Scale-up of two-step acid-catalysed glycerol pretreatment for production of oleaginous yeast biomass from sugarcane bagasse by *Rhodosporidium toruloides*

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Abstract

Two-step dilute acid and acid-catalysed glycerol pretreatment was developed to maximise sugar yield from sugarcane bagasse. At the laboratory scale, dilute acid pretreatment at 130 ºC followed by acid-catalysed glycerol pretreatment at 170 ºC led to a total sugar (C5+C6) yield of 82%, 31% higher than that from one-step acid-catalysed glycerol pretreatment. At the pilot scale, the two-step dilute acid and acid-catalysed glycerol pretreatment led to a maximum sugar yield of 74%, 13% higher than that from one-step pretreatment with 52% reduction in glycerol usage. The enzymatic hydrolysate containing glucose and residual glycerol were used to produce microbial oils by a *Rhodosporidium toruloides* strain. A fed-batch cultivation strategy led to the production of 44.8 g/L cell mass, including 26.6 g/L oil, 8.6 g/L protein and 12.7 mg/L carotenoid. The cell mass and oil yields were 19% higher than those from batch cultivation as feedstock inhibition and catabolite repression were alleviated.

Keywords:

Glycerol; pretreatment; *Rhodosporidium toruloides*; lipid; carotenoid

1. Introduction

Environmental problems and the world's growing population have drawn attention to the development of microbial oil-focused lignocellulosic biorefineries for food, feed, and fuel production (Chandel et al., 2018; Jin et al., 2015). Similar to cellulosic ethanol-focused biorefineries, a microbial oil-focused biorefinery generally consists of a pretreatment step to deconstruct the lignocellulose structure, an enzymatic hydrolysis step to convert lignocellulosic polysaccharides into monomeric sugars and a cultivation step to convert monomeric sugars into microbial oils (Fei et al., 2016; Xue et al., 2015). Despite long-standing research, production of microbial oils from lignocellulosic biomass has not yet been implemented at industrial scale due to the lack of economically viable pretreatment strategies to maximise the yield of fermentable sugars, and highly efficient strains to achieve high oil yield and productivity (Fei et al., 2016; Nagappan & Nakkeeran, 2020; Sitepu et al., 2014; Tian et al., 2018).

In terms of pretreatment, dilute acid (DA), liquid hot water (LHW) and steam explosion (SE) are the most commonly studied pretreatment technologies for microbial oil production (Hsu et al., 2010; Jin et al., 2015; Xiaowei & Hongzhang, 2012; Yu et al., 2020). However, these pretreatments generally lead to moderate cellulose digestibilities (60-80%) and total sugar (C5+C6) yields (50-70%), as well as low microbial oil yields (40-60 kg/tonne of feedstock) (Hsu et al., 2010; Xiaowei & Hongzhang, 2012). These pretreatments also release high levels of sugar- and lignin-degradation products which inhibit microbial cultivation (Chen et al., 2009). For example, previous studies reported that 0.5 g/L furfural derived from dehydration of C5 sugars under acidic conditions completely inhibited the growth of *Lipomyces starkeyi* 2.1608*, Rhodotorula. glutinis* 2.704*,* and *Rhodosporidium toruloides* 2.1389 (Chen et al., 2009). In addition, the growth of *Trichosporon cutaneum* ACCC 20271 was reduced by 50%

in the presence of 0.5 g/L furfural but *T. dermatis* 32903 was able to withstand furfural up to 2 g/L (Yu et al., 2020). Therefore, effective pretreatment technologies to maximise the sugars yield with minimum inhibitors from sugars and lignin are still needed for the economic development of microbial oil-focused biorefineries.

Glycerol is green, renewable, abundant and low-cost, and millions of tonnes of glycerol are produced by the biodiesel industry every year (Garlapati et al., 2016; Okoye et al., 2017). Glycerol can be used as a carbon source for biosynthesis of many bioproducts, such as 1,3-proanediol, hydrogen, microbial oils, citric acid and biopolymers (Garlapati et al., 2016). Glycerol has also been used for pretreatment of lignocellulosic biomass (Pascal et al., 2019; Tang et al., 2019; Zhang et al., 2016). Acid-catalysed glycerol pretreatment is generally conducted at lower temperature (130 to 170 °C) and shorter time (15 - 30 min) compared to catalyst-free and alkaline-catalysed glycerol pretreatment (190 - 220 °C, 15 - 80 min) (Zhang et al., 2016). AG pretreatment of sugarcane bagasse has been studied at both the laboratory and pilot scales and led to high cellulose digestibilities of more than 90% and low inhibitor yields (Zhang et al., 2013; Zhang et al., 2015). Furthermore, it has also been demonstrated that residual glycerol of up to 5% does not affect enzymatic hydrolysis, and residual glycerol can be co-fermented with cellulosic sugars for microbial oil production (Hassanpour et al., 2019; Zhang et al., 2015). These features could significantly reduce the downstream cost associated with biomass wash and solvent recovery.

Despite the progress of AG pretreatment, it is still challenging to develop microbial oil-focused lignocellulosic biorefinery based on this pretreatment. During AG pretreatment, hemicellulose is hydrolysed in the glycerol-rich pretreatment hydrolysate. Separation and utilisation of hemicellulose sugars for value-adding as well as recovery of glycerol for recycling are the main technical and economic barriers associated with AG pretreatment. The

AG pretreatment leads to high enzymatic cellulose digestibility; however, without recovery of hemicellulose sugars from glycerol pretreatment hydrolysate, the total sugars (C5+C6) yield is moderate. Furthermore, enzymatic hydrolysis of AG pretreated biomass contains both glucose and residual glycerol (due to incomplete biomass wash). The presence of multi-carbon sources can cause catabolite repression and limit microbial oil production though co-utilisation of glycerol and cellulosic sugars for microbial oil production has been reported (Bommareddy et al., 2017).

It was expected that (1) the application of a two-step dilute acid (DA) and AG pretreatment strategy could improve the total sugar (C5+C6) yield, (2) the application of an oleaginous strain capable to co-utilise glucose and glycerol could alleviate catabolite repression for producing microbial oils, and (3) the selection of suitable cultivation strategy could also lead to the improvement of microbial oil production. Therefore, in this study a two-step pretreatment technology was investigated to enhance fermentable sugar yield for microbial oil production. Firstly, a two-step (DA-AG) pretreatment was developed at the laboratory scale and further optimised at the pilot scale with the aim of recovering the majority of hemicellulose sugar in the first step DA pretreatment, followed by the second step AG pretreatment to improve cellulose digestibility. Furthermore, the fermentable sugars derived from the pilot scale DA-AG pretreatment were used for producing microbial oils by *Rhodosporidium toruloides* RP15. This oleaginous yeast strain was recently discovered and showed capability for co-utilisation of glucose and glycerol in shake flasks (Gebbie et al., 2020; Hassanpour et al., 2019). In this study, the use of this strain for co-utilisation of cellulosic glucose and residual glycerol was scaled up to a stirred tank bioreactor and different cultivation strategies were studied to improve microbial oil production. The present study provided useful information towards economic development of microbial oil-focused lignocellulosic biorefineries at industrial scale.

