex Foster City, CA, USA). Briefly 10 µL samples directly infused in negative electron ionization mode [7]. Furthermore, a complete isomer profiling was carried out using a liquid chromatography system equipped with a C18 reversed-phase LC Column (Luna® 3 µm C18(2) 100 Å, LC Column 100 × 4.6 mm). A gradient of 45% mobile phase A (5 mmol/L ammonium acetate) and 55% mobile phase B (acetonitrile) was set from 0 to 2 min and increased to 90% mobile phase B. The flow rate was maintained at 0.3 mL/min with 10 µL sample injection volume. The MS conditions were programmed at −3.5 KV ion spray voltage, 80–95 V declustering potential, and 22–26 V collision energy at a temperature of 100 °C.

2.6.4. Measurement of Surface Tension of Produced Rhamnolipids

The surface tension measurement was performed by the BZY-B (BZY102) Automatic Surface Tensiometer using the du Noüy ring method. Subsequently, the different concentrations (0–1000 mg/L) of RLs dissolved in water were subjected to surface tension measurements for the estimation of the critical micelle concentration (CMC).

2.6.5. Emulsifying Capacity of Produced Rhamnolipids

The emulsifying activity (E_{24}) was calculated over 24 h by calculating the height of the emulsion layer as a percentage of the total height of the liquid. A solution of 1 mL RLs with desired concentrations (0–1000 mg/L) in 0.1 M Tris-HCl pH 8.0 was mixed with 1 mL of n-hexadecane through vigorous vortexing for 2 min to obtain maximum emulsification [20].

2.7. Nutrient Assessment

2.7.1. Total Carbohydrate

The assessment of total carbohydrate was performed using the phenol-sulphuric acid method. Briefly, 1 mL of an appropriately diluted sample was mixed with an equal volume of 5% phenol solution. This was followed by the addition of 5 mL of a 96% sulfuric acid solution. The samples were incubated at 55 °C for 10 min. The absorbance was measured at 490 nm, and the concentration was calculated against a standard calibration plot [21].

2.7.2. Free Alpha Amino Nitrogen

The free alpha-amino nitrogen was measured using the EBC-ninhydrin method. The samples were diluted and mixed with 1 mL of coloring reagent (Ninhydrin solution comprising of 10 g $Na_2HPO_4.12H_2O$, 6 g KH_2PO_4, 0.5 g ninhydrin, and 0.3 g fructose in 100 mL distilled water having a pH of 6.7). The mixture was kept in boiling water for 16 min followed by cooling in a water bath at 20 °C for 20 min. After cooling, 5 mL of diluting agent (2 g KIO_3 in 600 mL distilled water along with 400 mL of 96% ethanol) was added, and absorbance was measured at 570 nm. The concentration was calculated against a single time-point standard of glycine [22].

2.7.3. Volatile Fatty Acids

The availability of volatile fatty acids was quantified using gas chromatography with flame ionization detection (Agilent, Wilmington, DE, USA). A properly diluted sample was acidified to a final concentration of 10% with formic acid. Following this, samples were injected into a gas chromatograph fitted with a TraceGOLD TG-WaxMS column (ThermoFisher Scientific, Carlsbad, CA, USA) at a flame ionization detection temperature of 250 °C. The analysis was programmed as follows: 120 °C for 5 min, ramped to 180 °C at a rate of 5 °C/min, and maintained at 180 °C for 10 min [17].

2.7.4. Glycerol

The glycerol estimation was obtained using a standard assay performed in a microplate reader according to a commercial glycerol assay kit (Megazyme International Ireland, Wicklow, Ireland). The glycerol is phosphorylated in this assay using adenosine-5′-triphosphate (ATP). The formed adenosine-5′-diphosphate (ADP) is used for the phosphorylation of d-glucose, which is oxidized with the formation of nicotinamide-adenine dinucleotide (NADH). The amount of NADH is detected by measuring absorbance at 340 nm [23].

3. Results

3.1. Bioconversion of Different Substrates to Rhamnolipids: Screening Studies

The substrates screened for RLs production ranged from carbohydrates (glucose, fructose, and sucrose), polyols (glycerol), fatty acids (oleic acid), lipids (canola oil and WCO), and FWH. *B. thailandensis* E264 was able to grow on all supplemented carbon sources; however, the kinetic profiles were significantly different (Table 1). The complex lipid-based feedstocks induced high biomass formation, which reached 11.44 g/L and 10.68 g/L when grown on WCO and canola oil, respectively, as carbon sources. A similar trend was reported in another study that valorized used cooking oil (UCO) and generated a comparable biomass of 12.2 g/L [11]. On the contrary, RLs' concentration with this waste substrate (WCO) was relatively lower (0.3 g/L). The simpler free fatty acids (oleic acid) improved the RLs production to 1.21 g/L, indicating that a fatty acid-rich medium is more favorable for RLs synthesis. Moreover, water-soluble substrates, i.e., carbohydrates and polyols, in the present study resulted in significantly higher RLs yields of up to 0.046 g/g with glycerol. The RLs concentration was 1.85 g/L, proving polyols to be the most suitable in batch shake flasks. Similarly, using pure glycerol as substrate was preferred by *B. thailandensis* E264 in previous reports but required the presence of an expensive nutrient medium, which significantly elevates the cost of production.

