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Influence of bioprocess parameters on sophorolipid production from bakery waste oil

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Abstract

Secondary/waste streams have previously been used as feedstocks to produce sophorolipids (SLs), a biosurfactant with low eco-toxicity and high biodegradability, in order to reduce production costs and protect the environment. However, limited productivities and titres from these feedstocks remain as important challenges. Thus, the optimisation of fermentation medium using bakery waste oil (BWO) as a hydrophobic carbon source by *Starmerella bombicola* was investigated. The optimal conditions were determined by multiple linear regression. Inoculum concentration of 2% v v^{-1} and BWO and glucose concentrations of 60 g L^{-1} and 100 g L^{-1}, respectively, resulted in an increase of 19.6% in the lactonic SL (67.8 ± 11.5 g L^{-1}). Further optimisation revealed the profound influence of KOH in pH regulation, i.e., compared with NaOH, KOH led to higher concentrations of biomass ($p < 0.05$), more BWO consumption, and thus, an increase of 42.2% in SL titre (96.4 ± 9.1 g L^{-1}) and corresponding volumetric and specific productivities of 0.446 g L^{-1} h^{-1} and 0.027 g g CDW^{-1} h^{-1}, respectively. Multiple regression analysis demonstrated that pH and the concentration of BWO as the feeding medium were the most influential parameters in fermentative SL production. This study demonstrated that KOH offered additional benefit to improve SLs titre by maintaining high biomass during the bioprocess, displayed the importance of intracellular potassium in cell viability and improved the valorisation of BWO process.

Keywords

bakery waste oil; circular economy; lactonic sophorolipids; pH regulation; potassium hydroxide; *Starmerella bombicola*

Abbreviations

BWO: bakery waste oil, CDW: cell dry weight, ELSD: evaporative light scattering detector, HPLC: high-performance liquid chromatography, OD: optical density, PDA: potato dextrose agar, SLs: sophorolipids, YE: yeast extract

1. Introduction

Sophorolipids (SLs) are biosurfactants produced by several non-pathogenic yeast species, the best known of which is *Starmerella bombicola* [1,2,3]. The production of SLs is an entirely biological process. SLs have low ecotoxicity and are biodegradable and thus are considered environment friendly agents for use in oil recovery, bioremediation and cleansing [4]. SLs have also demonstrated antimicrobial, anti-inflammatory and anti-cancer properties, which warrant their exploration for use in medical and pharmaceutical applications [5].

SLs are composed of lipophilic and hydrophilic moieties, therefore they are amphiphilic and function as surfactants. Their hydrophilicity is conferred by a β-1,2′-glycosidic linkage between two glucose residues. This disaccharide is linked to a lipophilic moiety by a β-glycosidic bond to the terminal or sub-terminal carbon atom of a fatty acid [6]. The biosynthesis of SLs occurs mainly intracellularly, and the so-called acetylated acidic SLs, which have a free carboxylic moiety, are then transferred into the extracellular environment by adenosine triphosphate-binding cassette transporters. Therefore, most acidic SLs are transformed into lactonic SLs by lactone esterase [7]. The degree of acetylation of SLs at the C6′-atoms of the glucose disaccharide sophorose and the extent of saturation, hydroxylation and chain length of the incorporated fatty acid vary according to the type of feedstock [8,9].

Hydrophilic and lipophilic feedstocks are typically used together, as this results in an optimal SL production, and thus, a typical production medium contains glucose and vegetable oil. Although bioprocessing using refined feedstocks has resulted in high SL titres of up to 200 g L^{-1} [10], the use of such first-generation feedstocks results in relatively high production costs and has adverse environmental effects [8]. Therefore, the use of second-generation feedstocks, i.e. industrial residual biomass waste-streams, has been investigated.

A key hurdle in the practical biorefinery application of such waste feedstocks is their inherent heterogeneity. Moreover, the titres of SL generated from second-generation feedstocks such as sunflower oil, waste fried oil, jatropha oil and animal fat (as hydrophobic substrates), and sugarcane molasses, soy molasses and glycerol (as hydrophilic substrates) have been relatively low and typically less than 100 g L^{-1}, as reviewed by Jiménez et al. and Solaiman et al. [11,12]. Thus, to facilitate low-cost SL production, there is a need to increase the efficiency of SL production and the SL titre obtained from second-generation feedstocks to be comparable to those obtain from the first-generation feedstocks.

In this study, the bioprocess of SL production by *S. bombicola* from waste cooking oil, provided by a local industrial bakery in Hong Kong was investigated. As shown in Figure 1, the experimental scheme of the BWO optimization process were performed. First, various nitrogen feedstocks were tested to identify the optimal nitrogen source and concentration. Second, the inoculum concentration was optimised. Third, the concentrations of fed substrates (i.e., bakery waste oil (BWO) and glucose) were

optimised to achieve the highest possible SL production, with a focus on preventing the accumulation of residual oil and glucose to facilitate downstream processing and prevent substrate losses. Moreover, two modes of pH regulation were assessed to further enhance the final SL titre. Following this optimisation process, multiple linear regression was used to compare the influence of each parameter on process efficiency. Finally, a proof-of-concept bioprocess comprising the optimised conditions was assessed and subject to additional fine-tuning adjustment.

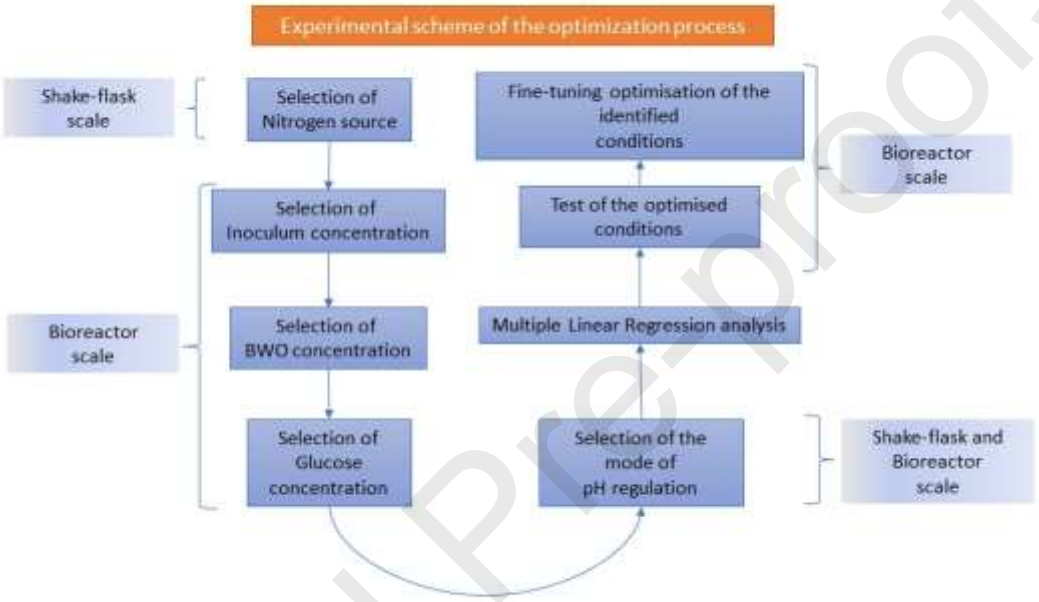


Figure 1. The experimental scheme of the optimization process (BWO = bakery waste oil).

2. Materials and methods

2.1 Chemicals

Yeast extract (LP0021B), peptone (LP0037) and potato dextrose agar (CM0139) were purchased from Oxoid Limited, UK. D-(+)-Glucose (A16828) was purchased from Alfa Aesar Chemical Co., UK. Sodium citrate dihydrate, citric acid (Cat. 100241), ammonium acetate (Cat. 101116), potassium dihydrogen phosphate (Cat. 104871), NaOH (Cat. 106469) and KOH (Cat. 105033) were purchased from Merck, Germany. Ammonium nitrate (A9642) was purchased from Sigma-Aldrich, USA.

2.2 Microorganism and culture conditions

The *S. bombicola* strain ATCC 22214 was purchased from the American Type Culture Collection, USA and cultivated as described in our previous study [2]. Briefly, a loopful of inoculum was streaked on a potato dextrose agar (PDA) plate from a cryovial stock culture in 10% glycerol that was stored at -80°C. The PDA plate was then incubated at 30°C for 48 h until single colonies were formed. A single colony was selected and transferred to a 14-mL round-bottomed culture tube that contained 3 mL of seed medium (i.e. 100 g L^{-1} glucose and 10 g L^{-1} yeast extract), and this glass tube was then agitated in an incubation shaker at 250 rpm and 30°C for 24 h. The resulting preculture was transferred to a 250-mL shake flask containing 50 mL of media at a 2% (v v^{-1}) inoculation ratio and agitated at 200 rpm at 30°C overnight for further propagation. The microbial growth was monitored by optical density (OD) measurements at 600 nm using a spectrophotometer (Jenway, Model 7300, USA). Samples were diluted 40-fold in deionised water before OD measurement. Cell dry weight (CDW) was calculated using a calibration curve of the correlation between biomass concentration (g mL^{-1}) and OD at 600 nm (see **Equation (1)**):

$$CDW\ (g\ mL^{-1}) = 0.002 \times OD - 0.0003 \qquad \textbf{Equation (1)}$$

Unless specified otherwise, a typical medium for the bioreactor (Sartorius, Biostat® B equipped with UniVessel Glass® 2 L) and shake-flask experiment contained 2% v v^{-1} of inoculum at a biomass concentration (CDW) of 20 g L^{-1}, 100 g L^{-1} of BWO, 100 g L^{-1} of glucose and 2.5 g L^{-1} of yeast extract. The working volume of the shake-flask and bioreactor experiments were 100 mL and 1.5 L, respectively. In the bioreactor experiment, aeration of compressed air, temperature and agitation speed were controlled at 2.67 vvm, 30°C and 600 rpm, respectively, and experiments were

conducted for 144 h. Shake-flask experiments were performed in an orbital shaker with temperature and agitation controlled at 30°C and 200 rpm, respectively, and were conducted for 240 h.

2.3 Bakery Waste Oil

BWO was kindly donated by the industrial plant of Kee Wah Bakery Limited (https://keewah.com/hk/) in Tai Po Industrial Estate, Hong Kong. BWO is the leftover oil from the bakery production process. As shown in Figure S1, the BWO is light brown in colour and contains suspended debris. It was kept overnight at room temperature to allow the debris to settle, and the debris-free oil was then separated by decantation. The clear oil was then kept at -20°C until further use and autoclaved at 121°C for 20 mins before use in bioprocess experiments.

2.4 Optimisation of the SL production medium and process variables

2.4.1 Selection of nitrogen source and optimisation of nitrogen concentration

Various nitrogen sources were tested in shake-flask experiments (as described in Section 2.2): yeast extract, peptone and ammonium nitrate. These were tested at three concentrations: 2.5, 5 and 10 g L^{-1}.

A carbon-to-nitrogen (C/N) ratio was calculated by **Equation (2)** to determine the mass ratio of the C source (i.e. glucose and BWO) to the N source being added in each condition:

$$C/N\ ratio = \frac{Total\ mass\ of\ the\ C\ source}{Total\ mass\ of\ the\ N\ source} \qquad \textbf{Equation (2)}$$

where C = carbon and N = nitrogen.