2. Materials and methods

2.1 Materials

Sugarcane bagasse used in this study were sourced from two sugar mills, depending on the availability to the trials at different scales. For laboratory scale pretreatment, sugarcane bagasse was collected from the Rocky Point Sugar Mill (Woongoolba, Australia), a sugar mill close to the Queensland University of Technology (QUT). It was completely washed with water, oven-dried at 45 °C to a constant weight and milled through a 2.0 mm sieve in a Retsch SM100 hammer mill (Retsch GmBH, Germany). The milled bagasse powders were sifted to collect the samples with particle sizes of 212 - 500 µm. Pilot scale trials were conducted at the QUT's Mackay Renewable Biocommodities Pilot Plant (MRBPP) located at the Racecourse Sugar Mill (Mackay, Australia), which is about 900 km away from QUT campus in Brisbane. Therefore, for the pilot scale trials, sugarcane bagasse with ~45% moisture was collected from the Racecourse Sugar Mill and was used directly for the pretreatments without further processing.

Sulfuric acid (98%) and pure glycerol were purchased from Merck Pty Ltd, Australia. Accellerase™ 1500 from Danisco (Danisco US Inc., USA) was purchased through Enzymes Solutions Pty Ltd, Australia. The protein concentration and the filter paper activity of Accellerase™ 1500 were 80 mg/mL (determined by the Qubit Protein Assay Kit) and 40 FPU/mL, respectively (Zhang et al., 2013). The oleaginous *R. toruloides* RP15 strain was previously isolated from a local sugarcane bagasse stockpile (Gebbie et al., 2020; Hassanpour et al., 2019). The strain was stored in 30% glycerol stock solution at -80 °C and streaked onto YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose and 20 g/L agar) three days before cultivation trials. All the chemicals used for cultivation were reagent grade or above, and purchased from Sigma, Australia.

2.2 Pretreatment

2.2.1 Laboratory scale pretreatment

Laboratory scale pretreatment conditions are shown in Table 1. Sugarcane bagasse was pretreated in one-step and two-step processes with a liquid:solid (L:S) weight ratio of 5:1. The two-step pretreatment consisted of a first step DA pretreatment at 130 °C and the second step AG pretreatment at temperatures of 130 °C, 150 °C and 170 °C (i.e. DA130-AG130, DA130-AG150 and DA130-AG170). The two-step pretreatments also included a second step DA at 170 °C (i.e. DA130-DA170). In addition, two separate one-step DA and one-step AG pretreatments were conducted at 170 °C for comparison.

In detail, for the first step DA130, 25 g dry sugarcane bagasse was mixed with 125 g acid solution containing 2.4 wt.% H_2SO_4 (based on fibre or 0.48% in liquid) in a 1.0 L lid-on bottle and incubated at 130 °C for 15 min in an autoclave (Tomy SX-500E, Australia). After pretreatment, 150 mL hot water was added to the mixture, mixed thoroughly, and filtered to separate pretreated biomass and hydrolysate. The filtrate was collected and stored at –20 °C for sugar and sugar degradation product analysis. The pretreated biomass was further washed and dried to calculate the recovery of pretreated biomass, compositional analysis, and used for the second step pretreatment. For the second step pretreatment, 9 g dry pretreated bagasse from the first step DA130 was mixed thoroughly with 45 g acidified aqueous glycerol solution containing 2.4 wt.% H_2SO_4 (on fibre or 0.48% in liquid) and 80 wt.% glycerol. The mixture was loaded into the three 316-stainless steel tubular reactors with an internal diameter of 15 mm, a length of 200 mm, and a wall thickness of 1.2 mm, and incubated at 130 °C, for 15 min in a fluidised sand-bath (i.e. DA130-AG130). The same was performed but with the second steps carried out at 150 °C, and 170 °C, to generate the DA130-AG150 and DA130-AG170 pretreatment conditions. After pretreatment, the reactor contents were

completely discharged into a beaker, mixed with 90 g of hot glycerol solution (60 wt.% and ~80 °C, to simulate the pilot scale liquid/solid separation process) and filtered to separate pretreated biomass from the hydrolysate. The pretreated biomass was further washed with 2 L water (4× 0.5 L/wash) to remove any residual solvent and loosely attached lignin and carbohydrates. The sample collections and analyses were similar to the previous step. For the two-step DA130-DA170 pretreatment, all the procedures were same except water instead of glycerol was used in the second step pretreatment. For one-step DA170 and one-step AG170 pretreatments (15 min), the pretreatments were started directly with raw bagasse at the same acid loading of 2.4 wt.% (on fibre) and a L:S weight ratio of 5:1 using the tubular reactors. All the pretreatments were conducted at least in triplicate (27 g initial biomass was used for each pretreatment condition in the second step to prepare sufficient biomass for analysis).

2.2.2 Pilot scale pretreatment

Pilot scale pretreatment conditions are also shown in Table 1. Sugarcane bagasse pretreatment was carried out in one-step and two-step processes using a horizontal reactor (Andritz, USA) at the MRBPP. Similar to the laboratory scale trials, two-step pretreatments consisted of a first step DA followed by second step AG. The first step DA pretreatment was conducted at 130 °C for 15 min with an initial L:S ratio of 3:1 (30 kg:10 kg) and 2.4 wt.% H_2SO_4 loading (on fibre). Following pretreatment, the liquid and solid phases were separated by *in-situ* press filtration at 80-90 ºC. The hydrolysate was collected for sugar analysis and the solid residue was washed twice with 20 kg water at 130 °C for 5 min. The wash liquids and solid were separated again by *in-situ* press filtration. A total of 10 batches of first step pretreatment were conducted and the solid residues were mixed thoroughly for the use in the second step AG pretreatment. Approximately a total of 172 kg solid residues (~58% moisture) were collected from the first step dilute acid pretreatment. The wash liquids were collected

for sugar analysis. Finally, pretreatment hydrolysate and wash liquids after the first step DA130 were mixed and concentrated to prepare seed cultures for growing *R. toruloides* RP15 cell mass.

For the pilot scale trials, solid residues after the first step pretreatment were used without drying in the second step pretreatment to mimic industrial operation. The second step pretreatments were conducted at 150 and 170 °C for 15 min. For each pretreatment, 17.6 kg solid residues (including 7.2 kg dry residue, corresponding to the 10 kg initial dry bagasse used in the first step DA pretreatment) from the first step pretreatment was used with the addition of 20 kg glycerol, 30 kg glycerol and 40 kg glycerol, corresponding to L:S ratios of 4.2:1, 5.6:1 and 7.0:1, respectively. For the second step pretreatment, an H_2SO_4 loading 2.4 wt.% was used based on 10 kg initial dry bagasse, corresponding an actual acid loading of 3.3 wt.% on dry biomass (while the concentration in liquid reduced from 0.79% at a L:S of 4.2:1 to 0.48% at a L:S of 7.0:1). The liquid and solids were collected by *in-situ* press filtration following pretreatment for future analysis and use. A benchmark one-step AG pretreatment conducted at 150 °C for 15 min was included as a control and this benchmark condition was based on the previous publication (Zhang et al., 2013). The initial L:S ratio was 5:1 (50 kg liquid:10 kg bagasse) and the liquid included ~42 kg glycerol and 2.4 wt.% H_2SO_4 on bagasse.

Following the AG pretreatments, a portion of solid residue (~2 kg) was collected and washed step-wise with 16 L water (4.0 L/wash×4 wash). The washed solid residue was collected for compositional analysis and enzymatic hydrolysis as well as calculating component recoveries and yields. The wash liquids were also collected and analysed to determine the residual glycerol content in the pretreated biomass.