Table 1. Comparative screening based on maximum cell dry mass produced (CDM_{max}), maximum rhamnolipid produced (RLs_{max}), biomass generated compared to initial substrate ($Y_{x/s}$ g/g), rhamnolipid yield related to initial substrate ($Y_{RLs/s}$ g/g), product yield related to biomass generated ($Y_{RLs/x}$ g/g) and volumetric productivity (P_{RLs} g/L/hr) for different substrates.

Fermentation	Medium	Substrate	Time (h)	$Y_{x/s}$ (g/g)	$Y_{RLs/s}$ (g/g)	$Y_{RLs/x}$ (g/g)	P_{RLs} (g/L/h)
Batch shake flask	Nutrient broth	Glycerol	72	0.12	0.046	0.370	0.026
		Sucrose		0.15	0.019	0.127	0.011
		Glucose		0.16	0.024	0.152	0.014
		Fructose		0.19	0.036	0.181	0.020
		Oleic Acid		0.23	0.030	0.127	0.017
		Canola Oil		0.26	0.025	0.096	0.014
		Waste Cooking Oil		0.28	0.007	0.026	0.004
		Food waste hydrolysate		0.08	0.040	0.469	0.011

The sustainability of *B. thailandensis* was monitored by employing FWH as a complete macronutrient and micronutrient source with no additional supplementation. During batch shake flask fermentation, the FWH supported a biomass production of 1.73 g/L with maximum RL concentrations of 0.81 g/L within 72 h of fermentation (Figure 1). Furthermore, yield of RLs was also higher (0.04 g/g) and more suitable than sole carbohydrates (glucose 0.024 g/g, fructose 0.036 g/g, sucrose 0.019 g/g). It is presumably due to the co-substrate availability in FWH, which allowed it to perform better as a feedstock.