2.4.2 Optimisation of the inoculum concentration

Inoculum with a biomass concentration of 20 g L^{-1} was inoculated into a 2.5-L bioreactor at an inoculation ratio of 2%, 4% and 6% (v v^{-1}). Other bioprocess parameters were as described in Section 2.2, and the yeast extract concentration was 2.5 g L^{-1} (as determined in Section 2.4.1).

2.4.3 Optimisation of the BWO concentration

The efficiency of SL production when using various initial concentrations of BWO (20, 40, 60, 80 and 100 g L^{-1}) combined with 2.5 g L^{-1} yeast extract and 2% v v^{-1} inoculum (as identified as optimal in Sections 2.4.1 and 2.4.2) in a 2.5-L bioreactor was examined. The pH was uncontrolled throughout the cultivation.

2.4.4 Optimisation of the initial glucose concentration

The efficiency of SL production when using various initial concentrations of glucose (50–150 g L^{-1}) combined with 2.5 g L^{-1} yeast extract, 2% v v^{-1} inoculum and 60 g L^{-1} BWO (as identified as optimal in Sections 2.4.1–2.4.3) in a 2.5-L bioreactor was examined. The pH was uncontrolled throughout the cultivation.

2.4.5 Regulation of pH

The regulation of pH using buffers and bases was performed as previously described by Ma et al. [13], with some modifications. First, pH regulation was evaluated in a shake-flask experiment, and the best mode of pH regulation was then tested in a 2.5-L bioreactor. The abovementioned optimised conditions in Sections 2.4.1 to 2.4.4 were used, i.e., 2.5 g L^{-1} yeast extract, 2% v v^{-1} inoculum, 100 g L^{-1} BWO and 100 g L^{-1} glucose. In pH-regulation experiments using buffers, sodium citrate dihydrate, ammonium acetate or potassium dihydrogen phosphate buffer was added to the media and the pH was adjusted to 3.5 at 0 h after inoculation. In pH-regulation experiments using bases, NaOH or KOH was added to the shake-flasks every 24 h, beginning from 48 h after inoculation, and the pH was maintained at 3.5. At 10 days after inoculation, an aliquot of the reaction mixture was taken for endpoint SL analysis.

2.4.6 Optimisation of bioprocess conditions in the bench-top bioreactor

The combined optimised conditions identified in Sections 2.4.1 to 2.4.5 were used for a 2.5-L bioreactor optimisation experiment. These optimised conditions were 60 g L^{-1} BWO, 2.5 g L^{-1} yeast extract, 100 g L^{-1} glucose and 2% v v^{-1} inoculum. KOH was added to the bioreactor at 24 h after inoculation to maintain the pH at 3.5. All fermentation experiments were carried with batch mode.

2.5 Sampling and analysis

2.5.1 High-performance liquid chromatography (HPLC) analysis

Analyses of lactonic SL and glucose were performed by the methods described in our previous study [3]. Lactonic SLs were separated on a Zorbax Eclipse Plus C18 column (4.6 × 100 mm, Agilent, USA) and were detected by a coupled evaporative light scattering detector (Waters®, 2424, USA). The lactonic SL standard was 1,4′ ′ -sophorolactone 6′,6″-diacetate with a purity of ≥ 80% (Cayman Chemical, USA), therefore the construction of standard curve was based on this purity. Chromatograms of 40 g L^{-1} Lactonic SL standard and four-fold diluted sample (i.e. 2% v v^{-1} Inoculum, 2.5 g L^{-1} YE, 100 g L^{-1} glucose, 100 g L^{-1} WCO and pH adjusted to 3.5 by KOH at the 144^{th} hour) are shown as Figure S2a and Figure S2b in the Supplementary Materials. Glucose was separated on a BIO-RAD 87H column (Aminex® HPX-87H column, 300 × 7.8 mm, USA) and was detected by a refractive index detector (Waters®, 2414, USA).

2.5.2 Analyses of Bakery Waste Oil and Cell Dry Weight

The gravimetric method was used for BWO detection. The procedure was similar to that described by Samad et al. [14], with minor modifications. Briefly, 1 mL of the sample containing BWO was extracted three times with an equal volume of hexane, and the combined extracts were centrifuged at 15,000 × *g* for 2 min. Then, the upper layer (hexane, containing BWO) was transferred to a 2-mL centrifuge tube, which was then placed in an oven and heated to 50°C. The residual oil was then weighed.

The lower layer (containing biomass) was used for CDW analysis. The biomass was washed three times with 1 mL of ethanol, vortexed and then centrifuged at 15,000 × g for 2 min. The supernatant was removed, and the pellet was dried at 50°C in an oven overnight. The dried pellet was then weighed to give the CDW.

2.5.3 Cell propagation analysis

S. bombicola growth was analysed by the colony-forming unit (CFU) method. Briefly, the collected sample was diluted 1×10^{-7}- to 10^{-8}-fold with potato dextrose broth, and a 1 mL aliquot of each of these dilutions was aspirated and then transferred to a PDA plate. The plates were incubated at 30°C for three days until colonies formed. The experiment was performed in triplicate, and the average number of CFUs was calculated.

2.5.4 Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS Inc., USA) software v16.0. Analysis of the test group was performed by a one-way analysis of variance. The paired *t*-test function in Microsoft Office Excel was used to identify differences in the CFU assay and between CDWs.

Multiple linear regression was used to quantify the relationship between multiple independent variables (i.e. Glucose, BWO, pH and inoculum concentration) and dependent variable (i.e. lactonic SL titre). A set of coefficients were generated from single linear regression that best describes the lactonic SL titre. Then, the resultant lactonic SL titre from each independent variable was calculated as summation of each molecular descriptor weighted by respective coefficients. A typical multilinear model can be represented in **Equation (3)**:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \cdots + \beta_k x_k + \varepsilon \qquad \textbf{Equation (3)}$$

Where k is the number of variables, β is the regression coefficient which represents the independent contributions of each calculated molecular descriptor, x and y are the independent and dependent variables, respectively.

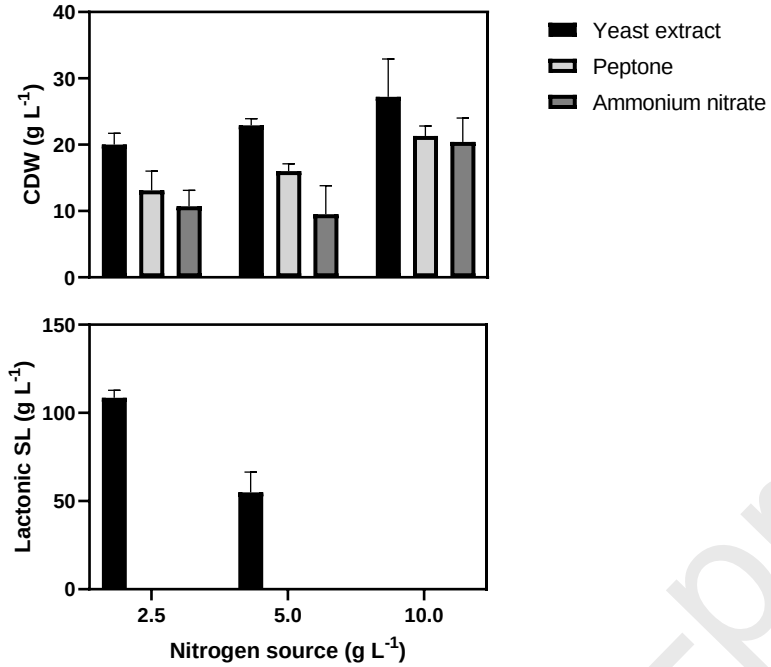
3. Results and discussion

3.1 Selection and optimisation of nitrogen (N) source

Although both organic and inorganic N sources can be utilised by SL producers, the type and concentration of nitrogen source are crucial parameters for SL production, as N limitation is a predominant condition involved in SL synthesis [14, 15]. Therefore, an optimisation of the appropriate N sources was performed to obtain the best possible BWO-derived SL titres, yields and productivities.

Three types of N sources were evaluated – ammonium nitrate, peptone and yeast extract – for their ability to produce lactonic SLs from BWO as the hydrophobic source. As shown in Figure 2a, no lactonic SLs were produced when ammonium nitrate or peptone was used, although all three types of N sources improved cell growth (Figure 2a). This lack of SL production may have been due to the inhibition of lactonic SL production by inorganic N (i.e., ammonium nitrate) [16]. In fact, Yu et al. successfully adopted ammonium nitrate as the N source to improve the total SLs production to 27.45 g L^{-1} which contained high ratio of acidic SL (i.e. 22.4%) [17]. In addition, Rispoli et al. [18] and Van Bogaert et al. [19] have stated that peptone may hinder glycolipid production. In contrast, we observed high CDWs and lactonic SL production when we used yeast extract, which indicated that it was a suitable N source for SL production with BWO as the hydrophobic source. This is consistent with the fact that yeast extract is the most common organic N source used for SL production and has been used at concentrations from 0.5 g L^{-1} to 10 g L^{-1} [2, 20, 21]. It has also been observed that SL titres decreased when yeast extract was substituted with other N sources, such as peptone and urea [19]. As shown in Figure 2b, increasing the yeast extract concentration from 0.625 to 2.5 g L^{-1} increased SL production, with the highest concentration of SLs being 108.47 ± 4.25 g L^{-1} ($p < 0.001$) resulting from the addition of 2.5 g L^{-1} of yeast extract. The SL titre decreased when the concentration of yeast extract was further increased from 2.5 g $L^{-1}$to 10 g L^{-1}. As shown in Figure 2b, the initial concentration of yeast extract was positively correlated with yeast growth, and a stepwise increase of CDW occurred when the yeast extract concentration was increased from 0.625 g L^{-1} to 10 g L^{-1}. A similar study performed by Bajaj et al. [22] also demonstrated that an optimised culture medium (100 g L^{-1} glucose, 100 g L^{-1} oleic acid and 100 g L^{-1} yeast extract) resulted in the best yields of SLs. The differences between the optimal concentration of yeast extract identified by Bajaj et al. [22] and that identified by us likely indicate that BWO has distinct requirements for N.

(a)



(b)

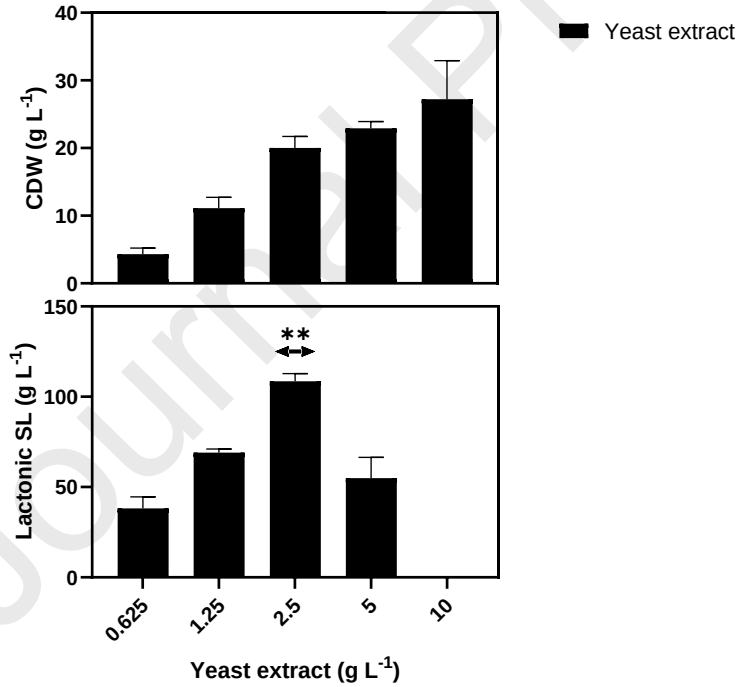


Figure 2. The optimisation of the nitrogen source (N) for lactonic sophorolipid (SL) production. Comparison of (a) SL production and cell dry weight (CDW) production using ammonium nitrate, peptone or yeast extract at 2.5 to 10 g L^{-1}. (b) Influence of concentration of yeast extract on SL production and CDW production. $^{**}p < 0.01$ relative to the different concentration.