2.3 Enzymatic hydrolysis of pretreated sugarcane bagasse for microbial oil production

Firstly, a series of enzymatic hydrolyses were conducted to evaluate the effect of pretreatment conditions on glucan digestibility. For laboratory scale trials, the enzymatic hydrolysis was performed in 20 mL scintillation vials with 5.0 g mixture of pretreated biomass, buffer and enzyme. For pilot scale trials, enzymatic hydrolysis was carried out in 250 mL shake flasks with 100.0 g reaction mixture. In both cases, the reaction mixture consisted of 3 wt.% glucan, 0.05 M citrate buffer (pH 4.8) and an enzyme dosage of 20 FPU/g glucan. Enzymatic hydrolysis trials were conducted at 50 °C and 150 rpm for 72 h in a shaking incubator. During hydrolysis, samples (~0.2 g for laboratory scale and ~1.5 g for pilot scale) were taken at different time intervals and were used for sugar analysis using high-performance liquid chromatography (HPLC). Enzymatic hydrolysis was conducted at least in duplicate.

Based on the total sugar yield, the two-step DA130-AG150 pretreatment at L:S ratio of 4.2:1 from the pilot scale was selected to prepare sugarcane bagasse media for microbial oil production. The xylose-rich hydrolysate from the first step of DA pretreatment was used as the seed culture medium to grow *R. toruloides* RP15. The incompletely washed pretreated bagasse (containing residual glycerol) from the second step AG150 was enzymatically hydrolysed by Accellerase 1500 to fermentable sugars for producing microbial oils. Enzymatic hydrolysis was conducted at pH 4.8, 50 °C and 150 rpm for 72 h in 500 mL shake flasks containing 200 g reaction mixture. The mixture contained 6% glucan (~12% biomass), 5% residual glycerol and 20 FPU/g glucan (~10 FPU/g solid) enzyme. Following hydrolysis, the enzymatic hydrolysate (containing ~5% glucose and 5% glycerol) was collected by vacuum filtration and concentrated up to 20% glucose and 20% glycerol for microbial oil production.

2.4 Production of oleaginous yeast cell mass by *R. toruloides* RP15

Seed cultures were firstly prepared prior to microbial oil production. To start the first seed culture, a loop of yeast cells from a single colony of *R. toruloides* RP15 was transferred from agar plate to 50 mL YPD broth (10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) in a 250 mL shake flask, incubated at 28 °C, 180 rpm, 72 h. For the second seed culture, 40 mL of the first seed culture of *R. toruloides* RP15 was inoculated into 200 mL xylose-rich hydrolysate from the two-step DA130-AG150 pretreatment. The hydrolysate contained 5.0 g/L glucose, 47.0 g/L xylose, 2.5 g/L galactose, 3.2 g/L arabinose and supplied with 10 g/L yeast extract and 10 g/L peptone. The second seed culture was incubated at 28 °C and 180 rpm in a shaking incubator. After 72 h cultivation, all the 200 mL second seed culture was collected, washed and centrifuged to collect the yeast cell mass.

Batch cultivation was performed with a 400 mL working volume while fed-batch cultivation started with a 400 mL working volume using the enzymatic hydrolysate derived from the second step AG pretreatment. All the cultivations were conducted in duplicate at 28 °C and pH 6.2 (by 2 M NaOH and 2 M H_2SO_4) for 96 h using Sartorius Biostat Q-plus stirred tank bioreactors. Aeration rates were fixed at 0.5 vvm and dissolved oxygen levels were controlled above 50% by adjusting the agitation speed in the range of 350 - 450 rpm. Batch cultivation started with enzymatic hydrolysate medium containing 60 g/L glucose and 60 g/L glycerol, and supplied with 4.0 g/L yeast extract, 4.0 g/L peptone, 7.0 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, 1.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 8 mg/L $FeCl_3.6\ H_2O$, 0.1 mg/L $ZnSO_4.7H_2O$, 0.1 mg/L $CuSO_4.5H_2O$, 0.1 mg/L $CoCl_2.H_2O$ and 0.1 mg/L $MnSO_4 \cdot 5H_2O$. The initial C:N ratio in batch cultivation was ~60:1 calculated based on the comparison of the total amounts of carbons of the two carbon sources with the total amounts of nitrogen of the two nitrogen sources. Washed and centrifuged yeast cell mass from the second seed culture was suspended in 400 mL enzymatic hydrolysate media to start the cultivation.

Fed-batch cultivation started with enzymatic hydrolysate medium having the same nutrient composition as that for batch cultivation but 15 g/L glucose and 15 g/L glycerol. Samples were withdrawn every few hours to determine the concentration of residual carbon sources. When the total carbon source concentration dropped below 5 g/L, ~30 g concentrated enzymatic hydrolysate containing 200 g/L glucose and 200 g/L glycerol was added to bring the total carbon source concentration back to ~30 g/L. The initial C:N ratio was ~15:1. Fed-batch cultivation was fed three times and a total of 120 g/L carbon sources (60 g/L glucose and 60 g/L glycerol) was used, equivalent to that used for the batch cultivation.

Both batch and fed-batch cultivations were terminated after 96 h when almost all the carbon sources were consumed.

2.5 Analyses

Analyses of untreated and pretreated sugarcane bagasse samples as well as and calculations of component recoveries and yields were referred to the previously reported methods (Zhang et al., 2013). For microbial oil production, residual sugars, microbial oil, carotenoid and fatty acid composition and yields were analysed using the methods published in a previous study (Hassanpour et al., 2019). The protein content was analysed using the Lowry method as described elsewhere (Hassanpour et al., 2015; Waterborg, 2009).

3. Results and discussion

3.1 Comparison of biomass composition and component recovery based on one-step and two-step pretreatments at laboratory and pilot scales

Table 2 shows biomass composition, component recovery and glucan digestibility of the solid residues at both laboratory and pilot scales. In terms of biomass composition, all pretreatments increased glucan and lignin contents compared to raw bagasse, due to significant removal of xylan and partial delignification. At the laboratory scale, one-step

AG170 and two-step DA130-AG170 pretreatments had slightly lower lignin content (~30%) compared to one-step DA170 and two-step DA130-DA170 pretreatments (~35%) because of the higher delignification capacity of glycerol compared to dilute acid processes (Zhang et al., 2013). Moreover, increasing pretreatment temperature in the second step of DA-AG pretreatments from 130 °C to 170 °C increased glucan content by ~5% and reduced xylan content by ~5% but the effect on lignin content was limited. At the pilot scale, increasing the pretreatment temperature in the second step AG pretreatment did not significantly affect the glucan and xylan contents but the lignin content increased by ~4% at the same L:S ratio. The increase in lignin content was due to the significant removal of glucan at 170 ºC as the glucan recoveries at 170 ºC were only 62%-65% compared to 85%-86% at 150 ºC. For the pilot scale pretreatment, increasing the L:S ratio from 4.2:1 to 7.0:1 also led to the removal of more biomass components, due to better mass and heat transfer.

In terms of component recovery, at both scales, increasing pretreatment temperature reduced the glucan and lignin recoveries. At the laboratory scale, increasing pretreatment temperature in the second step of DA-AG pretreatments from 130 °C to 170 °C reduced the glucan and lignin recoveries by ~5%. However, at the pilot scale, increasing pretreatment temperature from 150 °C to 170 °C reduced the glucan recoveries by 20-23% and lignin recoveries by 5-17%. At the pilot scale, increasing initial L:S ratio from 4.2:1 to 7.0 had a limited effect on glucan recoveries but reduced lignin recoveries by 5-12%. Furthermore, one-step AG150 led to higher glucan (88%), xylan (18.7%) and lignin (72%) recoveries compared to two-step DA130-AG150 pretreatments. This was due to the removal of some biomass components during the first step DA130 pretreatment.