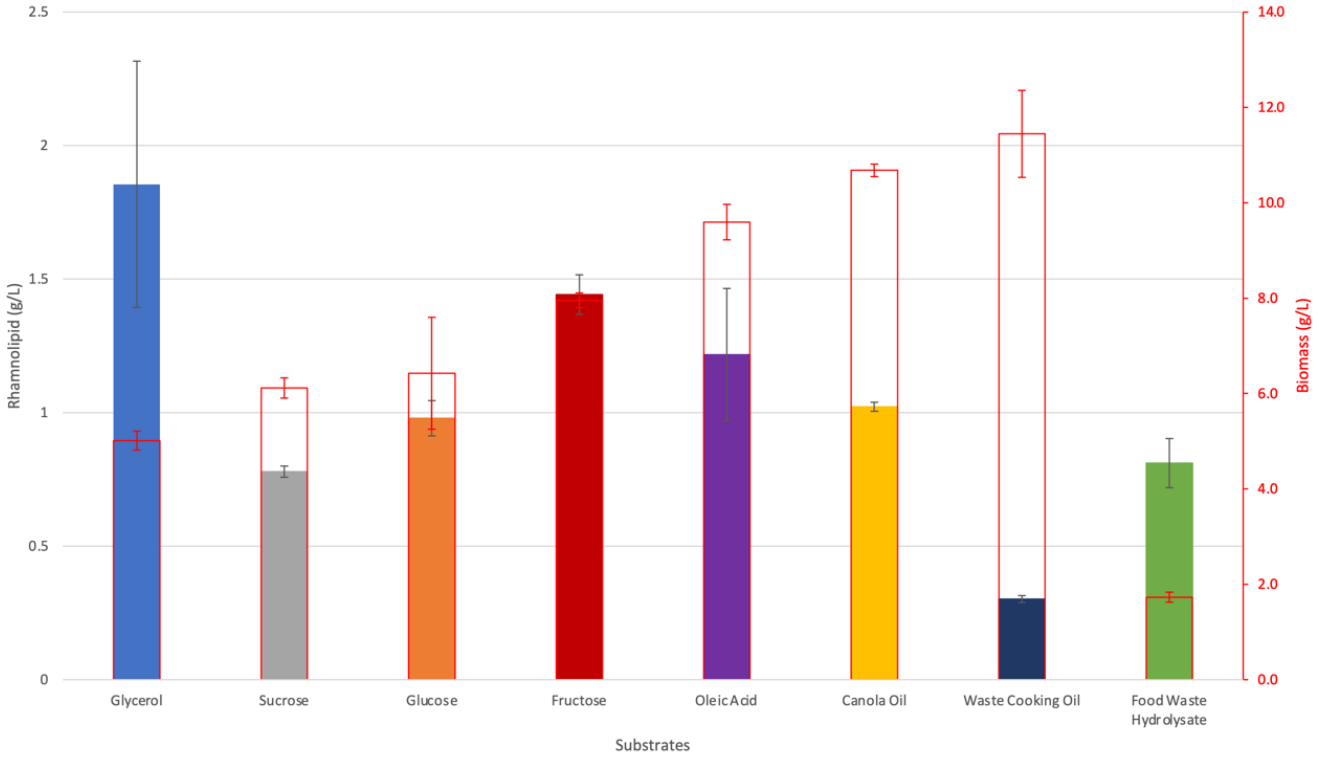


Figure 1. Differential substrate utilization based on rhamnolipids (g/L) and biomass concentrations.

3.2. Bioreactor Scale Fermentation with Food Waste Hydrolysate

On the basis of the shake flask performance of *B. thailandensis* E264, the RLs fermentation was scaled up in a bioreactor with the most preferred feedstocks, i.e., glycerol and FWH. To establish the fermentation feasibility, biochemical profiling of FWH was established in the form of available nutrients in the hydrolysate and monitored throughout the bioprocess (Figure 2). At the beginning of culture, total carbohydrates, volatile fatty acids (including alcohols), and amino nitrogen were 19.23 g/L, 2.23 g/L, and 0.083 g/L, respectively, and were actively consumed by *B. thailandensis*, as depicted in Figure 2a. The vertically extending line of standard deviation shows the distribution of data in relation to the mean. During the initial 15 h of active growth, the amino nitrogen was rapidly consumed, after which both cell dry mass production and nitrogen slowed, indicating the onset of the stationary phase. This stage is reported to be crucial for active carbon bioconversion, RLs biosynthesis, and the overall productivity of the system [24]. The FWH supports the formation of RLs' precursor by providing complex carbon sources that are consumed at a differential rate. Volatile fatty acids were valorized faster for generating major RL precursors, while sugars were utilized at a slower rate [8,10]. The order of VFA preference in the FWH was caproate > isovalerate > acetate > valerate, whereas butyrate was the least preferred (Figure 3). Alcohols were also consumed as a substrate during the bioprocess. At the end of fermentation, 77.5% of VFAs were utilized, and carbohydrates were reduced to 11.62 g/L. The observed biomass and RLs were 1.304 g/L and 0.616 g/L, respectively.

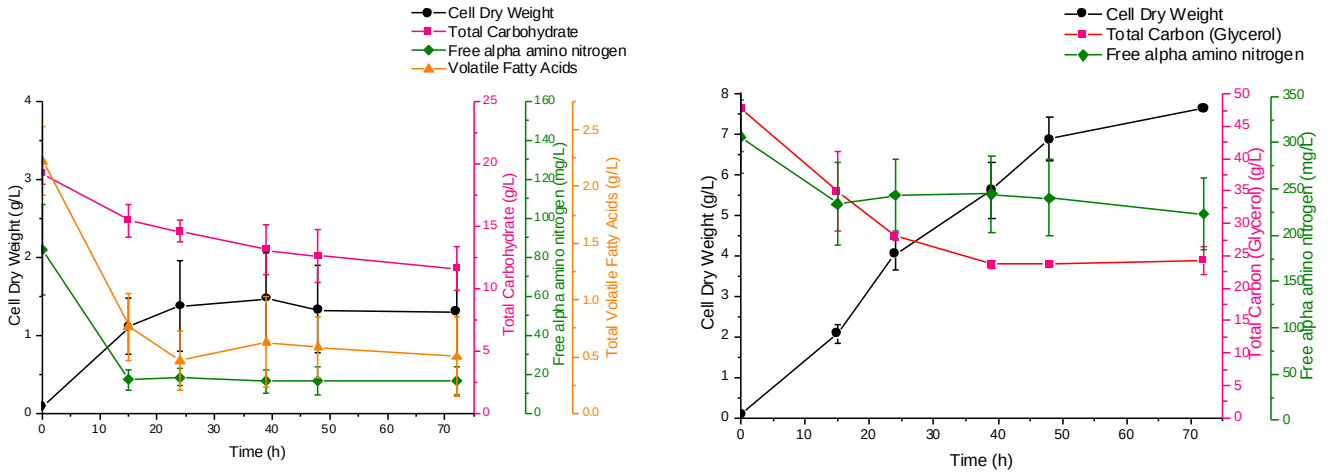


Figure 2. Time course profile for growth and utilization of nutrients by *B. thailandensis* during batch fermentation using: (**a**) Food waste hydrolysate; (**b**) Glycerol.