3.2 Optimisation of inoculum concentration

Various inoculum concentrations (i.e., 2% to 6% v v^{-1}) were evaluated to examine their effect on lactonic SL production. As shown in Figure 3 and Figure S3, an increase in the inoculum concentration did not lead to an obvious difference in the SL titre but caused an increase in the CDW. Specifically, at 144 h with 2% v v^{-1} inoculum, the CDW was 21.2 ± 4.7 g L^{-1}, whereas with 4% v v^{-1} inoculum it was 22.2 ± 8 g L^{-1} and with 6% v v^{-1} it further increased to 34.9 ± 2.4 g L^{-1}. As shown in Table 1, the lactonic SL titre at 144 h for 2%, 4% and 6% v v^{-1} inoculum were 60 ± 4.8 g L^{-1}, 52.1 ± 7.7 g L^{-1} and 52.9 ± 13.3 g L^{-1}, respectively. The results are consistent with those of a previous studies by Ribeiro et al. [23] and Daverey et al. [24], which reported that increases in inoculum concentration from 2.5% to 10% v v^{-1} had no effect on lactonic SL production. A high CDW may also limit the lactonic SL production due to oxygen limitation [25].

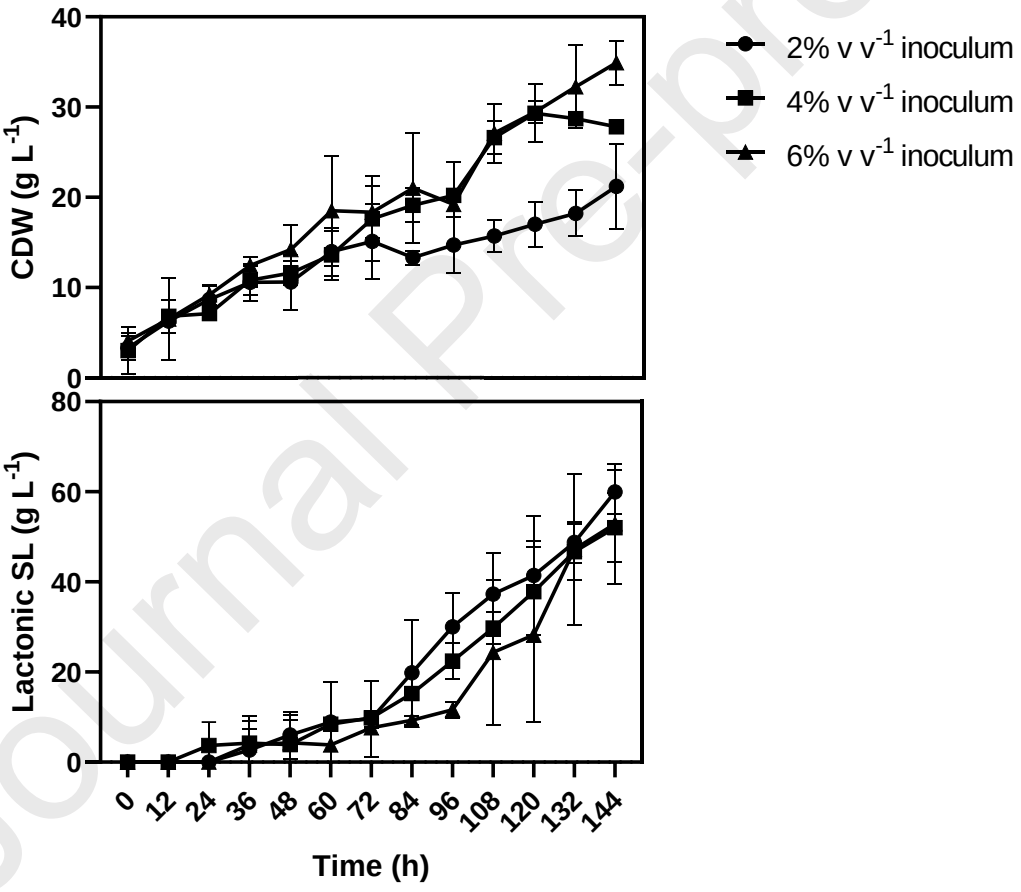


Figure 3. Comparison of effect of different inoculum concentrations on cell dry weight (CDW) and lactonic sophorolipid (SL) titre.

3.3 Optimisation of BWO concentration

Oleic acid is an important hydrophobic substrate for SL biosynthesis and is rich in many oil constituents. Much of the recent research have focused on comparing the use of secondary hydrophobic sources (e.g., oil refinery waste [26], residual oil cake [27] and oily sludge [28]) for SL production. However, only a few studies have focused on optimisation of the concentration of the secondary hydrophobic source in SL-forming bioprocesses [29]. This is a key research gap, as the use of excess concentrations of oil is a waste of the hydrophobic feedstock and entails additional downstream purification, which increases SL production costs. Insufficient hydrophilic carbon source in fermentation medium is also a problem, as it leads to decreases in SL production titres.

Therefore, BWO concentrations from 20 to 100 g L^{-1} were evaluated to identify the optimal concentration for SL production. As shown in Figure S4, increasing the BWO concentration from 20 g L^{-1} to 60 g L^{-1} had no effect on the CDW, but significantly increased the lactonic SL titre (at 144 h of cultivation) from 18.4 ± 5.9 g L^{-1} to 67.8 ± 11.5 g L^{-1}. However, further increases in BWO concentration (beyond 60 g L^{-1}) did not result in additional increase in lactonic SL production. This indicates that an increase in the initial BWO concentration can significantly improve the lactonic SL titre ($p < 0.05$) (Figure 4). As shown in Table 1, the highest SL volumetric productivity of 0.314 g L^{-1} h^{-1} was achieved from an initial BWO concentration of 60 g L^{-1}. This finding was also in line with Bajaj et al. [22], who demonstrated that increasing the oleic acid concentration from 0 to 100 g L^{-1} increased the SL titre generated by their system, but further increase in the oleic acid concentration decreased the SL production.

Variations in the BWO concentration may cause a delay in the lactonic SL biosynthesis. As shown in Figure S4, at a low BWO concentration of 20 g L^{-1}, lactonic SLs were produced from 12 h to 24 h. However, at higher BWO concentrations of 40 g L^{-1}, 60 g L^{-1} and 80 g L^{-1}, lactonic SLs were first detected at 36, 60 and 72 h, respectively. The trend in SL production throughout the experiment was also concordant with the glucose consumption, i.e., the higher the initial concentration of BWO, the higher the SL titre and therefore the more rapidly the glucose was consumed.

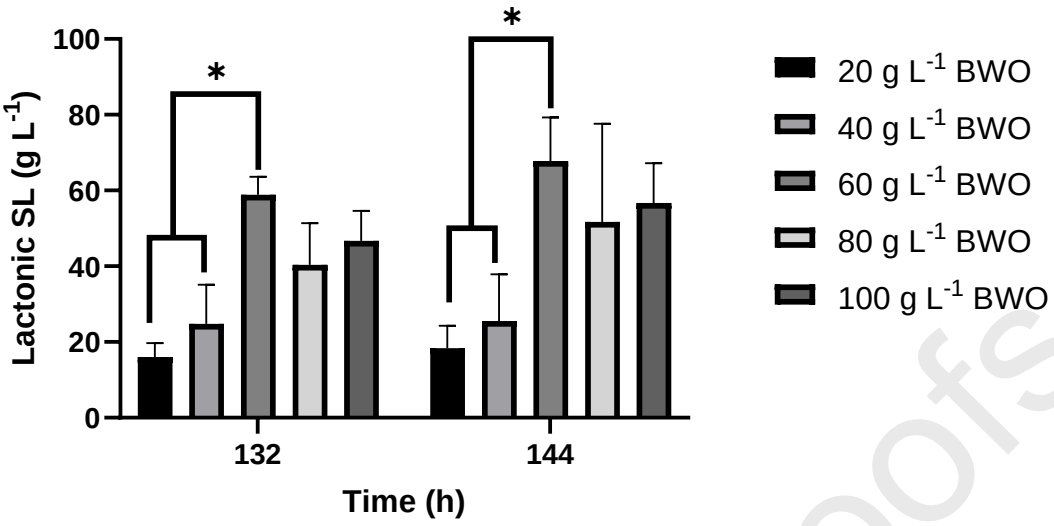


Figure 4. Comparison of batch lactonic sophorolipid (SL) production at 132 and 144 h using various concentrations of bakery waste oil (BWO). * $p < 0.05$ relative to the respective groups.

3.4 Optimisation of the initial glucose concentration

Glucose is an essential carbon (C) source in the biosynthesis of the hydrophilic moiety of lactonic SLs, and the glucose concentration has a combined effect with the hydrophobic substrate concentration and the N concentration on the efficiency of SL production. Thus, the C/N ratio is an essential bioprocess parameter that should be examined. It was found that at high C/N ratios, SL biosynthesis was triggered, and SLs accumulated extracellularly.

Three initial glucose concentrations (50 g L^{-1}, 100 g L^{-1} and 150 g L^{-1}) were used for further optimisation in the presence of 60 g L^{-1} BWO. Figure 5 shows that at an initial yeast extract concentration of 2.5 g L^{-1}, the maximum lactonic SL production (67.8 ± 11.5 g L^{-1}) occurred when the glucose concentration was increased from 50 g L^{-1} to 100 g L^{-1} (i.e. an C/N ratio increase from 44 to 64). However, the titres of lactonic SL decreased to 33.1 ± 11.4 g L^{-1} when the initial glucose concentration was further increased from 100 g L^{-1} to 150 g L^{-1} (i.e. C/N ratio increase from 44 to 64). The onset of SL production was also affected by the initial glucose concentration (Figure S5). Specifically, when the initial glucose concentration was 50 g L^{-1}, SL production began at 72 h, whereas at initial glucose concentrations of 100 g L^{-1} and 150 g L^{-1}, SL production began at 60 h. In addition, relatively higher CDWs were produced from 50 g L^{-1} and 150 g L^{-1} of glucose, which accounts for the low specific productivity under these conditions (Table 1).

The overall glucose consumption in various bioprocesses was calculated. At 144 h, the residual glucose from bioprocesses with initial glucose concentrations of 50 g L^{-1} and 100 g L^{-1} were 0.75 ± 0.07 g L^{-1} and 53.7 ± 7.8 g L^{-1}, respectively, which indicates that 49.3 g L^{-1} and 46.3 g L^{-1} of glucose had been consumed, respectively. In contrast, the overall consumption of glucose was reduced (to approximately 20 g L^{-1}) in the bioprocess with an initial glucose concentration of 150 g L^{-1}.