3.2 Comparison of fermentable sugars production based on one-step and two-step AG pretreatments at laboratory and pilot scales

Table 2 shows the glucan digestibility of pretreated biomass from one-step and two-step pretreatments at both scales. As shown in Table 2, two-step DA130-AG150 pretreatment at the pilot scale led to higher glucan digestibilities (82%-86%) compared to that at the laboratory scale with the same temperature of 150 °C (78.0%), possibly due to the better mass and heat transfers. It has been previously observed that pretreatment at the pilot scale with reduced reaction time and increased biomass loading achieved comparable glucan digestibility to that at the laboratory scale (Zhang et al., 2013). However, in the present study, the difference might also be due to the higher acid loading on fibre at the pilot scale and the use of different sources of sugarcane bagasse. Decreasing the L:S ratio from 7:0 to 4.2:1 at the pilot scale resulted in a slight decrease in glucan digestion (82.4%) but reduced the glycerol usage by ~52% (20 kg glycerol for initial 10 kg dry bagasse) compared to one-step pretreatment (42 kg glycerol for initial 10 kg dry bagasse). At both scales, increasing the pretreatment temperature increased glucan digestibility and glucan was almost digested completely in the second step of the two-step DA130-AG170 pretreatments.

In terms of one-step or two-step pretreatments at the laboratory scale, glucan digestibility (~99%) was very similar for the one-step AG170 and two-step DA130-AG170 pretreatments. However, at the pilot scale, a one-step AG150 pretreatment led to a higher digestibility (92.5%) compared to two-step DA130-AG150 pretreatments (86.4%) despite the higher acid loading (3.33% on fibre) in the AG step, possibly due to lignin condensation during the first step DA130. Similarly, at laboratory scale, one-step DA170 pretreatment resulted in a higher glucan digestibility (~68%) than two-step DA130-DA170 pretreatment (64%), due to higher lignin condensation in the latter case. Both one-step and two-step DA pretreatments led to much lower glucan digestibilities (64%-68%) compared to one-step and two-step AG pretreatments (~99%) at the laboratory scale.

Fig. 1 shows glucose, xylose and total sugar yields from both enzymatic hydrolysis of pretreated solid residues and sugars from pretreatment hydrolysates, which can be used directly for microbial cultivation. For the AG170, AG150 and the second step of DA-AG pretreatments, sugars from pretreatment hydrolysates were not included as these sugars were in the glycerol-rich pretreatment solutions and could not be directly used. For the laboratory scale pretreatment (Fig. 1A), the highest glucose yield of ~91% was achieved with the two-step DA130-AG170 pretreatment, followed by 82.3% with one-step AG170 pretreatment. In contrast, the glucose yields based on one-step DA170 and two-step DA130-DA170 were only 69%. The xylose yield based on the DA130-AG170 pretreatment was 68%, only 5% lower than that based on DA130-DA170 pretreatment. In terms of total sugar yield, the highest yield of ~82% was from the two-step DA130-AG170, ~18% and ~15% higher than that with DA pretreatments (DA170 and DA130-DA170), respectively, and ~31% higher than that from the one-step AG170 pretreatment. The major sugar degradation products, furfural and HMF were also determined from the laboratory scale pretreatment hydrolysates. For one-step AG170 pretreatment, both furfural and HMF were not detected, possibly due to the formation of glyceryl xylosides and glyceryl glucosides as indicated in the previous study (Zhang et al., 2013). For DA170 and DA130-DA170, ~11% and ~14% of the lignocellulosic polysaccharides were converted to sugar degradation products, respectively.

For the pilot scale pretreatment (Fig 1B), the highest glucose yield of 84.9% was achieved from the one-step AG150 pretreatment. However, because hemicellulose sugars were dissolved in the glycerol-rich pretreatment solution and could not be directly used for microbial cultivation, the total sugar (C5+C6) yield was 61.4%. The highest total sugar yields of 74%-77% were achieved with two-step DA130-AG150 pretreatments, which were higher than those (61%-69%) based on one-step AG and other two-step DA-AG pretreatments. Despite the high glucan digestibility, the DA130-AG170 pretreatments did not lead to the

higher glucose yields than DA130-AG150 pretreatments due to the solubilisation of glucose into the pretreatment hydrolysates, which could not be directly used for microbial cultivation. Based on the results shown in Fig. 1B, the DA130-AG150 pretreatment at a L:S ratio of 4.2:1 was selected for further study, which produced ~13% more total sugars with ~52% less glycerol used as pointed out previously.

Laboratory scale two-step solvent pretreatments were previously reported using ethanol, dioxane and ionic liquids (An et al., 2017; Mesa et al., 2011; Panagiotopoulos et al., 2013; Toscan et al., 2019; Zhang et al., 2018; Zhu et al., 2015). These pretreatments generally used high L:S ratios (~10:1) and high catalyst loadings (5-10% based on fibre). For example, in one study, it was reported that liquid hot water (LHW) pretreatment of *Eucommia ulmoides* wood at 180 °C for 30 min followed by 10% HCl-catalysed ethanol pretreatment at the same conditions led to the yields of glucose, xylose and total sugar of ~89%, ~56% and 78%, respectively (Zhu et al., 2015). In another study, 10% H_2SO_4 pretreatment of sugarcane bagasse at 120 °C for 30 min followed by 5% NaOH-catalysed ethanol pretreatment resulted in the yields of glucose, xylose, and total sugar of 66%, 15.5% and 49.4%, respectively (Zhang et al., 2018).

Pilot scale two-step solvent pretreatments have not been previously reported. In the present study, the pilot scale two-step pretreatment with a higher biomass loading and a lower catalyst dosage achieved higher yields of glucose, xylose and total sugars than those reported at the laboratory scale (An et al., 2017; Mesa et al., 2011; Panagiotopoulos et al., 2013; Toscan et al., 2019; Zhang et al., 2018; Zhu et al., 2015). At the pilot scale, only a low glycerol solvent dosage of 2 kg/kg biomass was used. These results and comparison indicate that the two-step DA-AG pretreatment is an economically promising pretreatment technology for maximising the sugars yield with low glycerol usage.

3.3 Production of carotenoid-rich oleaginous cell mass

3.3.1 Batch cultivation

In this study, the novel *R. toruloides* RP15 was grown on enzymatic hydrolysate (with glucose: glycerol ratio 1:1) derived from the DA130-AG150 pretreatment under batch and fed-batch modes to assess microbial oil production from pretreated sugarcane bagasse.

Fig 2A-2C shows the kinetics of carbon consumption, the changes of metabolite contents in cell mass as well as the kinetics of cell mass and metabolite production under batch mode. As shown in Fig 2A, the strain co-consumed 92% of the total carbon sources (~57 g/L glucose and ~53 g/L glycerol) over 96 h and consumption of glucose was slightly faster than glycerol. Fig 2B shows the oil, protein and carotenoid contents based on cell dry weight. In the first 24 h of cultivation with all the nutrients available, cell mass rapidly increased with no or little increase in oil and carotenoid contents. The protein content first decreased and then increased to 39%, possibly due to the production of nucleic acids and/or accumulation of intracellular carbohydrates in the early phase of growth (Hassanpour et al., 2015). As cultivation progressed, the nitrogen source was consumed and the higher C:N ratio led to accumulation of more oil and carotenoid in cell mass (Yu et al., 2020).The oil and carotenoid contents reached 60% and 285 µg/g while protein content decreased to 21% at the end of the cultivation. The overall cell mass reached 37.5 g/L, including an oil concentration of 22.4 g/L, a protein concentration of 8.0 g/L and a total carotenoids concentration of 10.7 mg/L (Fig 2C). As a result, an oil yield of 0.20 g/g (g of oil per g of consumed carbon) and productivity of 0.23 g/L/h were achieved under the batch cultivation mode.