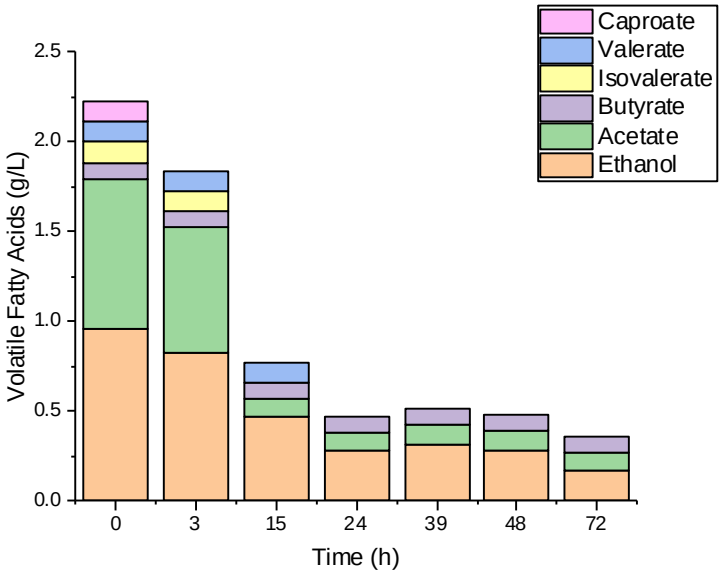


Figure 3. Volatile fatty acids (including alcohols) utilized during food waste hydrolysate fermentation.

The fermentation profile on pure glycerol as substrate (Figure 2b) was also established for process comparison and to identify the key advantages of using FWH as a potential feedstock. The growth pattern of *B. thailandensis* E264 in a glycerol-enriched medium was significantly different from the one employing FWH. A consistent increase in cell biomass (6.204 g/L) was observed during fermentation for 48 h which prolonged the onset of the early stationary process. For RLs synthesis with this strain on a pure substrate, typical fermentation has been reported to be carried out for extended periods [8,24]. This affects the economic feasibility of RLs, with a longer time being proportional to the higher cost of production. The overall RLs concentration was 1.037 g/L, which is higher than that obtained on FWH. However, a deeper analysis of fermentation kinetics is additionally required for production and process predictions as it provides a better overview of substrate utilization, biomass formation, and product recovery by biological routes. A comparison of the fermentation runs in the present study and other reports revealed variable performance kinetics (Table 2), which highlights a differential performance of FWH utilization by *B. thailandensis*.

Table 2. Comparison of rhamnolipid production metrics for different upscaled batch and shake flask fermentations carried out for *B. thailandensis* E264.

Parameters	This Study		Study Comparisons			
			Kourmentza et al. [11]	Funston et al. [24]	Dubeau et al. [8]	
Fermentation type	Batch bioreactor		Batch bioreactor	Batch bioreactor	Batch shake flask	
Working volume	0.6 L	0.6 L	8.0 L	4.0 L	0.2 L	0.2 L
Nutrient Medium	Nutrient broth	Food waste	Nutrient broth	Nutrient broth	Nutrient broth	
Substrate	Glycerol	hydrolysate	Used cooking oil	Glycerol	Glycerol	Canola oil
Temperature	30 °C		37 °C	25 °C	37 °C	
Time	72 h (3 days)		120 h (5 days)	264 h (11 days)	312 h (13 days)	
CDM_{max} (g/L)	6.204	1.304	12.6	7.98		
RLs_{max} (g/L)	1.037	0.616	2.20	2.06	0.42	1.47
$Y_{x/s}$ (g/g)	0.124	0.068	0.23	0.156		
$Y_{RLs/s}$ (g/g)	0.021	0.034	0.10	0.19		
$Y_{RLs/x}$ (g/g)	0.167	0.472	0.43	0.258		
P_{RLs} (g/L/h)	0.014	0.009	0.018	0.007	0.001	0.004

3.3. Characterization of Rhamnolipids Produced on Food Waste Hydrolysate

The RLs synthesized by *B. thailandensis* from different feedstocks had similar chemical signatures. Figure 4 shows typical FT-IR analysis of extracted RLs, which revealed the presence of conspicuous absorbance bands forming fingerprint regions specific to glycoplipids. Both glycerol and FWH-based biosurfactants have typical bands at 3299 cm^{-1} for the O-H group, amide N-H functions, and 2924 cm^{-1} for the aliphatic C-H group. The specific band at 2854 cm^{-1} infers to -C-H- vibrations of lipids. The specific band at 1637 cm^{-1} corresponds to C=O of ester and carboxylic acid groups. Further broad bands from 1320 cm^{-1} to 1500 cm^{-1} resemble δ(CH), asymmetric COO-, and broad C-H of CH_2 and CH_3 groups. Another strong absorption band between 1000 and 1100 cm^{-1} attributes to the -COC- group in rhamnose structure. This confirms the presence of rhamnose-type glycolipids as the extracted biosurfactant. Numerous other studies have also reported similar results for RLs [19,25].