A previous study by Cutler and Light [30] of a SL bioprocess with a defined medium showed that 2 g L^{-1} of SL was produced when fixed concentrations of glucose and nitrogen (30 g L^{-1} and 1.5 g L^{-1}, respectively) were used. In addition, the decrease in SL production could be modulated by decreasing the glucose concentration or increasing the nitrogen concentration. Another study conducted previously by our research group utilised food waste hydrolysate as a nutrient source and also demonstrated that a high C/N ratio (41) would lead to a high SL titre and high productivity (i.e., 115.2 g L^{-1} SL and 1.25 g L^{-1} h^{-1}) under optimised experimental conditions [2]. A similar study by Pekin et al. [20] showed that high SL production can be obtained from a high C/N ratio (20) from Turkish corn oil and honey.

In this study, the highest lactonic SL concentration (67.8 ± 11.5 g L^{-1}) ($p < 0.05$) was generated from 100 g L^{-1} glucose (Figure 5), and lactonic SL concentrations reduced to 44.5 ± 8.2 g L^{-1} and 33.1 ± 11.3 g L^{-1} when 50 and 150 g L^{-1} glucose were used, respectively. These results indicate that the N proportion at a total C/N ratio of 44 to 64 is better than that in a total C/N ratio of 64 to 84, and further increases in glucose

concentration adversely affect lactonic SL production and specific productivity, as shown in Table 1.

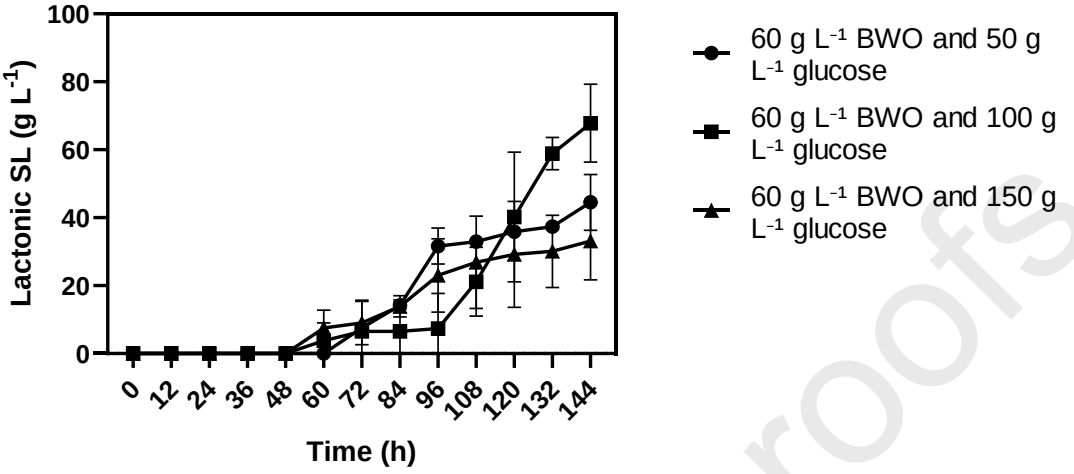


Figure 5. Comparison of batch lactonic sophorolipid (SL) production using 60 g L^{-1} bakery waste oil (BWO) with different initial glucose concentrations.

3.5 Regulation of pH

The rate of lactonic SL production can be altered by the acidity of the media, and a previous study found that a pH of 3.5 resulted in the highest titre of lactonic SLs [13]. In this study, a different mode of pH regulation was used to sustain high biomass and simultaneously produce high concentrations of lactonic SLs from BWO. Controlling of pH 3.5 was achieved by adding citrate, phosphate or ammonium acetate buffer before fermentation and by adding bases (KOH or NaOH) during fermentation (i.e., at 48 h in the shake-flask experiment). Several buffers and bases were evaluated in a medium containing 100 g L^{-1} glucose, 100 g L^{-1} BWO and 2.5 g L^{-1} yeast extract. After 10 days of cultivation, SL concentrations of 89.3 ± 5.0 g L^{-1} and 90.4 ± 11.1 g L^{-1} were obtained when 15 g L^{-1} of citrate and phosphate buffer were used, respectively, but these concentrations were not markedly different from that obtained in the control (i.e., 108.5 ± 4.3 g L^{-1}) and buffer groups. However, no SLs were detected when ammonium acetate buffer was used, as no cell growth occurred.

In contrast, higher SL production was observed when bases were used to maintain a bioprocess pH of 3.5 every 24 h. Thus, the use of KOH or NaOH resulted in the production of 140 ± 16.5 g L^{-1} and 101.6 ± 2.62 g L^{-1} lactonic SLs, respectively, in a 10-day shake-flask bioprocess (Figure 6b), and the difference between these concentrations was statistically significant ($p < 0.05$). Further analysis showed that the CFU mL^{-1} of the KOH group was five times of that for the NaOH group ($p < 0.001$; paired *t*-test; Figure 6c). In summary, the addition of bases would facilitate higher SL production as compared to the addition of buffers. After the bioprocess entered the

stationary phase, production of the lactonic SLs began, and a pH of 3.5 was optimal for this production.

Table 1. Summary of lactonic sophorolipid (SL) bioprocess parameters and results. These data are produced from bioreactor experiments.

Test group	Inoculum % (v v^{-1})	YE (g L^{-1})	Glucose (g L^{-1})	pH regulation	BWO (g L^{-1})	Lactonic SL titre at the 144th hour (g L^{-1})	Volumetric productivity (g L^{-1} h^{-1})	Specific productivity (g g CDW^{-1} h^{-1})	Yield (SL per gram of substrate) g g^{-1})
Inoculum proportion	2%	2.5	100	Nil	100	56.7 ± 10.5	0.262	0.022	0.3
	4%					52.1 ± 7.7	0.241	0.016	0.26
	6%					52.9 ± 13.3	0.245	0.011	0.26
BWO proportion	2%	2.5	100	Nil	20	18.4 ± 5.9	0.085	0.008	0.15
					40	25.5 ± 12.4	0.118	0.013	0.18
					60	67.8 ± 11.5	0.314	0.041	0.42
					80	51.7 ± 25.9	0.239	0.031	0.29
					100	56.7 ± 10.5	0.262	0.022	0.3
Glucose proportion	2%	2.5	50	Nil	60	44.5 ± 8.2	0.206	0.012	0.4
			100			67.8 ± 11.5	0.314	0.041	0.42
			150			33.1 ± 11.4	0.153	0.009	0.16
pH regulation	2%	2.5	100	KOH	100	96.4 ± 9.1	0.446	0.027	0.48
				NaOH		45.4 ± 2.5	0.210	0.026	0.23
				Nil		56.7 ± 10.5	0.262	0.022	0.3
Optimization of BWO in optimised condition	2%	2.5	100	KOH	60	29.6 ± 0.1	0.137	0.009	0.19
					80	59.7 ± 0.8	0.276	0.02	0.33
					100	96.4 ± 9.1	0.446	0.027	0.48

BWO and YE refer to as bakery waste oil and yeast extract respectively.

The results from the shake-flask experiments were confirmed by repeating the same condition in a 2.5-L bench-top bioreactor experiment with continuous pH regulation starting from 24 h (i.e., the start of the stationary phase). As shown in Figure S6a and b, the addition of KOH resulted in a higher CDW compared with the addition of NaOH, and this occurred in the KOH group from 48 h to 144 h. The difference in the CDW was also more significant in the KOH group, especially at 132 h and 144 h ($p < 0.05$, paired *t*-test). At 144 h, the titre of lactonic SLs in the KOH group was 96.4 ± 9.1 g L^{-1}, corresponding to a volumetric productivity and specific productivity of 0.446 g L^{-1} h^{-1} and 0.027 g g CDW^{-1} h^{-1}, respectively, which was more than double of the NaOH group ($p < 0.05$).

Notably, there were no obvious differences in the lactonic SL titre between the NaOH group and the no-pH-control group, as shown in Table 1 (i.e., 45.4 ± 2.5 g L^{-1} vs 56.7 ± 10.5 g L^{-1}). This could be explained by an observation reported by Ma et al. [13], who found that when excessive N was present, the effect of NaOH on lactonic SL production was diminished.

The increase in the lactonic SL production in the KOH group might be due to intracellular K^+ homeostasis leading to an increased viability of yeast cells during the bioprocess. Although NaOH is the most popular pH regulator in lactonic SL production [31, 32, 33], However, other pH regulators are seldomly used. Similar experiment was conducted by Liu et al. [34] to examine the influence of a range of pH regulators to the SLs production in *Wickerhamiella domercqiae var.* and found that the resultant total SL titer and lactonic SL ratio varies with different pH regulator. Furthermore, the influence of those pH regulators on biomass production was not obvious which was possibly due to the experimental set up in which a single dose of pH regulator was added at the beginning of the bioprocess, whereas continuous addition of KOH to maintain the pH at 3.5 was performed in this study.

The effect of K^+ on yeast viability has been studied previously by Kudo et al. [35], who stated that K^+ deficiency can either affect the ethanol bioprocess capacity or reduce ethanol bioprocess capacity by decreasing cell viability. Kahm et al. [36] showed that the control of intracellular K^+ concentrations was essential for the viability of yeast cells, and that K^+ is involved in many physiological functions, such as the maintenance of cell potential, protein synthesis and enzyme activation [37]. In contrast, Na^+ was toxic to yeast cells, as it inhibited their enzymatic functions and exerted osmotic stress [38].