Simultaneous co-utilisation of glycerol and glucose is not common as generally microorganisms exhibit diauxic growth on dual carbon-sources (Bommareddy et al., 2017). Nevertheless, one previous study showed that *Yarrowia lipolytica* strains co-consumed

glucose and glycerol but glycerol was the preferred carbon source but with limited intracellular oil accumulation (Workman et al., 2013). Previously, it was observed that *R. toruloides* RP15 could co-utilise glucose and glycerol simultaneously, but glucose consumption was much faster than glycerol and the depletion of dual carbon sources consisting of ~30 g/L glucose and ~20 g/L glycerol took 14 days in a shake flask trial (Hassanpour et al., 2019). However, in the present trial, it was observed that consumption of glycerol was only slightly slower than glucose and it took only 4 days to consume ~90% of a total of ~120 g/L carbon sources. The different observations were likely attributed to the use of low C:N ratio (~60:1) and improved oxygen supply (>50% dissolved oxygen level by aeration and agitation) in the present study compared to the previous shake flask trial (C:N=140:1 and no aeration but agitation only) (Hassanpour et al., 2019). In addition, C:N ratio is the most important factor affecting oil production and a higher C:N ratio generally leads to the accumulation of more oils in cell mass (Hassanpour et al., 2019; Yu et al., 2020). However, the C:N ratio optimised at shake flask trials on a single carbon source may not be applicable to the trials with changed cultivation conditions (e.g., oxygen supply, the use of dual carbon sources, etc) as shown in the previous study (Hassanpour et al., 2019) and the present study.

3.3.2 Fed-batch cultivation

In order to reduce the possible growth inhibition from the high concentration of total carbon source and to improve oil yield and productivity, a fed-batch cultivation based on off-line analysis of carbon sources was developed. The fed-batch cultivation was started with a low initial carbon concentration of 30 g/L (15 g/L glucose and 15 g/L glycerol).

Fig 2D-2F shows the kinetics of carbon consumption, the changes of metabolite contents in cell mass as well as the kinetics of cell mass and metabolite production under fed-batch mode.

As shown in Fig 2D, overall, carbon sources equivalent to 58 g/L glucose and 56 g/L glycerol were co-consumed over 96 h in the fed-batch cultivation. Interestingly, except for the initial cultivation in the first 23 h, with the fed carbon sources, glucose and glycerol were consumed simultaneously almost at the same rate. In the first 23 h, *R. toruloides* RP15 showed a diauxic growth pattern on glycerol and glucose and glucose was the preferred carbon source. It was estimated that the initial C:N ratio was ~15:1 compared to ~60:1 in the batch mode. These results indicate that co-consumption of glucose and glycerol was significantly affected by C:N ratio and controlling C:N ratio could alleviate the catabolite inhibition. Fig 2E shows the oil, protein and carotenoid contents based on cell dry weight during fed-batch cultivation. Overall, the changes of the metabolite contents were similar to those observed in the batch cultivation; namely, the oil and carotenoid contents increased with prolonged cultivation time while protein content peaked in 23 h but decreased afterwards. The final contents of oil, carotenoid and protein were 59%, 283 μg/g and 19%, respectively, similar to those in batch cultivation. As a result, the overall cell mass, oil, protein and carotenoid reached 44.8 g/L, 26.7 g/L, 8.6 g/L and 12.7 mg/L, respectively, in 96 h of fed-batch cultivation (Fig 2F), with an oil yield of 0.24 g/g (77% of theoretical yield) and oil productivity of 0.28 g/L/h. The overall production (yields and productivities) of cell mass, microbial oil and carotenoid were improved by ~20% and protein production was improved by 8% compared to batch cultivation. It was previously reported that the specific growth rate of *R. toruloides* strains decreased when the initial glucose concentration was more than 40 g/L (Li et al., 2007). The improved production was likely due to the reduced inhibition with the use of carbon sources at low concentration.

Previous studies mostly focused on the production of microbial oils from lignocellulosic sugars (glucose and xylose) based on dilute acid or alkaline pretreatments (Chang et al., 2015; Fei et al., 2016; Slininger et al., 2016; Yu et al., 2011; Yu et al., 2020). In one study, two-

stage cultivations led to 26-29 g/L microbial oils from lignocellulosic hydrolysate (111 g/L glucose, 77 g/L xylose and 11 g/L arabinose) derived from dilute acid pretreatments of switch grass by *L. tetrasporus, L. kononenkoae and R. toruloides*, respectively, corresponding to oil yields of ~0.16 g/g sugars and oil productivities of 0.13-0.22 g/L/h (Slininger et al., 2016). In another study, batch cultivation of a *T. dermatis* strain on lignocellulosic sugars (89 g/L glucose, 29 g/L xylose, 2 g/L arabinose and 11 g/L cellobiose) derived from two-step dilute acid and dilute alkaline pretreatment of corn stover produced 20.2 g/L oils with an oil yield of 0.16 g/g sugars and oil productivity of 0.21 g/L/h, respectively (Yu et al., 2020). In addition, fed-batch cultivation of a *R. toruloides* strain on lignocellulosic hydrolysate (100 g/L glucose and 10 g/L xylose) from two-step dilute acid and dilute alkaline pretreatment of corn stover with automated online sugar control produced 32 g/L oils with an oil yield of 0.32 g/g and oil productivity of 0.40 g/L/h, respectively (Fei et al., 2016).

Previously, glycerol was mixed with glucose or added into lignocellulosic biomass hydrolysates for microbial oil production by various oleaginous yeast strains, such as *Rhodotorula glutinis* (Gong et al., 2016), *Rhodosporidiobolus fluvialis* (Poontawee et al., 2018), *Cryptococcus curvatus* (Yen et al., 2015) and *R. toruloides* (Bommareddy et al., 2017). Although co-utilisation of glycerol and glucose was generally observed, the consumption of glucose was slower than consumption of glucose (Gong et al., 2016; Poontawee et al., 2018; Yen et al., 2015). In addition, catabolite repression was also observed with a *R. toruloides* strain and consumption of glycerol only occurred with the depletion of glucose (Bommareddy et al., 2017). In these studies, the microbial oil concentrations were less than 20 g/L with low productivities due to the low feedstock concentrations (no more than 90 g/L) and slow consumption of glycerol.

In the present study, fed-batch cultivation of *R. toruloides* RP15 on bagasse glucose and residual glycerol led to the production of 26.6 g/L microbial oils with an oil yield of 0.24

based on consumed carbon sources. These results were comparable to those achieved using biomass sugars derived from conventional dilute acid or alkaline pretreatments. The oil concentration and oil yield were much higher than those obtained with the use of sugar-glycerol mixture because of the use of a higher carbon source concentration of ~120 g/L and the allevaition of feedstock inhibition and catabolite repression. The oil yield of 0.24 g/g consumed carbon sources was 77% of the theoretical oil yield 0.31 g/g, estimated based on a 1:1 mixture of glucose and glycerol (Papanikolaou & Aggelis, 2011).