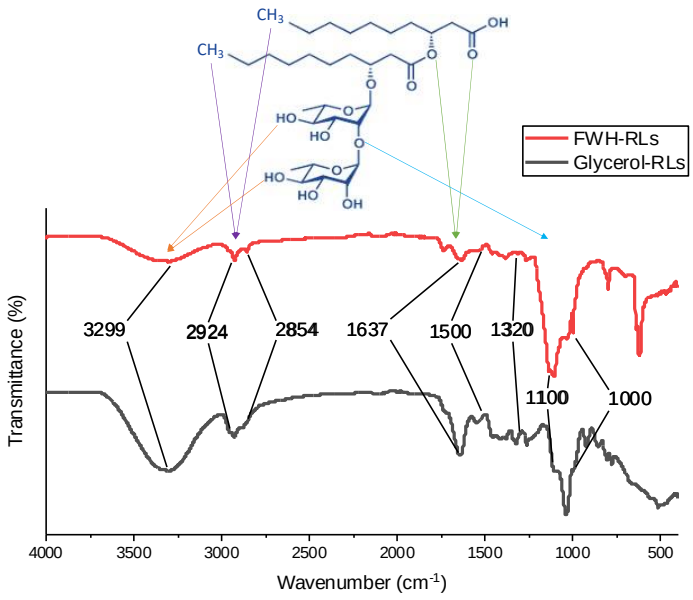
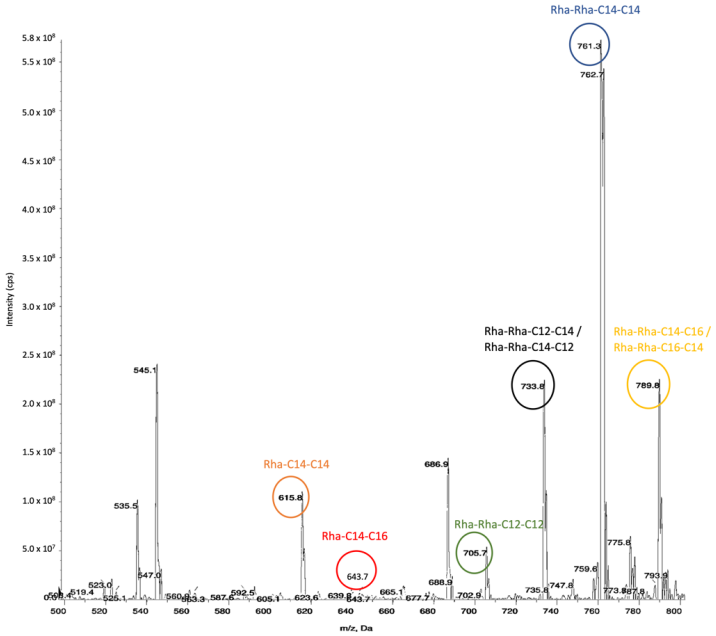


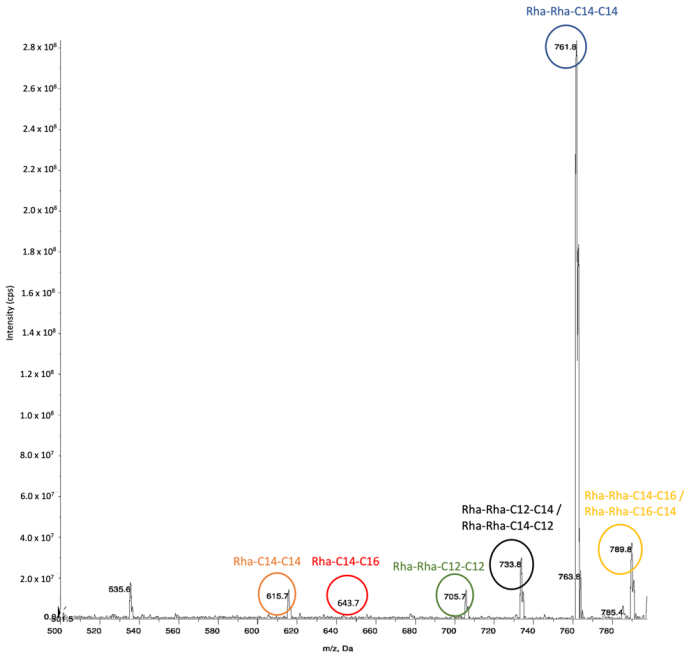
Figure 4. Fourier transform infrared spectrum showing chemical fingerprints specific to rhamnolipid synthesized by *B. thailandensis* using glycerol and food waste hydrolysate as feedstock. Orange arrows indicate O-H groups at 3299 cm^{-1}, purple arrows indicate aliphatic C-H group at 2924 cm^{-1}, green arrows indicate C=O of ester and carboxylic acid groups at 1637 cm^{-1} and blue arow indicates rhamnose -COC- group between 1000 cm^{-1} and 1100 cm^{-1}.