3.6 Multiple regression analysis

Multiple regression analysis was performed to examine the effect of various parameters on the SL titre and **Equation (4)** shows the calculation of Lactonic SL:

$$Lactonic\ SL = 31.104 - (1.467 \times Glucose\ concentration) + (5.229 \times BWO\ concentration) + (16.549 \times pH\ regulation) - (2.779 \times Inoculum\ concentration) \quad \textbf{Equation (4)}$$

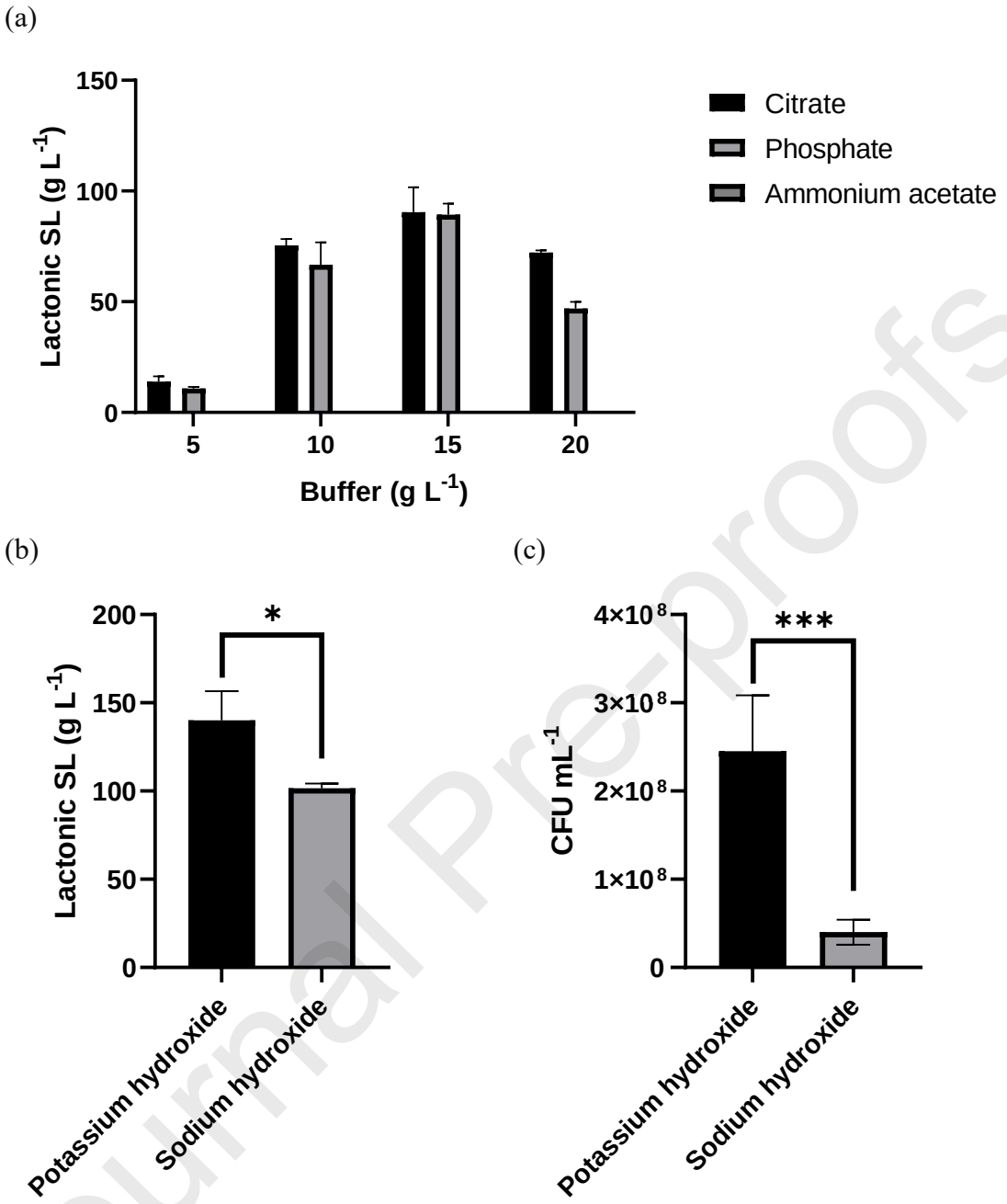


Figure 6. The effect of various buffers and bases as pH regulators at pH 3.5 on cell growth and lactonic sophorolipid (SL) production by *Starmerella bombicola* in a shake-flask experiment. **(a)** pH buffers were used to control the pH of the bioprocess broth. **(b)** NaOH and KOH were used to adjust the pH of the bioprocess broth every 24 h. **(c)** Colony-forming units (CFUs) obtained after 10 days of shake-flask bioprocess with NaOH or KOH. ***$p < 0.001$ relative to the respective groups.

The positive value in the non-standardised coefficients implies that the indicated descriptor contributed positively to the Lactonic SL titer (i.e. BWO and pH have the regression coefficients of 5.229 and 16.549), whereas negative values in glucose concentration and inoculum percentage (i.e. -1.467 and -2.779) lead to reduction of Lactonic SL titer, respectively.

As shown in Table S1, the *R*-value of 0.857 and *F*-ratio of $F(4, 25) = 17.245$, $p < 0.0005$, indicate that the regression model fits the data well. The *t*-value indicates that the pH regulation and BWO concentration have a greater effect on SL production ($p < 0.0005$) than initial glucose concentration and inoculum concentration.
Furthermore, the individual linear regression analysis performed (Table S2) have also shown that the inoculum, glucose and BWO proportion and potassium hydroxide exerted 1%, 2%, 48% and 45% of influence respectively on the SL production.

3.7 Bench-top bioprocess using the optimised condition

The optimised conditions were tested to identify the improvement in the lactonic SL titre at bioreactor scale. A bioprocess was performed using the optimal conditions (2.5 g L^{-1} yeast extract, 60 g L^{-1} BWO, 100 g L^{-1} glucose, 2% v v^{-1} inoculum and continuous pH control by KOH at 3.5 starting from 24 h), as shown in Figure S6a. Interestingly, after 144 h of incubation the optimised conditions resulted in the titre of lactonic SLs (29.6 ± 0.1 g L^{-1}) being lower than that under no pH control (67.8 ± 11.5 g L^{-1}), although the CDW was greater (23.1 ± 4.6 g L^{-1}) than that under no pH control (11.6 ± 1.6 g L^{-1}). This reduction in the production of lactonic SLs was closely related to the addition of KOH, as proven by the control experiment (Figure S5b), in which relatively higher concentrations of lactonic SLs were produced (67.8 ± 11.5 g L^{-1}) with no pH adjustment. It is also notable that the residual oil and residual glucose concentrations at 144 h were 8.6 ± 0.1 g L^{-1} and 63.3 ± 1.0 g L^{-1}, respectively, indicating that a BWO concentration of 60 g L^{-1} might not be enough to support SL biosynthesis using KOH as alkali for pH adjustment.
To further improve the lactonic SL production, bioprocesses were performed using BWO concentrations of 80 g L^{-1} and 100 g L^{-1}. As shown in Figure 7, an increase in BWO to 80 g L^{-1} and 100 g L^{-1} resulted in lactonic SL concentrations of 59.7 ± 0.8 g L^{-1} and 96.4 ± 9.1 g L^{-1}, respectively, and increased the overall glucose consumption from 40.5 g L^{-1} to 77 g L^{-1} (Figure S7 b and c). In addition, the residual glucose concentrations at 144 h reduced to 63.3 ± 1.0 g L^{-1} and 23.0 ±7.8 g L^{-1}, respectively, and the residual BWO concentrations were 14.1 ± 1.7 g L^{-1} and 17.0 ± 4.0 g L^{-1}, respectively.

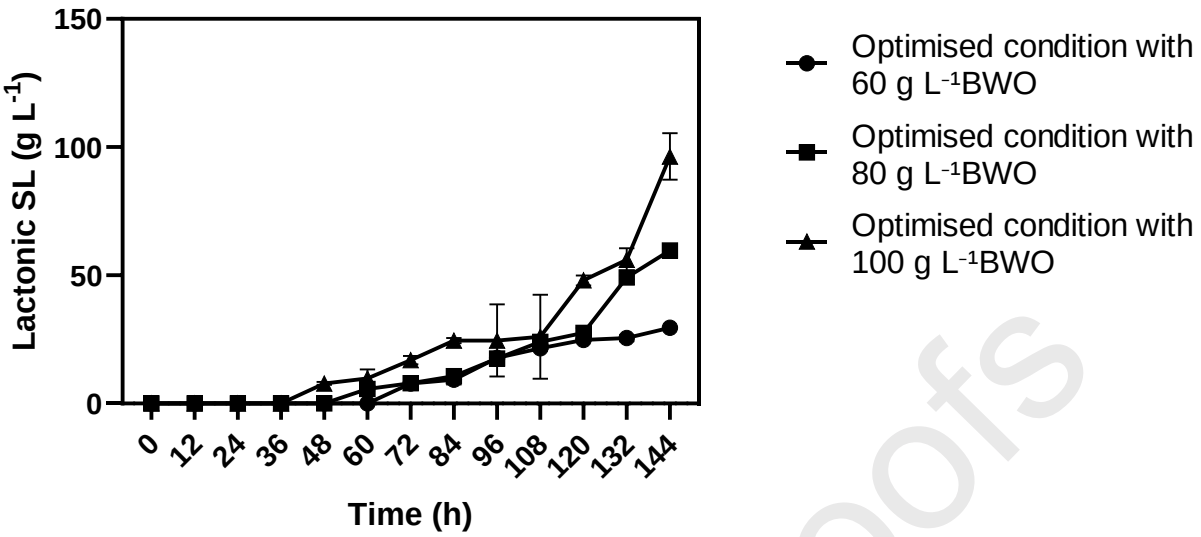


Figure 7. The influence of initial bakery waste oil (BWO) concentration on lactonic sophorolipid (SL) production.

After the fine-tuning of the experimental conditions, an optimised cultivation medium was identified, as comprising 2.5 g L^{-1} yeast extract, 100 g L^{-1} BWO, 100 g L^{-1} glucose and 2% (v v^{-1}) inoculum, with KOH used to adjust the pH to 3.5 from 24 h of cultivation. These optimised conditions resulted in a lactonic SL titre of 96.4 ± 9.1 g L^{-1}, which corresponds to a volumetric productivity and specific productivity of 0.446 g L^{-1} h^{-1} and 0.027 g g CDW^{-1} h^{-1}, respectively.

Table 3 lists the reported titres of SLs produced using different secondary hydrophobic feedstocks. From the tabulated data, it is clear that the SL titres vary with the type of hydrophobic carbon source and that the SL titres obtained in this study are comparable to those that have been previously reported from similar hydrophobic carbon sources (i.e., waste cooking oil, waste frying oil and restaurant waste oil, with respective volumetric productivities of 0.045, 0.119 and 0.141 g L^{-1} h^{-1}). The improved lactonic SL production in the current study (the highest SL titre of 96.4 ± 9.1 g L^{-1} and a volumetric productivity of 0.446 g L^{-1} h^{-1}) could be due to the optimisation of the experimental conditions and the pronounced effect of the KOH.

Table 3. Comparison of sophorolipid production with *Starmerella bombicola* using various secondary hydrophobic feedstocks.

Substrate	Mode	SL titre ($g\ L^{-1}$)	Volumetric productivity ($g\ L^{-1}\ h^{-1}$)	Specific productivity ($g\ g\ CDW^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	Reference
Glucose and bakery waste oil	Batch	96.4 [a]	0.446	0.027	0.48	This study
Glucose and waste cooking oil	Batch	24.5 [a]	0.045	0.015	0.31	[39]
Glucose and sunflower acid oil	Batch	51.5 [a]	0.210	0.015	0.21	[26]
Glucose and waste frying oil	Batch	50 [a]	0.119	0.042	0.35	[40]
Glucose and restaurant waste oil	Batch	34 [b]	0.141	-	0.24	[41]
Glucose and tallow fatty acid	Batch	90 [b]	-	0.027	0.64	[42]

[a] The reported SL titre contained the lactonic sophorolipid only.

[b] The reported SL titre represented the total sophorolipids (i.e. acidic and lactonic sophorolipids).

4. Conclusions

BWO was found to be a valuable hydrophobic carbon source for the production of lactonic SLs by *S. bombicola*. A bioprocess using BWO resulted in a lactonic SL concentration of 56.7 ± 10.5 g L^{-1}, which indicates that it is an ideal hydrophobic feedstock for use in SLs production. With further optimisation using KOH as a pH regulator, the titre of lactonic SLs was increased to 96.4 ± 9.1 g L^{-1}, corresponding to a volumetric productivity and specific productivity of 0.446 g L^{-1} h^{-1} and 0.027 g g CDW^{-1} h^{-1}, respectively [ab]. Additional benefit of KOH has been demonstrated in this study, when NaOH was replaced with KOH, higher biomass production (24.5 ± 0.5 g L^{-1}) was observed ($p < 0.05$) which was possibly ascribed by modulation of intracellular potassium balance. The results from this study indicate that BWO is feasible for use in the industrial production of SLs and furthermore, KOH can be used to enhance SLs production by maintaining high biomass concentration.