Fatty acid profile is an important factor affecting application of microbial oils. Despite different oil yields and productivities, the fatty acid profiles of batch and fed-batch cultivations were similar with oleic acid (C18:1, ~45%), palmitic acid (C16:0, 23-25%), linoleic acid (C18:2, 12-14%) and stearic acid (C18:0, ~13%) being the main fatty acids. The fatty acid profile was consistent with a previous study with the use of a different *R. toruloides* strain (Fei et al., 2016). The saturated fatty acids (SFA) were ~39% in *R. toruloides* RP15 oil, which were higher than that in sunflower oil (9.4%), rice bran oil (22.5%) and rapeseed oil (6.3%) and lower than palm oil (49.4%) (Fei et al., 2016; Orsavova et al., 2015). Monounsaturated fatty acids (MUFA) in *R. toruloides* RP15 oil were ~46%, comparable to rice bran oil (44.0%), higher than palm oil (38.9%) and sunflower oil (28.3%), but lower than rapeseed oil (72.8%) (Fei et al., 2016; Orsavova et al., 2015). Moreover, polyunsaturated fatty acids (PUFA) accounted for ~14% of *R. toruloides* RP15 oil, which were comparable to palm (11.7%) and lower than sunflower (62.4%), rice bran (33.6%) and rapeseed (20.9%) (Fei et al., 2016; Orsavova et al., 2015).

Microbial oils are generally considered as alternatives to vegetable oils for biofuel production (Jin et al., 2015; Papanikolaou & Aggelis, 2011; Slininger et al., 2016). However, higher value applications for human and animal consumption are preferred and can make microbial oil-focused biorefineries more economically attractive. Generally oils with low contents of

SFAs are more preferred for human consumption as SFAs increase the level of 'bad' LDL cholesterol in the blood, causing coronary heart disease (Orsavova et al., 2015). Hence, *R. toruloides* RP15 oil may not be suitable for human consumption because of the relatively high content of SFAs. Nevertheless, *R. toruloides* RP15 cell mass may be used as a feed supplement. Previously, palm oil containing high level of palmitic acid (C16:0) was used as a cow feed supplement and increased milk yield without adverse effects on milk protein and dry matter intake by cows (Mosley et al., 2007). The *R. toruloides* RP15 oil has much lower proportions of SFA and may be a better oil supplement to feed. Furthermore, yeasts such as *Saccharomyces cerevisiae* are widely used as feed protein sources. Moreover, carotenoid-producing yeast such as *Phaffia rhodozyma* is used to feed salmon and trout to increase the red pigmentation of the fillet (Shurson, 2018). These examples on the use of palm oil, yeast protein and carotenoid as feed supplements indicate that *R. toruloides* RP15 may be a promising and valuable feed supplement.

3.4 Mass balance

Fig 3 shows mass balance calculations of two-step DA130-AG150, with a L:S ratio of 4.2:1 followed by fed-batch cultivation of enzymatic hydrolysate containing glucose and residual glycerol for microbial oil production. From 1 tonne of raw bagasse, a total of 317 kg glucose and 136 kg xylose were produced at a glucose yield of 73.4%, a xylose yield of 75.3% and a total sugar yield of 74.3%. Pretreated bagasse was partially washed and the residual glycerol left on bagasse did not affect enzymatic hydrolysis. Fed-batch cultivation of enzymatic hydrolysate with 1:1 ratio of glucose to residual glycerol concentrations led to 233 kg cell mass containing 140 kg oil, 45 kg protein and 68 g carotenoid. The oleaginous cell mass may be used as a feed supplement. A xylose-rich pretreatment hydrolysate containing 169 kg of total sugar is also a by-product of this process and could be used for value-adding in addition to the use of as a seed culture carbon source for growing cell mass.

4. Conclusion

Sugarcane bagasse was pretreated by two-step DA-AG at the laboratory and pilot scales. The two-step pretreatment improved the yields of fermentable sugars and reduced glycerol usage. A fed-batch cultivation was used to produce oleaginous cell mass by a novel *Rhodosporidium toruloides* strain, which co-consumed cellulosic glucose and residual glycerol and alleviated catabolite inhibition and feedstock inhibition. overall, 233 kg of oleaginous cell mass containing 140 kg oils, 45 kg protein and 68 g carotenoid could be produced from 1 tonne of bagasse and 0.3 tonnes of glycerol. The oleaginous cell mass may be used as a valuable feed supplement.

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Figures and Tables Captions

Table 1. Pretreatment conditions in the laboratory and pilot scale trials

Table 2. Biomass composition, component recovery and glucan digestibility of pretreated bagasse samples at both laboratory and pilot scales.

Fig 1. Sugars yields from laboratory (A) and pilot (B) scale pretreatments compared to total sugar equivalent in raw sugarcane bagasse. The results are the means of three pretreatment and enzymatic hydrolysis experiments and the relative standard deviation was <5%.

Fig 2. Microbial oil production using bagasse pretreated by DA130-AG150 in batch (A-C) and fed-batch (B-F) cultivations. (A, D) Kinetics of carbon source consumption, (B, E) Contents of oil, protein and carotenoid, (C, F) Kinetics of microbial oil, protein and carotenoid production.

Fig 3. Schematic diagram of mass balance for microbial oil production based on 100 kg raw bagasse based on DA130-AG150 pretreatment at pilot scale.

Table 1.

Trial name	First step				Second step			
	L:S ratio	Glycerol (wt.%)	T (°C)	Time (min)	L:S ratio	Glycerol (wt.%)	T (°C)	Time (min)
Laboratory trials								
DA130	5:1	-	130	15	-	-	-	-
DA130-AG130	5:1	-	130	15	5:1	80	130	15
DA130-AG150	5:1	-	130	15	5:1	80	150	15
DA130-AG170	5:1	-	130	15	5:1	80	170	15
DA130-DA170	5:1	-	130	15	5:1	-	170	15
AG170	5:1	80	130	15	-	-	-	-
DA170	5:1	-	130	15	-	-	-	-
Pilot trials								
DA130	3:1	-	130	15	-	-	-	-
DA130-AG150	3:1	-	130	15	4.2:1	66	150	15
DA130-AG150	3:1	-	130	15	5.6:1	75	150	15
DA130-AG150	3:1	-	130	15	7.0:1	80	150	15
DA130-AG170	3:1	-	130	15	4.2:1	66	170	15
DA130-AG170	3:1	-	130	15	5.6:1	75	170	15
DA130-AG170	3:1	-	130	15	7.0:1	80	170	15
AG150	5:1	84	150	15	-	-	-	-

Table 2.