Further, the profiling of the RLs was performed with MS, which revealed the type and proportion of RL isoforms. However, the RLs congeners known to be synthesized by *B. thai-*

landesis E264 were identified in both glycerol and FWH fermentation. Initial MS spectrum for both fermentation types display presence of all major mono-rhamnolipids Rha-C_{14}-C_{14}, Rha-C_{14}-C_{16} and di-rhamnolipids Rha-Rha-C_{12}-C_{12}, Rha-Rha-C_{12}-C_{14}, Rha-Rha-C_{14}-C_{14}, and Rha-Rha-C_{14}-C_{16} (Figure 5). Dubeau et al. 2009 [8] and Elshikh et al. 2017 [26] screened similar high-chain-length RLs from *B. thailandensis* E264. The observations from the LC-MS-MS analysis of Figure S1 and summarized in Table 3, using standard glycerol led to the synthesis of Rha-Rha-C_{14}-C_{14} as the most dominant congener (32.59%). On the contrary, FWH allowed the synthesis of Rha-Rha-C_{12}-C_{14} as the most abundant congener (44.91%). The mono-rhamnolipid congeners displayed similar relative abundance among fermenters.



(**a**)



(**b**)

Figure 5. MS spectrum of rhamnolipids recovered from: (**a**) food waste hydrolysate; (**b**) pure glycerol fermentation broths.

Table 3. LC-MS-MS-based comparison of relative abundance (%) for rhamnolipid congeners recovered from pure substrate (glycerol) and food waste hydrolysate batch fermentations using *B. thailandednsis* E264.

Rhamnolipid Congeners	Pseudomolecular Ion (m/z)	Relative Abundance (%)	
		Pure Substrate (Glycerol)	Food Waste Hydrolysate
Mono-rhamnolipids (total)		12.89	15.65
Rha-C_{14}-C_{14}	615	12.35	15.57
Rha-C_{14}-C_{16}	643	0.54	0.08
Di-rhamnolipids (total)		87.11	84.35
Rha-Rha-C_{12}-C_{12}	705	2.39	19.33
Rha-Rha-C_{12}-C_{14}	733	29.22	44.91
Rha-Rha-C_{14}-C_{14}	761	32.59	16.22
Rha-Rha-C_{14}-C_{16}	789	22.91	3.89

3.4. Economical Rhamnolipid Properties and Performance

To evaluate the surfactant performance of FWH-derived RLs, the surface activity of the RLs mixture was measured. The CMC at which FWH-derived RL demonstrated maximum surface activity was 800 mg/L (Figure 6). In addition, the solution also achieved a surface tension reduction from 71.14 to 53.15 mN/m. Furthermore, the emulsification activity was also investigated, where the increase in RL concentration gradually improved the E_{24} and maximized at 54.6% at 800 mg/L concentration (Figure 7).

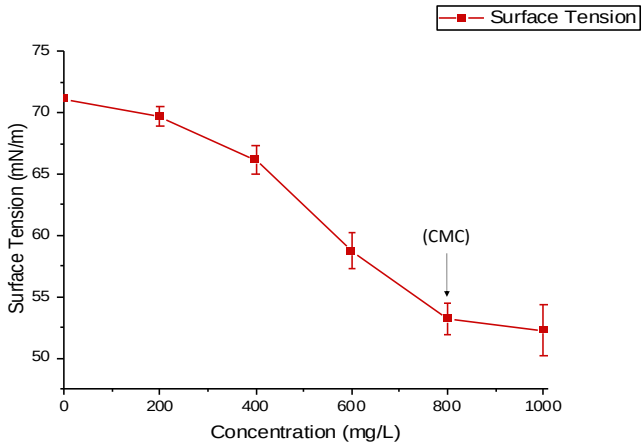


Figure 6. Surface tension reduction and critical micelle concentration (CMC) for food waste hydrolysate rhamnolipids at different concentrations.

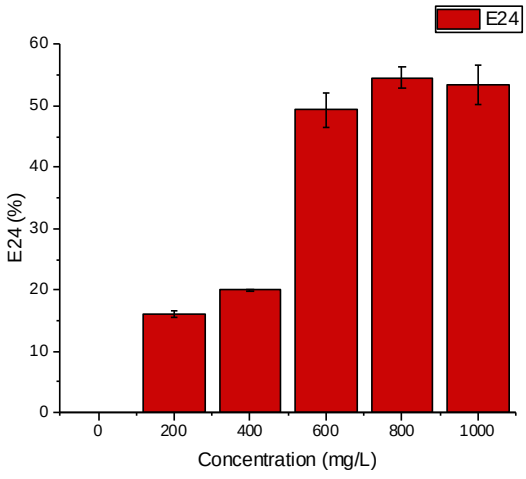


Figure 7. Emulsifying index (E_{24}) at different concentrations of food waste hydrolysate-derived rhamnolipids.

4. Discussion

This research has shown that both RL quality and quantity are influenced by the choice of substrate along with the producer strain. *Burkholderia thailandensis* E264 is emerging as an environmentally safe, non-pathogenic bacterium with the ability to synthesize longer chain length RLs [27,28]. This has promoted their application to numerous industrial, environmental, and biomedical sectors [7,26,29]. Moreover, to meet the high demands and economically upscale high value RLs from *B. thailandensis* E264, previous works have utilized both pure and agro-industrial substrates for RL biosynthesis. A brief comparison has been provided in Table 2 for the RL production metrics of some batch fermentations with *B. thailandensis* E264. Several studies have used pure glycerol as a carbon source, with maximum RL production reported to be between 1.47–2.06 g/L [8,24]. Additional nutrients necessary to support growth and RL synthesis are provided by the nutrient-rich synthetic medium. Furthermore, economical bioproduction studies have used WCO as a potential substrate for bioconversion to RLs with a concentration of 2.2 g/L [11]. However, the synthetic nutrient broth used as a fermentation base makes it an unviable option due to the inherently high price of pure nutrients or supplements. Moreover, fermentation feedstocks contribute 50–80% of the total fermentation cost, thereby hindering the downstream industrial manufacture of microbial products such as RLs [30]. Another study used the supplementation of raw soluble fractions of agricultural residues from the white winemaking process and olive oil extraction for the biosynthesis of RLs from *B. thalandensis*. The derived nonfermented grape marcs (NF) and olive pomace residues (OMP) both resulted in RL production reaching 1.07 g/L and 0.3 g/L, respectively [13]. However, nitrate salts and minerals were added as a supplement to fulfill the required nitrogen and micronutrients during fermentation. Correia et al. (2022) reported the use of a co-nutrient medium supplemented with corn steep liquor (CSL) and olive mill wastewater (OMW) as carbon sources. The RLs produced in the CSL + OMW medium reached 0.253 g/L after 96 h of fermentation [12].

This research is novel in its attempt to prove post-consumption food waste as a viable feedstock without additional supplementation for *B. thailandensis* fermentation. The general hydrolysis technology involves the use of harsh acids/bases or expensive enzymes to release the trapped nutrients in the food waste substrate [22,31,32]. Hydrolysis using mixed cultures from wastewater sludge provides an alternate biological platform that is both environmentally friendly, inexpensive, and toxic by-product-free [33]. This observation led us to examine the contents of FWH upon hydrolysis in an LBR, which yielded complex carbohydrates (19.23 g/L), volatile fatty acids (with alcohols) (2.23 g/L), and free amino acids (0.083 g/L) as the major constituents. All these macronutrients are known to be essential for sustaining growth and RL biosynthesis [8]. In contrast to the conventional post-treatment methods, the conditions used for the processing of FWH in the present study also did not promote the formation of inhibitory compounds such as Maillard reaction products (MRPs). In addition, an in-depth substrate screening was also carried out in the present work to dictate the fermentation RLs kinetics with pure as well as homogeneous waste residues. The pure complex lipidic substrates (canola oil & WCO) induced biomass production in *B. thailandensis*, while RLs were considerably low. Furthermore, the simpler fatty acids (oleic acid) improved the RL production to 1.21 g/L, indicating that a fatty acid-rich medium is more favorable for *B. thailandensis* RLs synthesis. The water-soluble carbon substrates, i.e., fructose and glycerol, increased RL to 1.44–1.85 g/L and outperformed all other hydrophilic substrates. Previous literature have utilized glycerol as one of the most consistent carbon sources during fermentation development for high RL synthesis by *B. thailandensis* E264, but in the presence of synthetic nutrients [24,34,35].

To overcome nutrient supplementation prerequisites, batch fermentation was upscaled in a bioreactor with FWH, and kinetics parameters were compared for both FWH and pure glycerol. Contrary to past research reporting an extended stationary phase (264–312 h) [8,24], FWH fermentation gained its highest RLs production within 72 h of fermentation. The achieved maximum cell dry weight and RLs concentration were 1.304 g/L and 0.616 g/L,

respectively. In addition, the fermenter FWH RLs concentration was slightly lower than the shake flask FWH RLs (0.81 g/L). This is probably due to the diversion of carbon flux towards other metabolite pathways such as polyhydroxyalkanoates which also hold high bio-industrial value [36], The overall fermentation performance with FWH exceeded that of pure substrates (sugars, lipids, and fatty acids). Furthermore, RLs production yields with FWH substrates were elevated by 1.6 times (34 mg/g) the amount obtained with standard glycerol medium (21 mg/g). These observations are in the higher range as RLs yields reported with other agro-industrial feedstocks are: CSL (16.5 mg/g), CSL + OMW (23 mg/g), NF (13.37 mg/g), and OMP (15 mg/g) [11–13].

Furthermore, the choice of carbon feedstock also influences the type of RLs isoform (mono- or di-), fatty acyl chain lengths (C_{10}, C_{12}, C_{14}, and C_{16}), and subsequent downstream applications. The high-chain di-rhamnolipids are considered superior surface-active agents with uses in various domains such as bioremediation, enhanced oil recovery, pharmacology, dermatology, therapeutics, cosmetics, cleaning, and agriculture [26,29,37]. In addition, the structural fingerprints of FWH-produced RLs were similar to other literature reports, which shed evidence on the elevated RL quality obtained from the use of FWH feedstock [19,25]. Subsequent chemical characterization revealed mainly di-RLs (Rha-Rha-C_{12}-C_{12}, Rha-Rha-C_{12}-C_{14}, Rha-Rha-C_{14}-C_{14}, and Rha-Rha-C_{14}-C_{16}), with Rha-Rha-C_{12}-C_{14} as the most abundant congener (44.91%). The RLs fatty acyl chain of C_{12}-C_{14} was also synthesized by *B. thailandensis* using CSL + OMW but mostly as mono-rhamnolipids Rha-C_{12}-C_{14} (29.79%) [12]. Other studies have reported Rha-Rha-C_{14}-C_{14} as being primarily synthesized from pure substrates [24,26,34]. Particularly in FWH, the heterogenous substrate consumption trend reflected a fast utilization of VFAs over other complex carbohydrates. This allowed mixed VFAs to supply different chain-length precursors to the RLs synthesis enzyme (RhlC) through the inherent FAS II pathway as observed in RLs producers [10,38]. This observation implies that there is potential for tailoring the synthesis of unique congener profiles for broader applicability.

The measurement of surface tension data was used to identify the FWH RL critical micelle concentration at which surfactant complexation occurs. Under the equilibrium condition, CMC was approximately 800 mg/L and acted as the most probable surface tension break-point concentration, which is slightly higher than other reported values. The extraction of RLs from *B. thailandensis* is known to co-recover proteins, but the emulsifiability and sensitivity of di-rhamnolipids remain unchanged [39]. To confirm this, aqueous RL solutions were used against alkane hydrocarbons to form stable emulsions. The emulsion index of this mixture was above 50% (54.6%), which denotes complete emulsification of the organic phase. The *B. thailandensis* RLs produced by Correia et al. [12] with agro-industrial by-products had E_{24} below 20%. Thus, FWH RLs are highly favorable for industrial applications as low-cost biosurfactants.

5. Conclusions

The post-consumption food waste was demonstrated to be superior no-cost feedstock for biosynthesis among other hydrophilic and hydrophobic substrates used in the present investigation. The observations revealed that *B. thailandensis* E264 is a strong biocatalyst, efficiently performing batch fermentative conversion of food waste while achieving unique congener profiles and product titers. In addition, the upscaled fed-batch strategies could further improve the performance and RLs biosynthesis while monitoring the effects of using alternative waste streams during the feeding phase. This research also demonstrated the utility of fermentative feedstock selection as a waste management strategy and workflow for downstream bioconversion to value-added RLs bioprocess development. In conclusion, this research showed a better biological, eco-friendly method for synthesizing industrially relevant biochemicals and could direct possible future studies for addressing advanced bioprocess strategies with *B. thailandensis* E264 in a sustainable manner.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15010059/s1. Figure S1: LC-MS-MS spectra of (a) food waste hydrolysate and (b) pure glycerol-derived rhamnolipids.

Author Contributions: Conceptualization, R.K. and G.K.; methodology, R.K. and G.K.; investigation, R.K., R.D.P. and D.J.; data curation, R.K.; writing—original draft preparation, R.K.; writing—review and editing, R.K. and G.K.; visualization, R.K.; resources, J.W.C.W. and G.K.; supervision, G.K.; funding acquisition, G.K. All authors have read and agreed to the published version of the manuscript.

Funding: The research was funded by the Innovation and Technology Commission (ITC), Hong Kong (Grant No. ITS/176/18).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available within the article or Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

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