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Competing interests
The authors declare that they have no competing interests.

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Highlights

Sophorolipid (SL) biosynthesis from bakery waste oil (BWO) was optimized

Enhancement of lactonic SL production via pH control with potassium hydroxide (KOH)

KOH and BWO concentration had the greatest effect on SL production

The highest SL titre was 96.4 ± 9.1 g L^{-1} using BWO-based cultivation medium

Figure S1. Bakery waste oil obtained from Kee Wah Bakery Limited in Tai Po Industrial Estate, Hong Kong.

Figure S2. The HPLC chromatogram for Lactonic SL. (a) The 40 g L^{-1} Lactonic SL standard and (b) the SL bioprocess with 2% v v^{-1} Inoculum, 2.5 g L^{-1} YE, 100 g L^{-1} glucose, 100 g L^{-1} WCO and pH adjusted to 3.5 by KOH at the 144th hour, the sample was diluted fourfold before analysis.

Figure S3. Comparison of batch SL bioprocess using WCO with different amount of inoculum. (a) 2% (v v^{-1}), (b) 4% (v v^{-1}) and (c) 6% (v v^{-1}).

Figure S4. Comparison of batch SL bioprocess using BWO in different concentration. (a) 20 g L^{-1}, (b) 40 g L^{-1} (c) 60 g L^{-1}, (d) 80 g L^{-1} and (e) 100 g L^{-1}.

Figure S5. Comparison of batch SL bioprocess using 6%(w/v) WCO with different glucose percentage. (a) 5% (w v^{-1}) glucose, (b) 10% (w v^{-1}) glucose and (c) 15% (w v^{-1}) glucose. (d) indicate the SL production in different time interval.

Figure S6. (a) NaOH and (b) KOH to maintain the pH of the bioprocess broth at 3.5.

Figure S7. Batch bioprocess of the optimized condition and fine adjustment of initial BWO concentration in bench-top bioreactor. (a) Bioprocess of optimized conditions with 60 g L^{-1} of BWO. (b) Fine adjustment of BWO to 80 g L^{-1} and (c) Fine adjustment of BWO to 100 g L^{-1}.

Figure S1.

Figure S2.

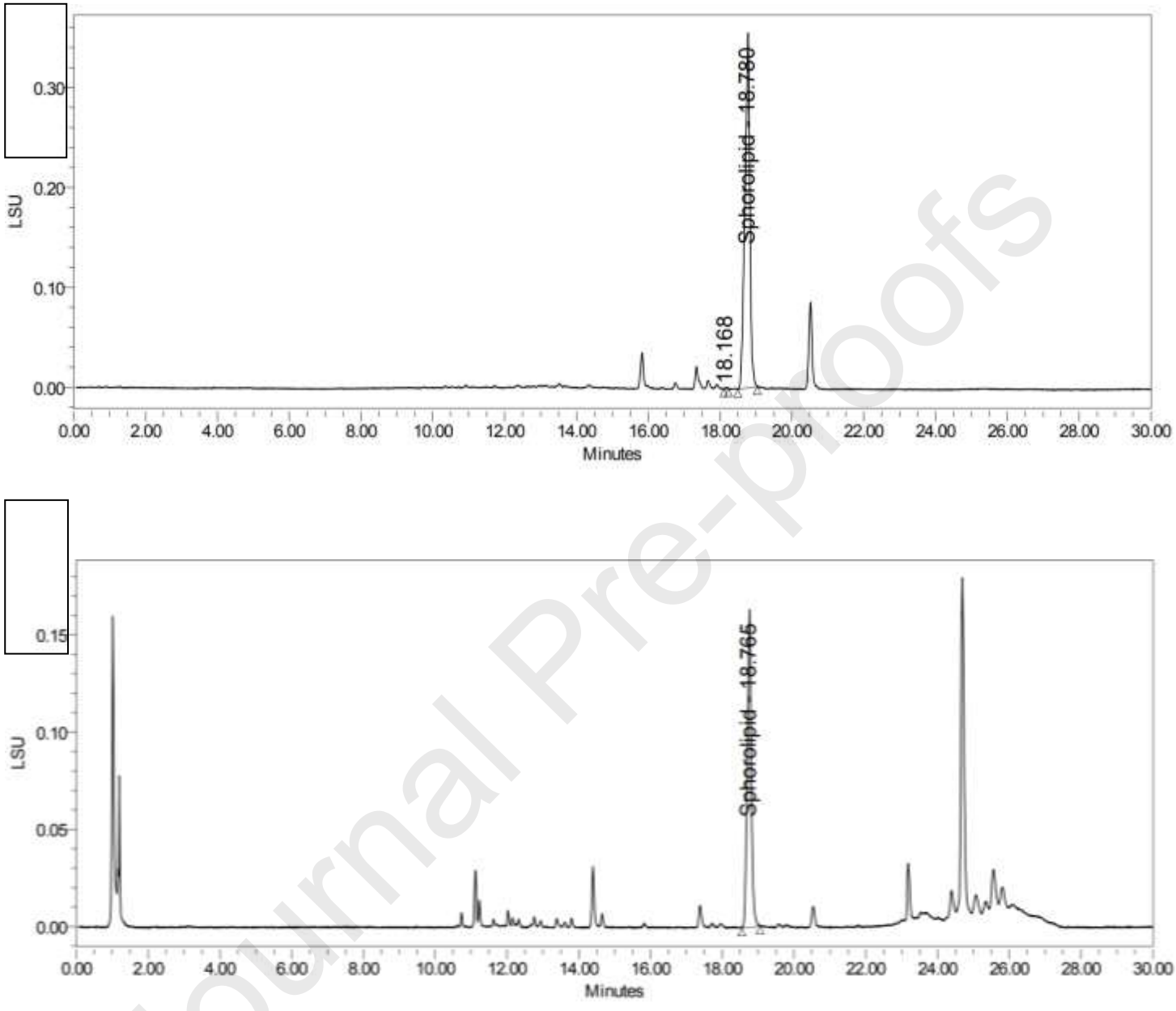
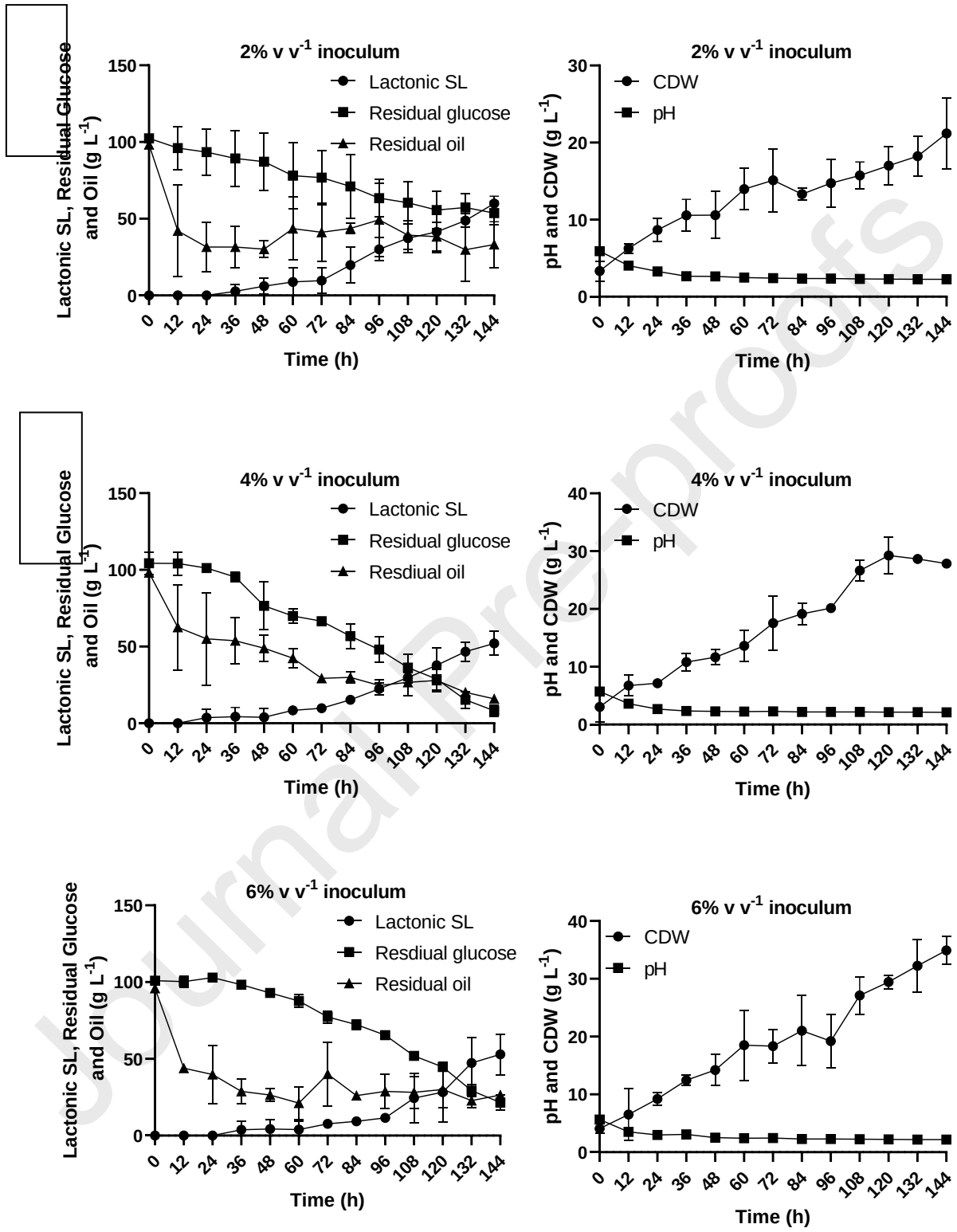


Figure S3.