Trial	L:S	Solid yield (%)	Biomass composition (%)			Component recovery (%)			Glucan digestibility (%)
			Glucan	Xylan	Lignin	Glucan	Xylan	Lignin	
Laboratory trials[1]									
DA130	5:1	72.1	51.3	8.9	30.7	89.9	27.8	87.7	34.2
DA130-AG130	5:1	59.9	58.0	7.2	29.7	84.5	16.1	70.7	52.6
DA130-AG150	5:1	56.2	58.6	4.5	29.5	80.2	9.5	65.9	78.0
DA130-AG170	5:1	52.5	63.3	1.5	31.4	80.8	3.4	65.4	99.2
DA130-DA170	5:1	49.7	61.4	0.9	35.4	74.1	2.0	69.7	64.6
AG170	5:1	53.1	64.2	2.5	29.1	82.9	5.8	61.4	99.3
DA170	5:1	58.3	60.5	2.0	34.7	85.9	4.9	80.5	68.4
Raw bagasse	-	100	41.1	23.1	25.2	100	100	100	12.8
Pilot trials									
DA130[2]	3.0:1	72.2	48.8	4.5	33.3	89.5	18.7	78.6	50.1
DA130-AG150[3]	4.2:1	66.0	50.7	1.7	35.3	84.9	7.9	69.8	82.4
DA130-AG150[4]	5.6:1	65.1	52.1	2.1	32.8	86.1	7.9	69.8	85.1
DA130-AG150[5]	7.0:1	63.6	53.1	2.2	31.2	85.8	8.1	64.9	86.4
DA130-AG170[3]	4.2:1	51.3	50.1	1.3	38.5	65.2	3.9	64.6	93.5
DA130-AG170[4]	5.6:1	49.0	50.8	1.9	37.1	62.0	5.4	59.5	92.5
DA130-AG170[5]	7.0:1	46.7	52.8	1.7	34.7	62.6	4.6	52.9	99.4
AG150[6]	5.0:1	67.1	51.7	4.8	32.8	88.0	18.7	72.0	92.5
Raw bagasse	-	100.0	39.4	17.0	30.6	100.0	100.0	100.0	14.0

1. 2.4% H_2SO_4 on dry biomass or 0.48% H_2SO_4 in liquid, 2. 2.4% H_2SO_4 on dry biomass or 0.8% H_2SO_4 in liquid, 3. 3.33% H_2SO_4 on dry biomass or 0.79% H_2SO_4 in liquid, 4. 3.33% H_2SO_4 on dry biomass or 0.79% H_2SO_4 in liquid, 5. 3.33% H_2SO_4 on dry biomass or 0.79% H_2SO_4 in liquid, 6. 2.4% H_2SO_4 on dry biomass or 0.48% H_2SO_4 in liquid.

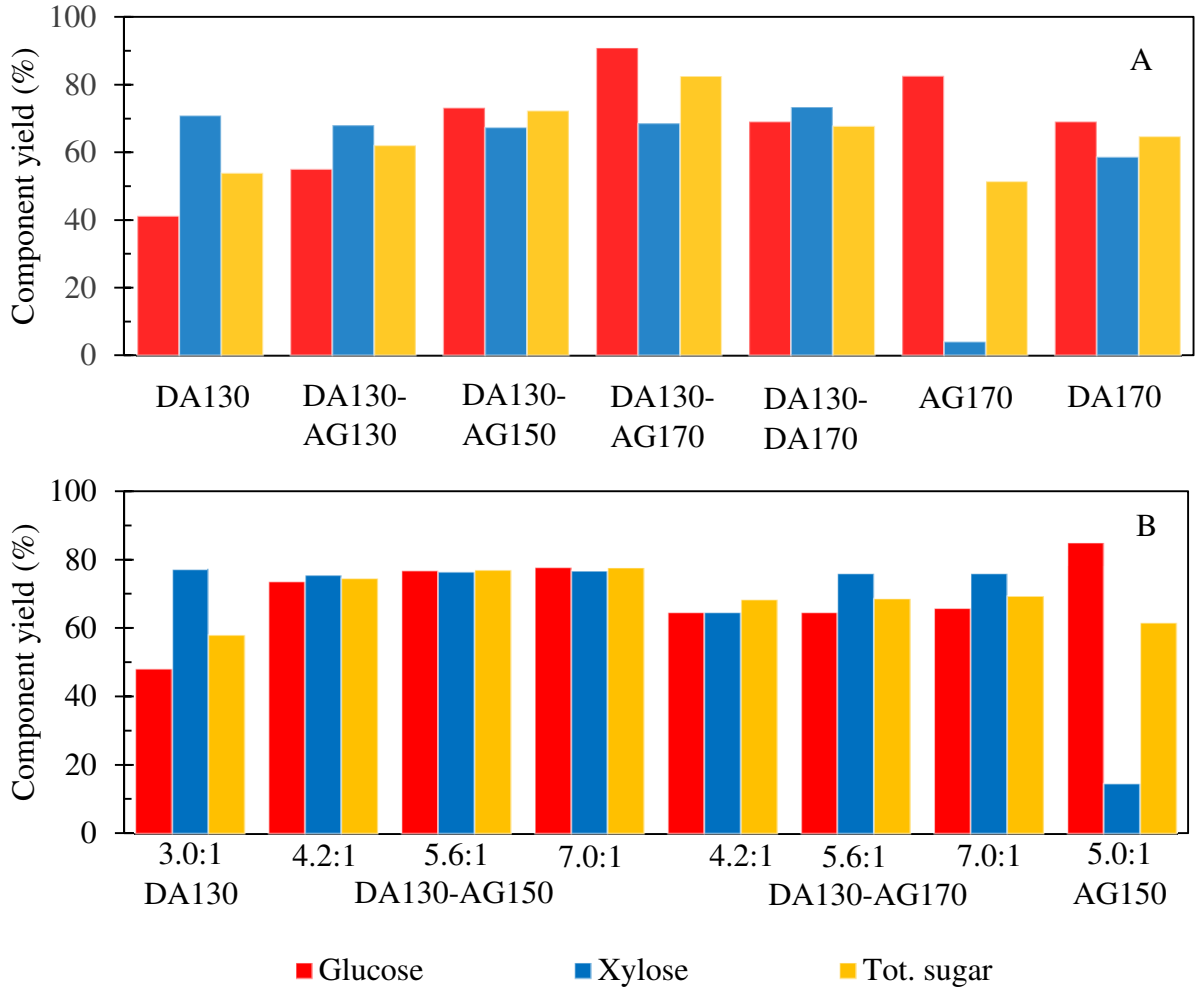
A
Component yield (%)
100
80
60
40
20
0
DA130
DA130-AG130
DA130-AG150
DA130-AG170
DA130-DA170
AG170
DA170
B
Component yield (%)
100
80
60
40
20
0
3.0:1
4.2:1
5.6:1
7.0:1
4.2:1
5.6:1
7.0:1
5.0:1
DA130
DA130-AG150
DA130-AG170
AG150
Glucose
Xylose
Tot. sugar

Fig 1.

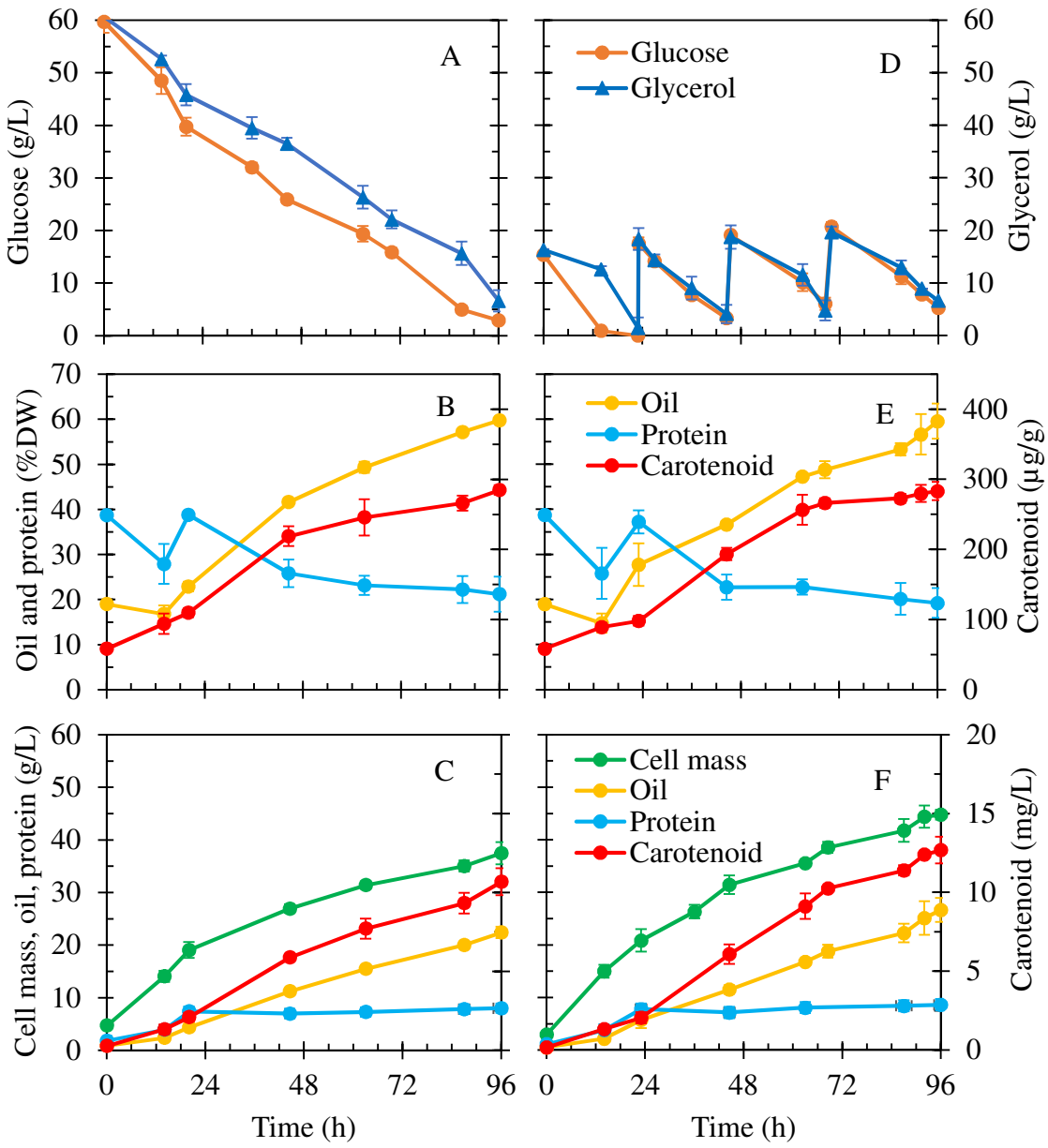
A
B
C
D
E
F
Glucose
Glycerol
Oil
Protein
Carotenoid
Cell mass
Glucose (g/L)
Glycerol (g/L)
Oil and protein (%DW)
Carotenoid (μg/g)
Cell mass, oil, protein (g/L)
Carotenoid (mg/L)
Time (h)
Time (h)

Fig 2.

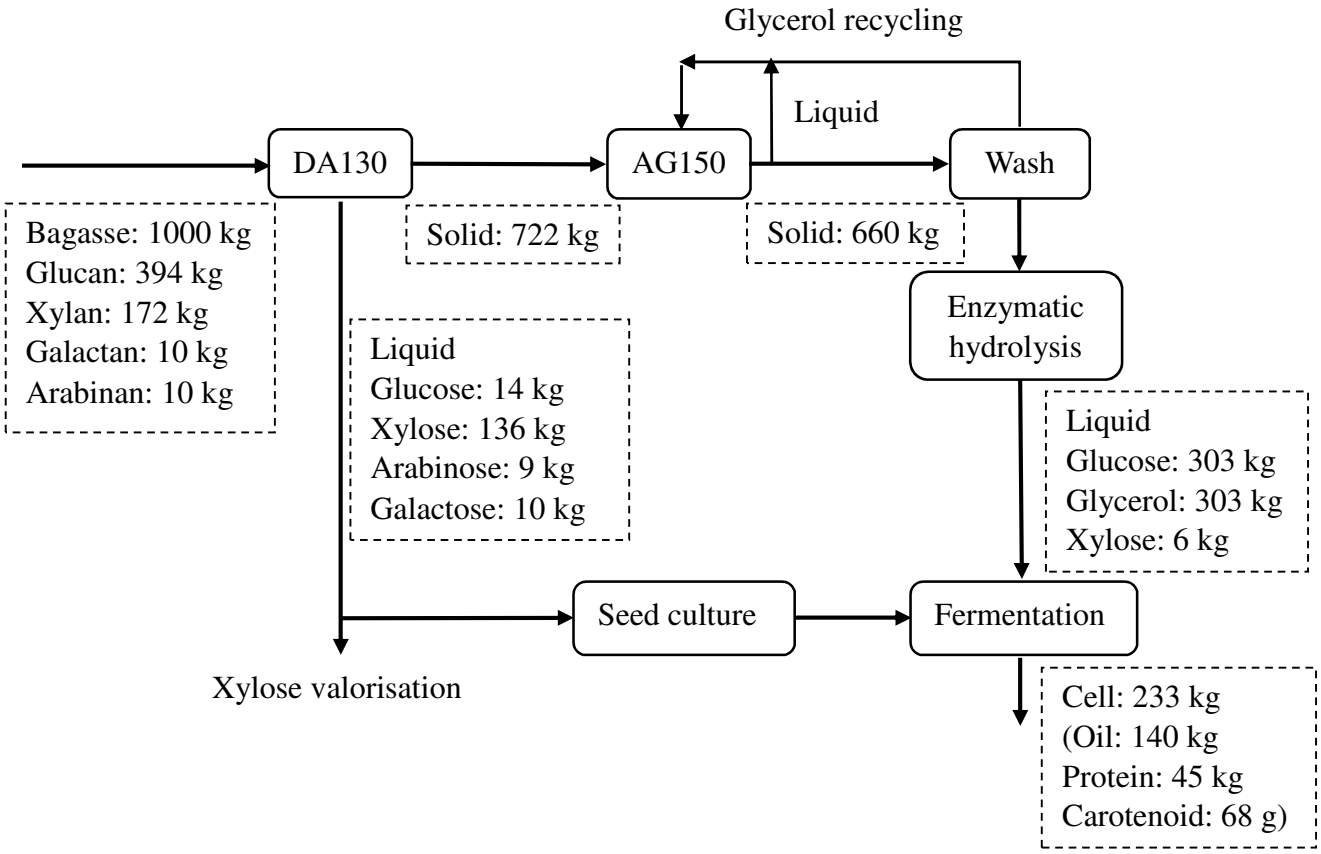
Glycerol recycling
Liquid
DA130
AG150
Wash
Bagasse: 1000 kg
Glucan: 394 kg
Xylan: 172 kg
Galactan: 10 kg
Arabinan: 10 kg
Solid: 722 kg
Solid: 660 kg
Enzymatic hydrolysis
Liquid
Glucose: 14 kg
Xylose: 136 kg
Arabinose: 9 kg
Galactose: 10 kg
Liquid
Glucose: 303 kg
Glycerol: 303 kg
Xylose: 6 kg
Seed culture
Fermentation
Xylose valorisation
Cell: 233 kg
(Oil: 140 kg
Protein: 45 kg
Carotenoid: 68 g)

Fig 3.

Graphical abstract

Two-step acid-catalysed glycerol pretreatment improved sugar production while fed-batch cultivation enhanced microbial oil production on bagasse glucose and residual glycerol.