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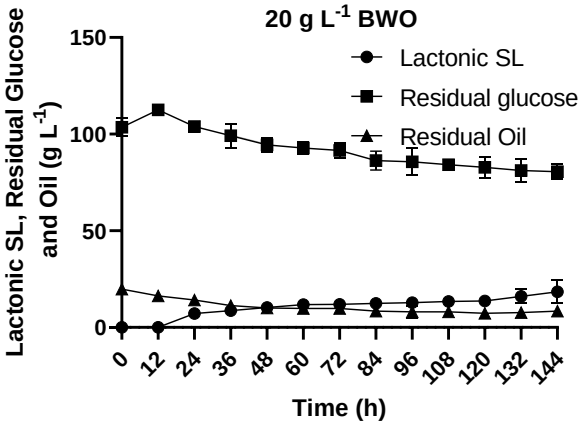

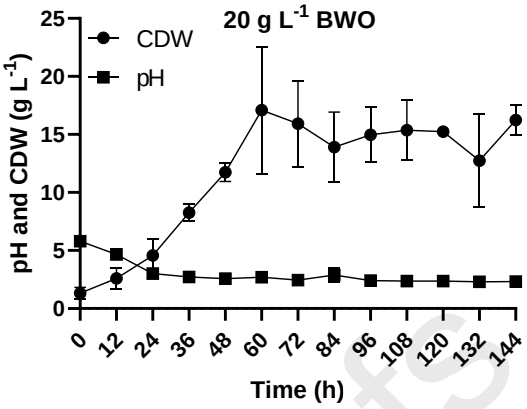

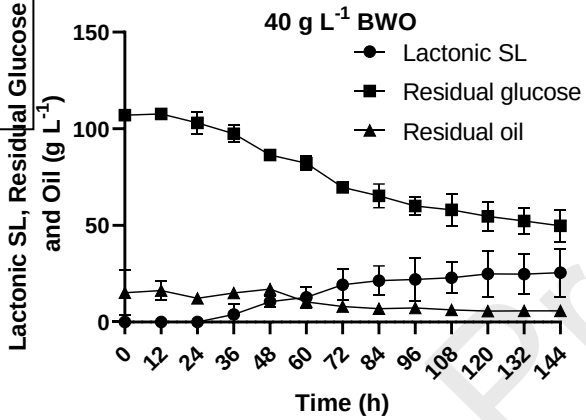

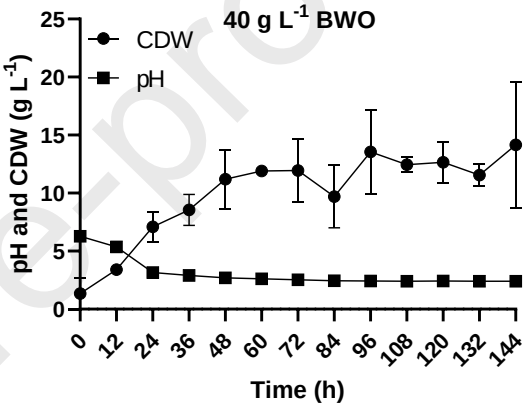

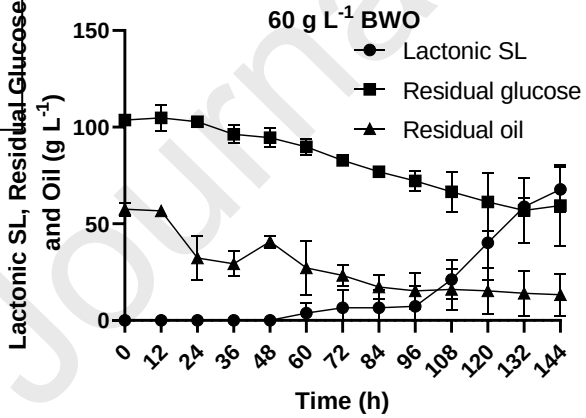

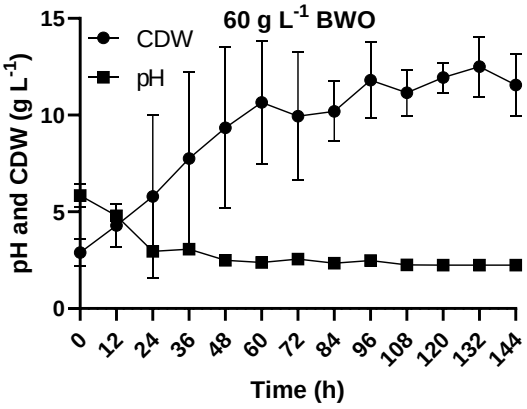

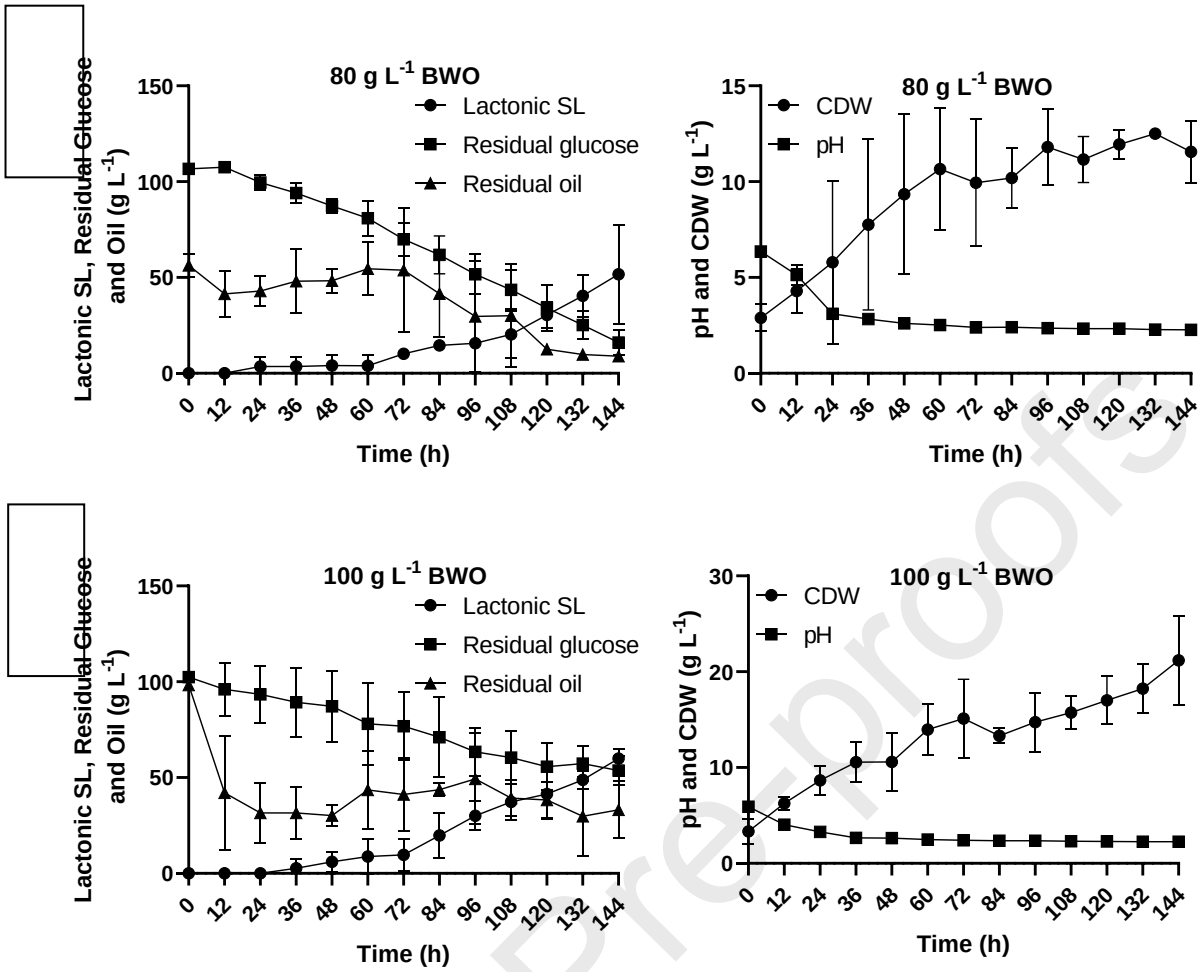

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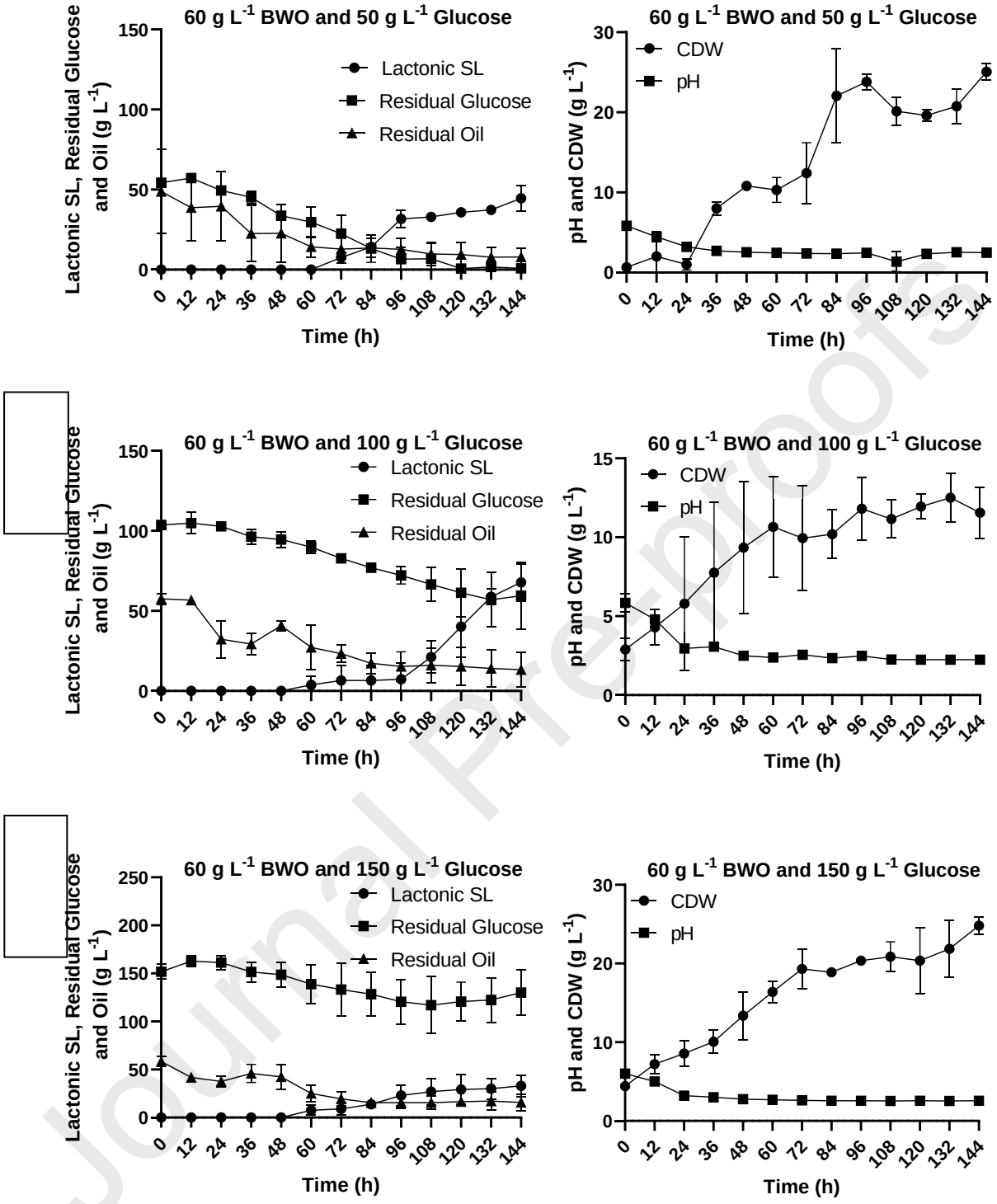

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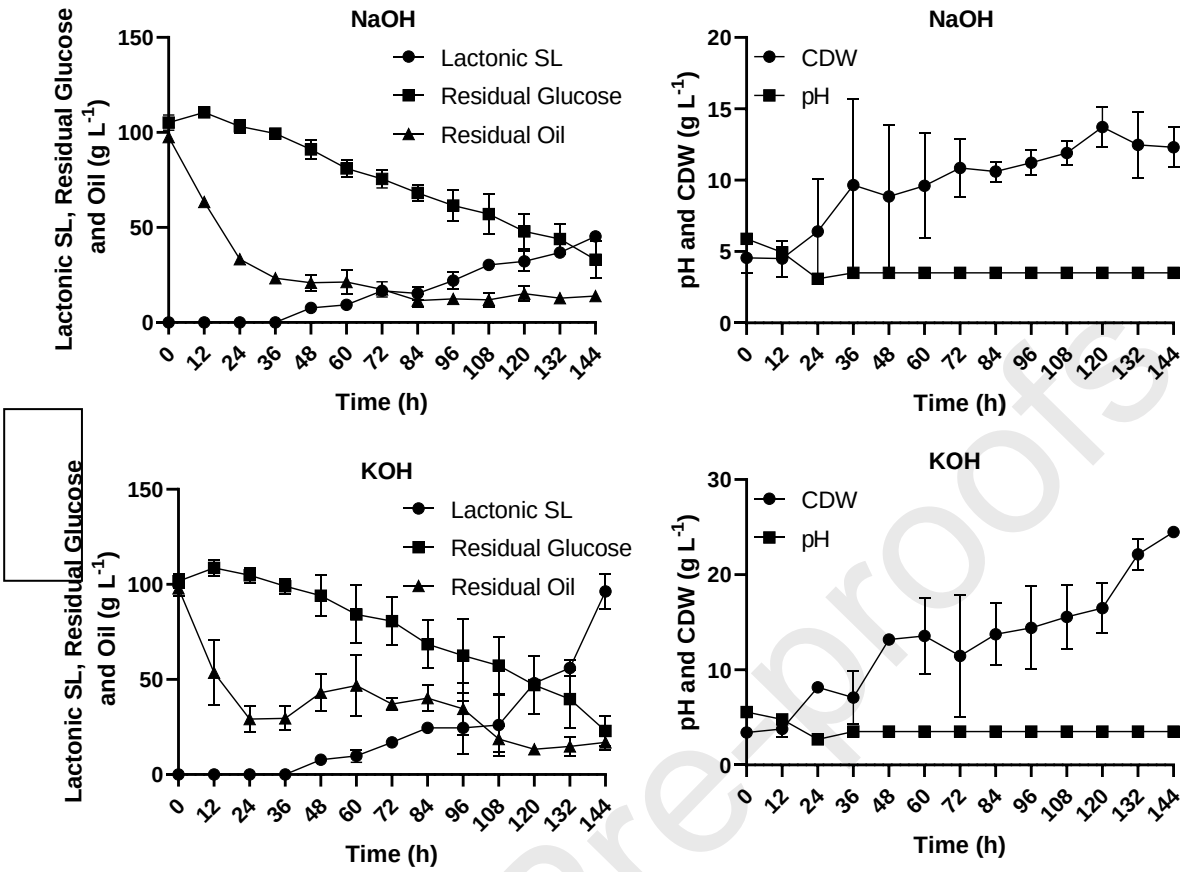

Figure S7.

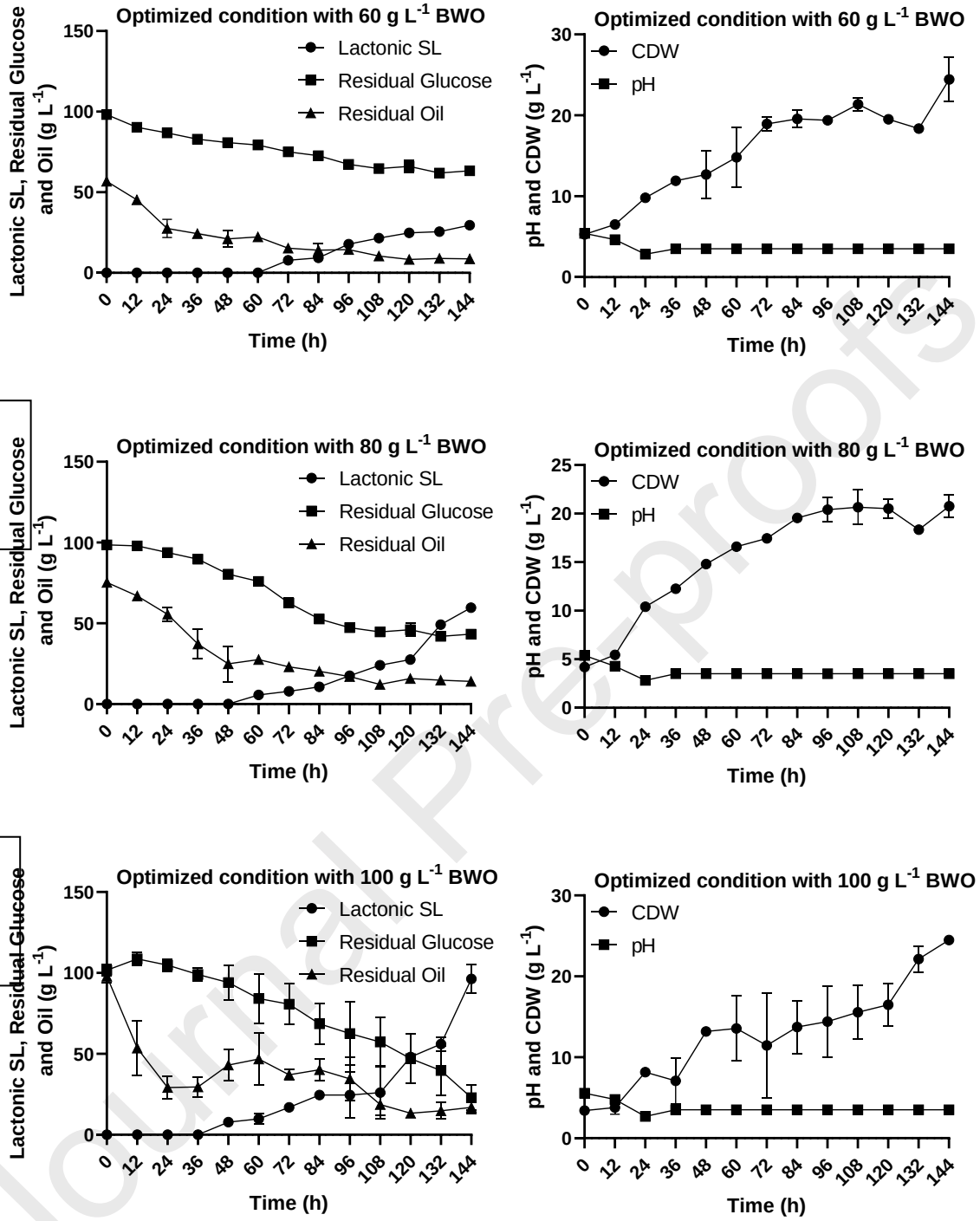

Table S1. Multiple regression analysis of the lactonic sophorolipid (SL) titre.

Table S2. Linear regression analysis of the lactonic sophorolipid (SL) titre. (a) Glucose. (b) Inoculum. (c) pH and (d) BWO.

Table S1.

Model Summary

Model	R	R^2	Adjusted R^2	Std. Error of the Estimate
1	0.857[a]	0.734	0.691	12.91122

a. Predictors: (Constant), Inoculum, glucose, pH, BWO

Analysis of variance[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	11498.691	4	2874.673	17.245	0.000[a]
	Residual	4167.491	25	166.700		
	Total	15666.182	29			

a. Predictors: (Constant), Inoculum, glucose, pH, BWO

b. Dependent variable: Lactonic SL

Coefficients[a]

		Non-Standardised Coefficients		Standardised Coefficients		
	Model	B	Std. Error	Beta	*t*	Sig.
1	(Constant)	31.104	12.665		2.456	0.021
	Glucose	-1.467	1.054	-0.144	-1.391	0.176
	BWO	5.229	1.135	0.621	4.606	0.000
	pH	16.597	4.549	0.436	3.648	0.001
	Inoculum	-2.779	2.286	-0.156	-1.215	0.236

a. Dependent variable: Lactonic SL

BWO = bakery waste oil

Table S2.

Model Summary

Model	R	R^2	Adjusted R^2	Std. Error of the Estimate
1	0.144[a]	0.021	-0.014	23.40903

a. Predictors: (Constant), Glucose

Analysis of variance[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	322.667	1	322.667	0.599	0.449[a]

	Residual	15343.515	28	547.983		
	Total	15666.182	29			

a. Predictors: (Constant), glucose

b. Dependent variable: Lactonic SL

Coefficients[a]

		Non-Standardised Coefficients		Standardised Coefficients		
	Model	B	Std. Error	Beta	*t*	Sig.
1	(Constant)	31.104	12.665		2.456	0.021
	Glucose	-1.467	1.054	-0.144	-1.391	0.176

a. Dependent variable: Lactonic SL

Model Summary

Model	*R*	R^2	Adjusted R^2	Std. Error of the Estimate
1	0.76[a]	0.006	-0.03	23.58470

a. Predictors: (Constant), inoculum

Analysis of variance[b]

	Model	Sum of Squares	df	Mean Square	F	Sig.
1	Regression	91.512	1	91.512	0.165	0.688[a]
	Residual	15574.669	28	556.238		
	Total	15666.182	29			

a. Predictors: (Constant), Inoculum

b. Dependent variable: Lactonic SL

Coefficients[a]

		Non-Standardised Coefficients		Standardised Coefficients		
	Model	B	Std. Error	Beta	*t*	Sig.
1	(Constant)	46.637	9.745		4.786	0.000
	Inoculum	1.364	3.362	0.076	0.406	0.688

a. Dependent variable: Lactonic SL

Model Summary

Model	*R*	R^2	Adjusted R^2	Std. Error of the Estimate
1	0.674[a]	0.454	0.434	17.48113

a. Predictors: (Constant), pH

Analysis of variance[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	7109.668	1	7109.668	23.265	0.000[a]
	Residual	8556.514	28	305.590		
	Total	15666.182	29			

a. Predictors: (Constant), pH

b. Dependent variable: lactonic SL

Coefficients[a]

Model		Non-Standardised Coefficients		Standardised Coefficients		
		B	Std. Error	Beta	*t*	Sig.
1	(Constant)	45.052	3.364		13.391	0.000
	pH	25.657	5.319	0.674	4.823	0.000

a. Dependent variable: Lactonic SL

Model Summary

Model	*R*	R^2	Adjusted R^2	Std. Error of the Estimate

1	0.695[a]	0.484	0.465	16.99760

a. Predictors: (Constant), BWO

Analysis of variance[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	7576.465	1	7576.465	26.224	0.000[a]
	Residual	8089.716	28	288.918		
	Total	15666.182	29			

a. Predictors: (Constant), BWO

b. Dependent variable: lactonic SL

Coefficients[a]

		Non-Standardised Coefficients		Standardised Coefficients		
	Model	B	Std. Error	Beta	*t*	Sig.
1	(Constant)	8.007	8.801		0.910	0.371
	BWO	5.858	1.144	0.695	5.121	0.000

a. Dependent variable: Lactonic SL
BWO = bakery waste oil

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: