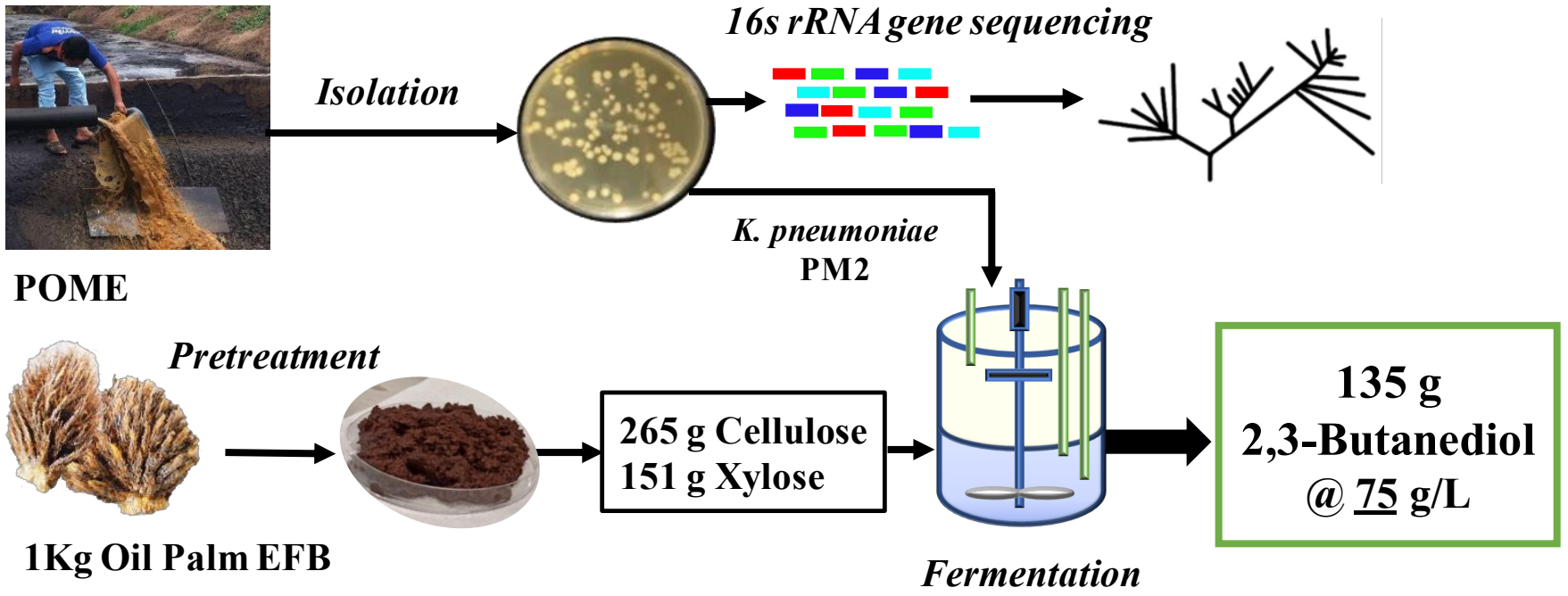
Graphical Abstract



Highlights

- A novel xylose-utilizing strain was newly isolated for 2,3-BDO bioproduction
- Sulfite-pretreated oil palm empty fruit bunches whole sugars were fermented
- High 2,3-BDO titer of 75 g/L was achieved via staged-fed batch SHF
- A maximum of 0.48 g/g 2,3-BDO on glucose and xylose was yielded
- 135 kg 2,3-BDO and 14.5 kg acetoin precursors from 1 tonne biomass

Whole Sugar 2,3-Butanediol Fermentation for Oil Palm Empty Fruit Bunches Biorefinery by a Newly Isolated *Klebsiella pneumoniae* PM2

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Abstract

Effective utilization of cellulose and hemicelluloses is a critical factor for sustainable bioconversion of lignocellulose. A newly isolated xylose-utilizing strain *Klebsiella pneumoniae* PM2 was introduced to convert the biomass "whole sugars" into high value 2,3-butanediol (2,3-BDO) in an integrated biorefinery process. The fermentation conditions were optimized (temperature 30 °C, initial pH 7, and 150 rpm agitation speed) using glucose for maximum 2,3-BDO production in batch systems. A sulfite pretreated oil palm empty fruit bunches (EFB) whole slurry (substrate hydrolysate mixed with pretreatment spent liquor) were fed to PM2 for mixed sugar fermentation. The optimized biorefinery process resulted in 75.03±3.17 g/L of 2,3-BDO with 0.78±0.33 g/L/h productivity and 0.43 g/g yield (87% of theoretical value) via a modified staged separate hydrolysis and fermentation process. This result is equivalent to approximately 135 kg 2,3-BDO and 14.5 kg acetoin precursors from 1 tonne EFB biomass without any wastage of both C_6 and C_5 sugars.

Keywords: 2,3-Butanediol; whole slurry; oil palm empty fruit bunches; fermentation; integrated biorefinery

1. Introduction

The finite fossil fuel resources coupled with instable market prices have reignited the interest of scientific community to find the alternate renewable reserves counteracting the petroleum-derived economy. Among such bio-based products from renewable resources, 2,3-butanediol (2,3-BDO) has been receiving enormous attentions for its multifarious industrial implications. It is a potential feedstock in polymer industry for the production of polyurethanes, polyesters, and synthetic rubber monomers. It has also been utilized as a derivative of fuel additive, anti-freeze agent, and in food and pharmaceutical industries (Song et al., 2019). The non-natural structural isomer of 2,3-BDO, 1,4-butanediol (1,4-BDO) is with high market value which offers similar functions as 2,3-BDO, but this compound is a petrochemical and can be only produced biologically with the aid of genetic engineering (Cortivo et al., 2019; Yim et al., 2011), while 2,3-BDO can be produced by many wild-type microorganisms. Due to the biodegradable nature, 2,3-BDO has minimal environmental and end user risks, hence is considered as a green chemical (Celińska and Grajek, 2009). The current market price of 2,3-BDO is higher than 1,4-BDO (Tinôco et al., 2020) hence many upgrading technologies have been implementing to ramp up the 2,3-BDO production industry.

Agro-industrial biomass is a sustainable source of biofuels and biochemicals, including 2,3-BDO production. Oil palm industry is a leading contributor with a production reaches 75.5 million metric tons in 2020/21 (USDA, 2021). Crude palm oil constitutes nearly 10% of the whole palm tree and remaining 80% of the plant biomass is wasted (Ofori-Boateng, 2013). A huge portion of the biowaste has been incinerated for the slash-and-burn activities thereby contributes to high greenhouse gas emissions (Dhandapani and Evers, 2020). A holistic approach should be developed for effective management of waste products from palm oil industry. Among all the oil palm biomass, empty fruit bunches (EFB) constitutes to 20-23% of the total production (Ofori-Boateng, 2013), and therefore has been selected as an example for our study. At present, only few studies have investigated the oil palm EFB and frond biorefinery for 2,3-BDO production (Hazeena et al., 2019; Kang et al., 2015).

Two significant challenges exist before a sustainable and economical production of 2,3-BDO in commercial scale. The modern biorefinery aims to utilize all the building block

chemicals (cellulose, hemicellulose, and lignin) while lignocellulosic biomass is recalcitrant to biodegradation and enzymatic hydrolysis (Islam et al., 2020). Pretreatment is essential to harvest the fermentable sugars (Leu and Zhu, 2013). Although many pretreatment processes have been developed for increasing the enzymatic digestibility of EFB (Ling et al., 2020; Nurfahmi et al., 2016; Tan et al., 2016), many of which focus only on the saccharification of cellulose but not the hemicellulose. Co-utilization of both six-carbon (*e.g*., glucose) and five-carbon sugars (*e.g*., xylose) is also a challenging step for strain selection (Guo et al., 2014; Li et al., 2010). Some 2,3-BDO producing strains such as *Enterobacter*, *Klebsiella*, *Bacillus,* etc. can utilize xylose naturally (Cortivo et al., 2019; Li et al., 2014), but their xylose tolerance is poor, leading to the incomplete utilization of high xylose containing substrate (Cai et al., 2012; Zhang et al., 2009). Furthermore, the microbial glucose preference over xylose via carbon catabolite repression (CCR) mechanism can also hinder the complete utilization of xylose.

This study aims to identify an efficient xylose-utilizing bacteria for 2,3-BDO fermentation with oil palm EFB whole slurry (mixture of pretreatment substrate and liquor). The targeted bacterial strains were isolated from the palm oil mill effluent (POME) and tested for their capability of 2,3-BDO production. A strain-specific process was optimized for the selected strain through bio-kinetics. Sulfite pretreatment was performed to fractionate the oil palm EFB, of which both the substrate and liquor were used in fermentation. Batch and fed-batch fermentations were performed for maximizing the 2,3-BDO yield under optimized conditions. An integrated biorefinery process with mass balance was presented in comparing with the productivity of recently published works for future development of 2,3-BDO bioindustry.

2. Experimental Section

The experiments of this study include isolation, characterization, and optimization of the 2,3-BDO producing bacteria; pretreatment and subsequent fermentations of the EFB substrate. The detailed procedures are provided as follows:

2.1. Isolation and maintenance of the 2,3-BDO producing bacteria from xylose

The POME samples were collected from a waste oil treatment plant in Thailand. For enrichment, 5 g of POME was added in 100 mL of mineral salt medium (MSM) containing 20 g/L xylose in Erlenmeyer flasks and incubated at 37°C for 12 h. The broth was serially diluted using 0.9% saline solution and plated on nutrient agar medium. After overnight incubation at 37°C, the representative xylose-utilizing colonies were selected based on their morphological and colonial characteristics and purified on agar plates. All the isolates were screened for 2,3-BDO fermentation in the basal (MSM) medium containing 20 g/L glucose and 20 g/L xylose (in separate flasks) at 37°C and 150 rpm. After 24 h of incubation, the metabolic products were analyzed and the strain produced the highest 2,3-BDO concentration from glucose and xylose was designated as PM2 and maintained in glycerol stocks at -80°C until further analysis.

2.2. 16s rRNA identification and phylogenetic analysis

For basic morphological characterization, Gram staining was performed which characterized PM2 as gram-negative bacteria. For molecular identification, the genomic DNA of PM2 was extracted using DNeasy PowerSoil Kit (Qiagen, USA). After extraction, DNA sample was sent to Macrogen (South Korea) for PCR amplification and sequencing. The universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'), and for sequencing, 785F (5' GGATTAGATACCCTGGTA 3') and 907R (5' CCGTCAATTCMTTTRAGTTT 3') primers were used for 16s rRNA gene amplification. The sequences were analyzed through BioEdit Sequence Alignment Editor (7.2.5, Ibis Biosciences. USA) and resultant nucleotide sequences were aligned with known sequences available in the NCBI database using BLASTn program. The MUSCLE program was used for multiple sequence alignment with the most closely related bacterial sequences. The evolutionary distance was computed using maximum composite likelihood method and a phylogenetic tree was constructed based on the neighbour-joining method with 1,000 bootstraps using Mega-X software.

2.3. Growth kinetics and culture conditions

To refresh the culture, PM2 strain from the cryopreservation vial was incubated overnight at 37 °C and 150 rpm in a nutrient broth (20 mL). To determine the mid-exponential phase, the growth profile of strain PM2 was observed through optical density (OD_{600}) measurements using UV-Vis spectrophotometer (Shimadzu-2600). Briefly, 2mL of the broth sample was taken after every 2 h and OD_{600} was measured against sterile nutrient broth as blank. All the samples were diluted before each measurement with the culture broth (if necessary) to get the absorbance range between 0.1 and 0.9. The growth curve for PM2 is shown in **Fig. S1**. For seed inoculum in a shake-flask, 5 mL of the refresh culture was transferred in 50 mL fermentation medium and incubated under same conditions. After 8 h, the culture during its mid-exponential phase (OD_{600}=1) was harvested via centrifugation (10,000 rpm, 4°C), resuspended in sterile medium, and used in the subsequent experiments.

The composition of the fermentation medium used in this study is listed in **Table S1**. The media and sugars were separately autoclaved (to avoid Maillard reaction) at 121 °C for 15 min. and mixed under aseptic conditions prior to use. The chemicals used in this study were procured from Sigma-Aldrich (MO, USA) and J&K Acros Organics (Beijing, China), except where otherwise specified. All the fermentation media, glasswares and consumables were autoclaved (121 °C; 15 min) before use.

2.4. Optimization of fermentation conditions

Batch fermentation of PM2 was performed by shake flask method to optimize the operational parameters for 2,3-BDO fermentation using one-factor-at-a-time experimental design. The procedures were performed in 130 mL wheaton glass serum bottles containing 50 mL of the fermentation media using glucose as a sole carbon source. The bottles were sealed with the butyl rubber stopper and aluminium seals to create microaerophilic conditions. First, the effect of temperature on cell growth and 2,3-BDO production was investigated by incubating the bottles at different temperatures (25, 30, 33, 37, and 40 °C) under initial pH 7 and agitation of 150 rpm. Second, the effect of initial pH of the medium was evaluated at six different pH levels ranging from 5.5 to 8.0 for 2,3-BDO production by PM2 strain at its optimized temperature 30 °C. The pH was adjusted before autoclaving the medium with 1N

NaOH/HCl solution. Third, the effect of agitation speed of the reactor was determined by providing a series of agitations from 100 to 350 rpm. All other conditions were remained same. Furthermore, the effect of initial substrate concentration was investigated by adding different initial concentrations of glucose (20-140 g/L) and xylose (20-120 g/L) in the fermentation media. All the optimization experiments were run in duplicate for 60 h and samples were taken after every 12 h for cell growth and fermentation products analysis.

2.5. 2,3-BDO tolerance assay

For product tolerance test, a refresh culture (2 mL) of PM2 in the mid-exponential phase was used to inoculate 20 mL of the nutrient media with increasing concentrations of 2,3-BDO, starting from 0 to 120 g/L. The initial optical density (OD_{600}) for the cultivations was recorded as 0.1. All the culture tubes were then incubated at 30 °C and 150 rpm for 72 h. The 2,3-BDO toxicity was determined by measuring the PM2 cell density via spectrophotometry.

2.6. 2,3-BDO production from pure sugars

The nutrient media was fed with a variety of pure sugars such as glucose, fructose, xylose, galactose, arabinose, mannose, sucrose with an initial concentration of 20 g/L. Cell growth was observed by colorimetric method during different time intervals and observed that PM2 utilized almost all the sugars, showing the diversified growth abilities (**Table S2**). The 2,3-BDO production performance by PM2 was then evaluated using glucose (G), xylose (X), and the mixture of both (G:X), in shake flasks experiments. The production medium was supplemented with 100 g/L of glucose, 80 g/L xylose, and a (G2:X1) ratio for the mixed sugars (*i.e.*, 70 g/L glucose and 30 g/L of xylose), separately. The fermentations were run for 72 h under optimized conditions, in serum bottles (100mL working volume). The samples were periodically drawn (after 12-h interval) and analyzed for 2,3-BDO production, residual sugars, and by-products formation. All fermentations were carried out in duplicate and the data points are their average values ± standard deviation (used as error bars in data plots).

2.7. 2,3-BDO production from oil palm EFB

EFB biomass was first subjected to sulfite pretreatment and the fractionated substrate was presented to different fermentation processes, which are detailed in this section.

2.7.1. Sulfite pretreatment of EFB (SpEFB)

The sulfite pretreatment was performed by following the protocol described in our previous study (Cheng et al., 2014). Briefly, 7% sodium bisulfite and 2.5% sulfuric acid were loaded directly to 1 kg (oven dry wt.) of EFB without any size reduction. The reaction digester was run at 165 °C for 75 minutes with a solid/liquid ratio of 1:4. After pretreatment, the reactor was cooled to room temperature. Small aliquots of pretreated solids and spent liquor were drawn for chemical composition analysis using NREL protocol (Sluiter et al., 2012). Then, the entire solid and liquid from the reactor were milled together to a slurry using a blender, without any extra water addition. The pH of the resultant whole slurry was neutralized to 6.0 with solid lime. Thereafter, both the fractions were separated via vacuum filtration and stored at 4°C for subsequent fermentation experiments.

2.7.2. Batch and fed-batch fermentation

Prior to fermentation, a preliminary trial was performed to determine the acetic acid (a major by-product after sulfite pretreatment) tolerance to *K. pneumoniae* PM2. The cultivations were carried out with ascending concentrations (0-14 g/L) of acetic acid in the fermentation broth at 30 °C and 150 rpm. The general scheme of the quasi-simultaneous saccharification and fermentation (Q-SSF) and staged fed-batch separate hydrolysis and fermentation (staged-SHF) procedures is displayed in **Fig. 1**. All the fermentations were performed in duplicate. The protocols were performed under strict sterile conditions to avoid bacterial and environmental contamination. The samples were periodically drawn and analyzed via spectrophotometry.

2.7.2.1. Quasi-simultaneous saccharification and fermentation (Q-SSF)

Sulfite-pretreated EFB (SpEFB) was used as a Q-SSF substrate with 8% of solid loadings in a 1L bioreactor with 100 ml working volume, under optimized conditions. A pre-hydrolysis step was performed using 15 FPU/g-dry substrate of commercial cellulase enzyme (Cellic®

CTec2; Novozyme), sodium acetate buffer (pH -4.8), and SpEFB substrate. The reactor was incubated at 50 °C, 200 rpm for 12 h and then was cooled down to room temperature. The pH of the partially-hydrolyzed EFB biomass was adjusted to neutral using solid lime. Afterwards, a 10% (*v/v)* PM2 seed culture was inoculated along with 1% tryptone and yeast extract solution and batch SSF was started at 30 °C, initial pH 7, and 150 rpm. The samples were drawn after 12-h interval up to 72 h for analysis. Fed-batch SSF was initiated similar to batch cultivations with 18% solid loadings at 200 mL total working volume. After pre-hydrolysis, 10% inoculum was added to the partially- hydrolysed substrate. Upon utilization of >80% of sugars in SSF broth, approximately 100 g of partially hydrolysed SpEFB (neutralized) was fed twice to the bioreactor. The initial pH of the medium was set at 7.0, with no pH control during the fermentation. The samples were collected and sequentially analyzed for sugar utilization, 2,3-BDO, and other products formation. The fed-batch process lasted for 144 hours.

2.7.2.2. Staged-separate hydrolysis and fermentation (staged-SHF)

The staged fed-batch SHF was also performed to counter the carbon catabolite repression (CCR) effect. At the first stage, fermentation was started with spent liquor containing ~80 g/L C_5 sugars as a sole carbon source. After >90% sugar utilization, enzymatic hydrolysate (prepared as described in section 2.6.2.1) of SpEFB substrate (~60 g/L C_6 sugars) was sequentially fed at the second stage. The sugar concentrations were adjusted by either diluting the spent liquor or vacuum concentrated the enzymatic hydrolysate using rotary evaporator. Other operating conditions were remained similar to Q-SSF cultivations.

2.8. Analytical methods

For biomass determination, the dry cell weight (DCW) was calculated using a conversion factor of $0.587 g_{dry\ cell}/L/OD_{600}$, obtained by the calibration curve between DCW and OD (**Fig. S2**). The concentrations of sugars and fermentation metabolites (i.e., organic acids, diols, and acetoin) were analyzed using high performance liquid chromatography (HPLC; Shimadzu, Japan) equipped with an Aminex HPX-87H column (Bio-Rad, USA) coupled to a refractive index detector (RID; Waters, USA). The HPLC was operated at 65 °C with a flow rate of 0.6

mL/min using 5mM H_2SO_4 as a mobile phase. Unless stated otherwise, 2,3-BDO concentrations (C_{BDO}; g/L) produced during this study denote the sum of all (*L-, D-, meso-*) stereoisomers. The 2,3-BDO yield (Y_{BDO}) was calculated as the amount (in grams) of produced 2,3-BDO over sugars consumed. Likewise, the volumetric productivity (Q_{BDO}) was determined as the amount of 2,3-BDO produced (g/L) per hour of the batch/fed-batch operation. All the analytical values presented are the average of the independent set of experiments, obtained against standard curves (R^2 >0.998) of the pure compounds used in this study.

3. Results and Discussions

3.1. Identification of the 2,3-BDO producing bacteria

Morphologically, PM2 was characterized as Gram-negative bacilli, facultative anaerobic, encapsulated, sessile, and non-spore forming bacteria (**Table S2**). The colonial characteristics are mucoid, large-sized, creamy coloured, and circular in shape. The full-length 16s rRNA gene identification disclosed that the strain PM2 shared sequence similarity with many *Klebsiella pneumoniae* strains with >99% sequence similarity. The PM2 gene sequence was aligned with other published 16s rRNA *Klebsiella* sequences available in GenBank database for phylogenetic tree construction via neighbour-joining method. The tree displayed the most closed relatedness of PM2 with *Klebsiella pneumoniae subsp. ozaenae* AR0096, hence designated as *Klebsiella pneumoniae* PM2 (**Fig. S3**). The partial gene sequence of 16s rRNA of strain PM2 had been deposited to GenBank with an accession number MT422266. Most of the species of *K. pneumoniae* are discovered as the potent 2,3-BDO producers from diversified sugars including plant-derived sugars (Cortivo et al., 2019; Ma et al., 2009; Wong et al., 2012). Hence, this study was designed to explore the 2,3-BDO producing ability of *K. pneumoniae* PM2 from hexoses and pentoses, obtained from valorization of lignocellulosic biomass.

3.2. Optimization of fermentation conditions for PM2 growth and 2,3- BDO production

Optimization studies were carried out to explore the maximum potential of the 2,3-BDO fermentation by PM2 using glucose as the sole carbon and energy donor in the system. The

effect of temperature, initial pH of the medium, agitation speed, substrate/product inhibition assays are summarized under the following sections:

3.2.1. Effect of temperature

The influences of incubation temperature were determined on PM2 growth and subsequent 2,3-BDO production. The strain remained active at all the tested temperatures (*i.e*., 25, 30, 33, 37, 40°C), indicating its high growth adaptability. The optimum fermentation temperature was detected at 30°C where the strain reached to exponential growth phase by utilizing 82.8% of glucose within 24 h of cultivation and produced 8.18±0.27 g/L 2,3-BDO. Whereas, at 25°C, PM2 displayed the similar quantitative 2,3-BDO production till 24 h and substantially decreased during the stationary phase (**Fig. 2a**). Most of the reported studies with *K. pneumoniae* displayed maximum growth and 2,3-BDO production at 37°C (Li et al., 2010; Ma et al., 2009). Nevertheless, at temperatures 33°C and 37°C, the growth rate of *K. pneumoniae* PM2 ramped down with decreased product yield and productivity (**Fig. 2f**). A further decline in cell growth and 2,3-BDO quantity was noticed at 40°C, which might be due to the thermal denaturation of the key catabolic enzymes responsible for regulating 2,3-BDO metabolic route, thus considered as an enzymatic controlled process (Perego et al., 2003). At this high temperature, the glucose consumption was >70% but it maximally converted into lactic acid (5.72±0.24g/L) but not 2,3-BDO. When the batch fermentation was conducted at 30°C, the concentrations of major by-products, both ethanol and lactic acid, were detected up to 3 g/L (at 48 h) and subsequently re-metabolized, leaving 2.3±0.23 and 1.7±0.08 g/L in the fermentation broth at 72 hours, respectively. Many of the *Klebsiella* strains reported the mixed acid fermentation route for 2,3-BDO production. However, in our study, only trace amount of acetic acid and succinic acid (<1 g/L) were produced, whereas pyruvic acid and formic acid were not detected due to anaerobic mode of fermentation (Guragain and Vadlani, 2017).

3.2.2. Effect of pH

Studies on microbial 2,3-BDO production described that initial pH of the fermentation media is a governing factor which has a direct effect on metabolites production and their

concentration (Petrov and Petrova, 2009). **Fig. 2b** shows the effects of changing initial pH (5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) during 2,3-BDO fermentation. At lower initial pH (*i.e.*, 5.0 and 6.0), cell growth and 2,3-BDO production (1.2-4.0 g/L) were both low, respectively (**Fig. S4a and b**). A sharp decline in pH was occurred in the initial 24 h of fermentation started with pH 6.5 and 7.0, indicating the production of acids and gases at the expense of rapid glucose utilization by PM2 cells. When glucose was almost depleted, the formation of organic acids ceased and increment in pH was recorded at 32-42 h. Another pH drop was observed when secondary metabolites were utilized as the carbon source by the bacteria. The 2,3-BDO production was slightly increased between 52 and 60 h of cultivation, which prevented acidification in the medium (**Fig. S4c and d**). Optimum concentrations for cell growth and 2,3-BDO production were also found at the pH levels. The maximum volumetric productions were 6.5±0.35 and 7.8±0.22 g/L with 82 and 98% of the maximum theoretical yield, respectively (**Fig. 2b and g**). Furthermore, the effect of alkaline pH was observed with 54.5% (@pH-7.5) to 81.8% (@pH-8.0) decline in BDO productivity (**Fig. 2g**). Although the glucose consumption at alkaline pH was higher than at low pH, it was converted into acetoin, lactic acid, succinic acid, and ethanol, leading the metabolic activities towards mixed-acid fermentation (**Fig. S4e and f**). Hence, an optimum initial pH together with a pH control/adjustment (~6.0-7.0-optimum pH level for major BDO producing enzymes) during fermentation may lead to the high BDO production (Celińska and Grajek, 2009).

3.2.3. Effect of agitation speed

Agitation rate of fermentation is a bacteria-specific function to affect 2,3-BDO production. In the present study with *K. pneumoniae* PM2, 150-200 rpm promoted the highest BDO production among all tested speeds, yielded 7.66±0.19 g/L and 7.08±0.22 g/L of 2,3-BDO after 24 h of fermentation. The speed out of the optimum range resulted in reduced BDO production, *i.e.*, 5.7±0.31 g/L @100 rpm, 4.8±0.19 g/L @250 rpm, and 4.1±0.42 g/L @300 rpm of agitation (**Fig. 2c**). These results might be due to the accumulation of other metabolites, *i.e.,* organic acids, ethanol, and acetoin. In a previous study, *K. pneumoniae* achieved very low amount of 2,3-BDO and acetoin (38.2 & 5.3 mM) without agitation, but both 2,3-BDO and acetoin

concentrations (129.9 & 62.5 mM) increased rapidly when 220 rpm of mixing was provided (Barrett et al., 1983). Another attempt using *K. oxytoca* M1 found that increasing the agitation speed also increased the glucose consumption, cell growth, and diol production, however, the diol yield declined with lowering of agitation speed (Cho et al., 2015). Likewise, in the current setup, bacterial growth was sequentially promoted by speeding up from 250 to 300 rpm. 2,3-BDO yields and productivities were decreased at the end of fermentation at the expense of other metabolites (**Fig. 2h**). An adequate agitation speed should be formulated for maximizing the BDO production.

3.2.4. Effect of initial substrate concentration

Batch fermentation is generally applied for high product recovery, but this process has some major limitations, such as the low productivities and high substrate inhibition. This study investigated the effects of changing initial concentrations of glucose (20-140 g/L) and xylose (20-120 g/L) on PM2 growth and 2,3-BDO production. The strain showed a great tolerance to high sugar concentrations. **Fig. 2d** shows that all the treatments with glucose feedings behaved similarly at the beginning of the log phase. The amount of 20 and 40 g/L initial glucose in the media were rapidly converted into 7.97±0.28 and 18.02±0.52 g/L 2,3-BDO after 24h of fermentation (>80% glucose consumption). Further increase in sugar concentrations up to 60, 80 and 100 g/L enhanced the glucose uptake rate and 2,3-BDO reached up to 21.8±0.19, 26.8±0.27, and 29.4±0.44 g/L after 36 h, respectively. For comparison in similar studies, *K. pneumoniae* SDM produced 34.6 g/L 2,3-BDO from initial glucose of 70 g/L at batch mode (Ma et al., 2009) and further high production was obtained via fed-batch fermentation. Contrarily, in this study, acetoin concentrations raised after 48 h of operation (data not shown) inferring the reversible action of 2,3-BDO to acetoin upon glucose depletion in the broth (Maina et al., 2019). As shown in **Fig. 2i**, the yield and productivity from 120 to 140 g/L of glucose were reduced, which might be due to the slower cell growth at higher substrate concentrations. Likewise, 2,3-BDO concentrations were relatively lower (30.2±0.24 and 28.5±0.35 g/L) and overall product yields got reduced to 81% and 70%, respectively.

In case of using xylose as the sole source of carbon, strain tolerance was limited as compared with glucose. **Fig. 2e** depicts that xylose utilization by PM2 required a longer lag phase (12 h), which may be due to the phases of xylose catabolism machinery undergoing the pentose phosphate pathway before pyruvate metabolism for 2,3-BDO pathway (Jansen and Tsao, 1983). The maximum 2,3-BDO titers were achieved after 36 h from 20 to 120 g/L xylose feedings. Among those, 80g/L was the highest tolerable amount with 25±0.39 g/L 2,3-BDO. However, higher xylose concentrations, *i.e.*, 100 and 120 g/L inhibited the cell growth as well as 2,3-BDO titers, yields and productivities (**Fig. 2j**). On the whole, substrate (sugar) level regulation has a prime importance due to its impacts on water activity and oxygen availability which determines the function of cell growth and product formation (Jansen et al., 1984).

3.2.5. 2,3-BDO tolerance test

End-product inhibition is amongst the most critical limitations for industrial 2,3-BDO fermentation although 2,3-BDO generally has minimum inhibitory effects on bacterial growth than other alcohols (Wang et al., 2012). The tolerance assay displayed a non-toxic behaviour of 2,3-BDO to PM2 up to 60 g/L when compared with control (no BDO added) in the culture media. However, the inhibitory effect was prominent from 80 g/L BDO, where only 47.5% cell growth was detected. The cell growth further reduced to 24% at 100 g/L BDO, and almost ceased at 120 g/L of 2,3-BDO (**Fig. 3a**). In a previous study with *L. lactis*, Kandasamy et al. (2016) reported the toxicity of acetoin (precursor of BDO) and 2,3-BDO on the cell growth. They found that acetoin was more toxic than 2,3-BDO and more prominent during aerobic cultivations. For 2,3-BDO, 100 g/L was the toxic level that reduced 64% of the specific growth rate at anaerobic, and 78% during aerobic cultivation. Whereas, for acetoin, only 60 g/L decreased the cell growth up to ~80% and 96%, under anaerobic and aerobic conditions, respectively (Kandasamy et al., 2016).

3.3. 2,3-BDO production from pure sugar(s)

Batch fermentations were performed using single- and mixed-sugars under optimized growing conditions. PM2 strain preferred glucose over xylose, of which 32.7±0.49 g/L (yield

0.47 g/g; productivity 1.36±0.22 g/L/h) and 28.3±0.51 g/L (yield 0.46 g/g; productivity 0.77±0.19 g/L/h) 2,3-BDO were produced from 100 g/L of glucose and 80 g/L xylose, respectively. Other metabolites were also found in smaller quantities in both cases, *i.e.*, lactic acid (9.5-10.7 g/L), succinic acid (5 g/L), and ethanol (5.8-9.8 g/L) (**Fig. S5a** and **S5b**). Co-fermentation (G:X) results showed a total of 36.3±0.55 g/L 2,3-BDO with a 6.5% increment in BDO yield (0.49 g/g) when compared with solely xylose-based fermentation. The maximum sugar uptake rate was 2.9±0.24, 1.7±0.18, and 1.5±0.39 g/L/h using glucose, xylose and G2:X1 (respectively). Xylose-uptake was principally enhanced after ~90% of glucose depletion and reached up to ~40% after 48 h of operation (**Fig. 4a**). Since 2,3-BDO production is a growth-associated operation, a decline in the yield was noticed after late stationary phase (72 h). Concomitantly, acetoin accumulation increased up to 6.84±0.27 g/L after 2,3-BDO concentration reached a plateau, indicating the reversible action of butanediol dehydrogenase for NAD^+/NADH redox balance (Maina et al., 2019). Ethanol (6.7±0.33 g/L), succinic acid (5.9±0.27 g/L), and lactic acid (8.8±0.54 g/L) might also have inhibitory effects on 2,3-BDO yield by diverting to mixed-acid fermentation pathway (Guo et al., 2014).

3.4. 2,3-BDO production from SpEFB

3.4.1. Chemical composition of the SpEFB and pretreatment spent liquor

The chemical composition of the oil palm SpEFB is shown in **Table 1**. The solid substrate contained 56.83±0.37% cellulose and 4.81±0.16% hemicellulose, which accounted for 61.6% of the dry EFB by weight. The remaining sugars were dissolved in the spent liquor, of which 0.64±0.03% cellulose, 11.75±0.14% hemicellulose, and 11.53±0.23% lignin. The sulfite pretreatment dissociated approximately 84% of hemicelluloses (xylan), among which 72% was detected as monomeric xylose in the spent liquor, 12% in the pretreated solids, and remaining xylose was either converted into acetic acid (6.5 g/L) and furfural (2.2 g/L). Cellulose (glucan) recovery was 98% in the pretreated substrate, which was then used in enzymatic saccharification process. Lignin removal was 52% (**Table S3**) and 5-hydroxymethylfurfural (glucose degradation by-product) was not detected. The furfural concentration was under the tolerable range and should not cause significant impact on the subsequent fermentation (Cheng

et al., 2014). However, inhibiting effects of acetic acid was determined before the cultivations and minimum inhibitory concentration was found at 4-6 g/L for PM2 (**Fig. 3b**). The pretreated substrate showed a high substrate enzymatic digestibility (≥ 98% at 72h), principally due to the increased xylan removal and delignification as reported previously (Cheng et al., 2014; Islam et al., 2021; Leu et al., 2013). Sulfite-pretreatment was suggested a suitable approach for high quantities of EFB mixed sugars to obtain higher 2,3-BDO yields.

3.4.2. Batch fermentation (Q-SSF)

The oil palm SpEFB whole slurry was used for co-utilization of both glucose and xylose. **Fig. 4b** shows the time course of sugar assimilation and 2,3-BDO production by PM2 during a 72 h fermentation period. From the initial 8% solid loading, approximately 46 g/L of the total sugars (glucose and xylose) were released through enzymatic saccharification in the first 24 hours. After PM2 addition, the reducing sugars were gradually consumed and reached at a maximum assimilation (~86%) after 48 h of operation. The highest 2,3-BDO titer was 18.98±0.69 g/L, with a productivity of 0.53±0.21 g/L/h and 0.48 g/g yield (96% of the maximum theoretical yield). The acetic acid (4 g/L) in the saccharified SpEFB whole slurry was also utilized, hence no accumulation was detected. It can be suggested that low concentrations of acetic acid did not result in the inhibitory effect, rather presented as a substrate and contributed to high 2,3-BDO yields. The acetic acid could activate the enzymes (α-acetoacetate synthase, and acetoin reductase) in 2,3-BDO metabolic pathway (Biswas et al., 2012; Joo et al., 2016). Two fermentation by-products, *i.e.,* lactic acid and ethanol, were detected as low as 1.61±0.25 g/L and 3.05±0.44 g/L, respectively. Previous studies reported that acetic acid could involve in blocking the metabolic pathways for intermediate compounds (succinic and formic acid) and prevented the acidic pH, thus favored the increased 2,3-BDO production (Cheng et al., 2010; Frazer and McCaskey, 1991). However, in our study, the substrate consumption was reduced at later stages of cultivation, concomitant to the lowered 2,3-BDO productivities and increased by-products in the fermentation medium.

3.4.3. Fed-batch Q-SSF

Fed-batch Q-SSF was performed with low initial slurry to circumvent the effect of substrate inhibition. As shown in **Fig 4c,** glucose was rapidly consumed during the first 24 h and caused a drop in pH, which favoured 2,3-BDO production. Once the glucose level dropped below 15 g/L, an additional batch of 55-60 g/L was fed to the fermentation broth. Unlike in the first cycle, glucose consumption rate was slower and 2,3-BDO productivity reached up to 0.58±0.16 g/L/h during 46-84 h. The process continued to reach the maximum 2,3-BDO titer of 51.9±3.05 g/L (0.54±0.13 g/L/h productivity) after 96 h of fermentation. The overall yield was 0.41 g/g (81.8% of the maximum theoretical value), which was 14.2% lower than the batch cycle (section 3.4.2). Xylose may not be consumed due to high glucose feed (CCR effect) or the higher acetoin production, *i.e.,* 11.17±2.07 g/L (0.07 g/g) reflecting to the loss of 2,3-BDO yield. Increased levels of organic acids and ethanol might also divert the carbon flux towards mixed acid fermentation. After 108 h of operation, fermentation was extremely reduced although the medium still contained enough sugars (both glucose and xylose). The unfinished consumption of remaining sugars might be due to the feed-back inhibition due to high concentrations of 2,3-BDO and/or other by-products. Okonkwo et al. (2021) described that 60 g/L 2,3-BDO can inhibit the growth of *Paenibacillus polymyxa*. Kandasamy et al. (2016) reported that 100 g/L 2,3-BDO was the inhibitory concentration for *Lactococcus lactis*, of which its growth rate was declined despite genetic alteration.

3.4.4. Staged fed-batch SHF

To overcome the strict CCR effect observed in the Q-SSF process, a staged-fed-batch SHF strategy was employed to consume xylose and glucose sequentially. During the first stage of the fed-batch SHF, the spent liquor with 80 g/L xylose was used as the sole carbon source of fermentation, and resulted in 33.16±1.35 g/L 2,3-BDO after 48 h of operation. When the residual sugar concentration dropped below 5 g/L, the enzymatic hydrolysate (~65 g/L glucose and ~20 g/L xylose) was fed to the system. Since glucose was more preferable than xylose, the second stage resulted in a higher sugar uptake rate (1.8±0.36 g/L/h) with a yield of 0.41-0.45 g/g (**Fig. 4d**). Interestingly, glucose and xylose were co-utilized to some extent when the residual glucose was <15 g/L and did not significantly repress the pentose metabolism. *K.*

pneumoniae PM2 was found to exhibit a relatively relaxed CCR effect at low glucose levels. During 96 h, 75.03±3.17 g/L 2,3-BDO was produced and found as the highest titer in this study. The productivity was 0.78±0.33 g/L/h and a yield of 0.43 g/g with respect to the total glucan and xylan in the pretreated solids and their monomeric forms in the liquor. At the end of fermentation, glucose was almost utilized completely and 15 g/L xylose remained unutilized.

As shown in **Fig. 4c** and **d**, the productivity of 2,3-BDO of staged-SHF was higher (0.78±0.33 g/L/h) than that of Q-SSF (0.54±0.13 g/L/h) at 96 h. Also, a 5.6% higher 2,3-BDO yield suggested that staged-feeding (xylose → glucose) enhanced the xylose conversion, which further improved the production capacity of PM2. Another probable reason can be noticed in the excess metabolites production. During staged-SHF, lactic acid and ethanol yields were only 0.03 g/g, whereas in Q-SSF, both the by-products reached up to 0.06 g/g, which might have aided to the loss of 2,3-BDO yield. Moreover, acetoin accumulation was increased up to 0.07±0.09 g/g, indicating the change in NADH/NAD^+ flux which converted 2,3-BDO into acetoin (Bao et al., 2015). Increment in the acetic acid concentration (2.47±0.31 g/L) might also have promoted the acetoin production pathway (Wang et al., 2021).

3.5. Comparison with other studies

Different 2,3-BDO producing strains (wild-type) have been studied for high yield 2,3-BDO production under different fermentation strategies and lignocellulosic substrates, which are summarized in **Table 2**. *K. pneumoniae* and *K. oxytoca* produced 21.9 g/L and 31.4 g/L 2,3-BDO via batch SHF using dilute acid pretreated substrates, *i.e.*, soybean hull and jatropha hull hydrolysates, respectively (Cortivo et al., 2019; Jiang et al., 2012), and alkaline-pretreated substrates (*i.e.*, sugarcane bagasse and oil palm frond) converted into 9.2 g/L and 30.7 g/L 2,3-BDO via batch SSF, respectively (Hazeena et al., 2019; Zhao et al., 2011). Higher BDO titers, *i.e.*, 82.5 g/L from *K. pneumoniae SDM* (Wang et al., 2010), 89.6 g/L from *B. subtilis CS13* (Wang et al., 2021), 50.6 g/L from *E. ludwigii* (Psaki et al., 2019) have also been reported but with either media engineering (corn steep liquor, urea, KOH) or process augmentation with sophisticatedly controlled aeration. Even genetic modification of the strains are quite adaptive, but the effects of genetic alteration are strain-dependent, yielding higher/lower/similar 2,3-

BDO yields as compared with the parent strains (Li et al., 2015; Um et al., 2017). Moreover, uncertainty is associated with the adaptability and reproducibility of engineered strains, which additionally causes a burden on the overall process cost and a menace to the natural ecosystem. The wild-type *K. pneumoniae* PM2 found and used in this study produced 51.9±3.05 and 75.03±3.17 g/L 2,3-BDO in fed-batch Q-SSF and staged-SHF processes from EFB, respectively, without addition of any nutrients or aeration control, making it a promising candidate for cost-effective 2,3-BDO production from lignocellulosic biomass.

3.6. Mass balance of the overall process

The mass balance for 2,3-BDO production from SpEFB in an integrated biorefinery was calculated and provided in **Fig. 5**, reflecting the carbon conversion of oil palm EFB among 2,3-BDO, acetoin and other by-products. The sulfite pretreatment process resulted in 257.2 g cellulose, 21.7g hemicellulose in the pretreated solids; and 7.04g cellulose and 129.2g hemicellulose in the spent liquor. The novel *K. pneumoniae* PM2 utilized both C_6 and C_5 sugars and resulted in a total 2,3-BDO production of 83.8 g (Q-SSF) and 134.8g (staged-SHF) based on 1 kg of EFB. The resultant lignosulfonate (LS: 126g, based on the klason lignin removal) can be recovered from the pretreatment liquor, which is a profitable industry product. The fed-batch process using our isolated wild-type strain has demonstrated to be a viable approach for high yield 2,3-BDO integrated biorefinery.

4. Conclusions

The newly isolated *K. pneumoniae* PM2 has proved to be an ideal candidate for high 2,3-BDO titer production (75.03±3.17 g/L) from oil palm EFB biomass. Without any addition of genetic modification or sophisticated aeration control during fermentation, the wild-type strain achieved an excellent utilization of both xylose and glucose which were provided from the sulfite pretreated EFB biomass whole slurry (substrate hydrolysate and pretreatment spent liquor). The integration of the fermentation strain, pretreatment process, and fed-batch staged-SHF process demonstrated significant potential of EFB biorefinery in supporting the sustainable development of the oil palm industries.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

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Figure Captions

Fig. 1. Scheme of 2,3-BDO fermentation using sulfite-pretreated empty fruit bunches. [Q-SSF: Quasi-simultaneous saccharification and fermentation, SHF: separate hydrolysis and fermentation].

Fig. 2. Optimization of 2,3-BDO fermentation conditions. **(a)** Effect of temperature **(b)** Effect of initial pH of the medium **(c)** Effect of agitation speed **(d)** Effect of initial glucose concentration **(e)** Effect of initial xylose concentration on 2,3-BDO production by *K. pneumoniae* strain PM2 **(f-j)** Corresponding yields and productivities of the processes. Lines and bars showing in red are the optimum conditions observed. All data points are the average of duplicate experiments ± standard deviations.

Fig. 3. Tolerance test by *K. pneumoniae* PM2. **(a)** 2,3-BDO tolerance **(b)** Acetic acid toxicity for sulfite pretreatment feasibility.

Fig. 4. Fermentation profile of *K. pneumoniae* PM2 using pure sugars and sulfite-pretreated EFB under optimum conditions (30°C, 150 rpm, initial pH 7, and initial inoculum of 10%). **(a)** Batch process with pure sugars (glucose 70g/L and xylose 30g/L), **(b)** Batch Q-SSF, **(c)** Fed-batch Q-SSF, and **(d)** Fed-batch staged-SHF. Error bars represent the mean ± S.D. of two independent cultivations.

Fig. 5. Preliminary mass balance of a 2,3-BDO integrated oil palm empty fruit bunches biorefinery.

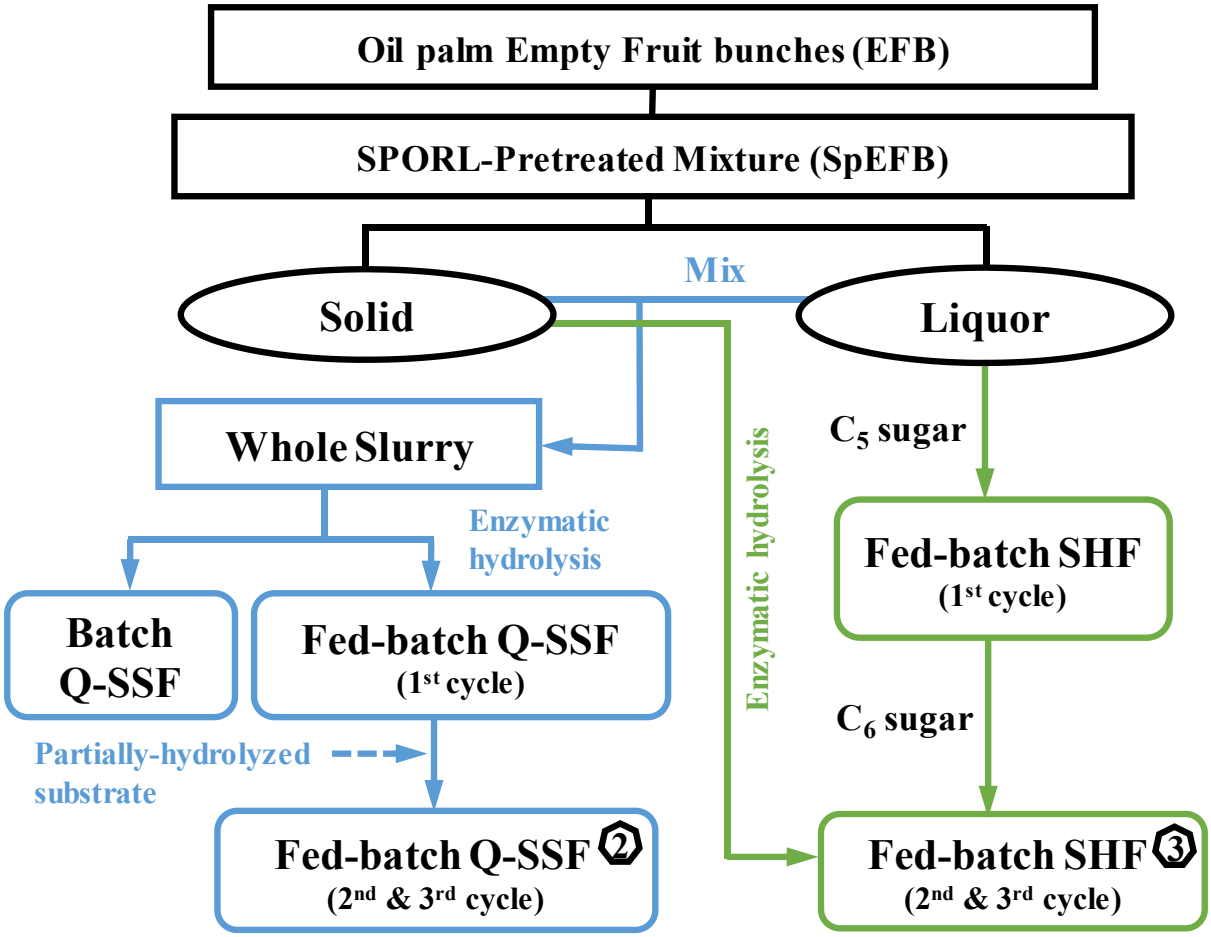
Oil palm Empty Fruit bunches (EFB)
SPORL-Pretreated Mixture (SpEFB)
Solid
Mix
Liquor
Whole Slurry
Enzymatic hydrolysis
Batch Q-SSF
Fed-batch Q-SSF (1st cycle)
Partially-hydrolyzed substrate
Fed-batch Q-SSF ② (2nd & 3rd cycle)
Enzymatic hydrolysis
C5 sugar
Fed-batch SHF (1st cycle)
C6 sugar
Fed-batch SHF ③ (2nd & 3rd cycle)

Fig. 1

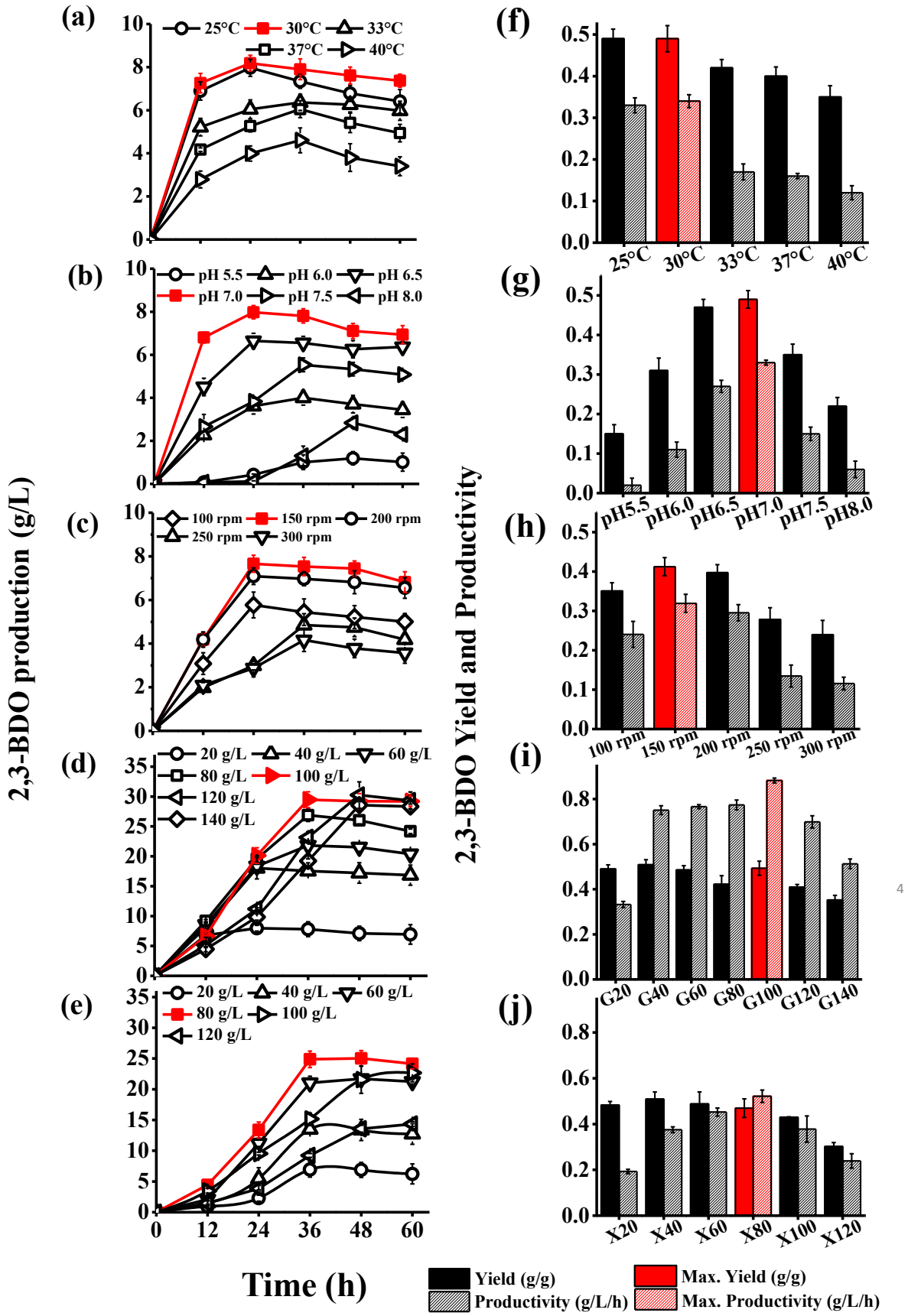
(a)
25°C
30°C
33°C
37°C
40°C
(b)
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 7.5
pH 8.0
(c)
100 rpm
150 rpm
200 rpm
250 rpm
300 rpm
(d)
20 g/L
40 g/L
60 g/L
80 g/L
100 g/L
120 g/L
140 g/L
(e)
20 g/L
40 g/L
60 g/L
80 g/L
100 g/L
120 g/L
2,3-BDO production (g/L)
Time (h)
(f)
25°C
30°C
33°C
37°C
40°C
(g)
pH5.5
pH6.0
pH6.5
pH7.0
pH7.5
pH8.0
(h)
100 rpm
150 rpm
200 rpm
250 rpm
300 rpm
(i)
G20
G40
G60
G80
G100
G120
G140
(j)
X20
X40
X60
X80
X100
X120
2,3-BDO Yield and Productivity
Yield (g/g)
Productivity (g/L/h)
Max. Yield (g/g)
Max. Productivity (g/L/h)

Fig. 2

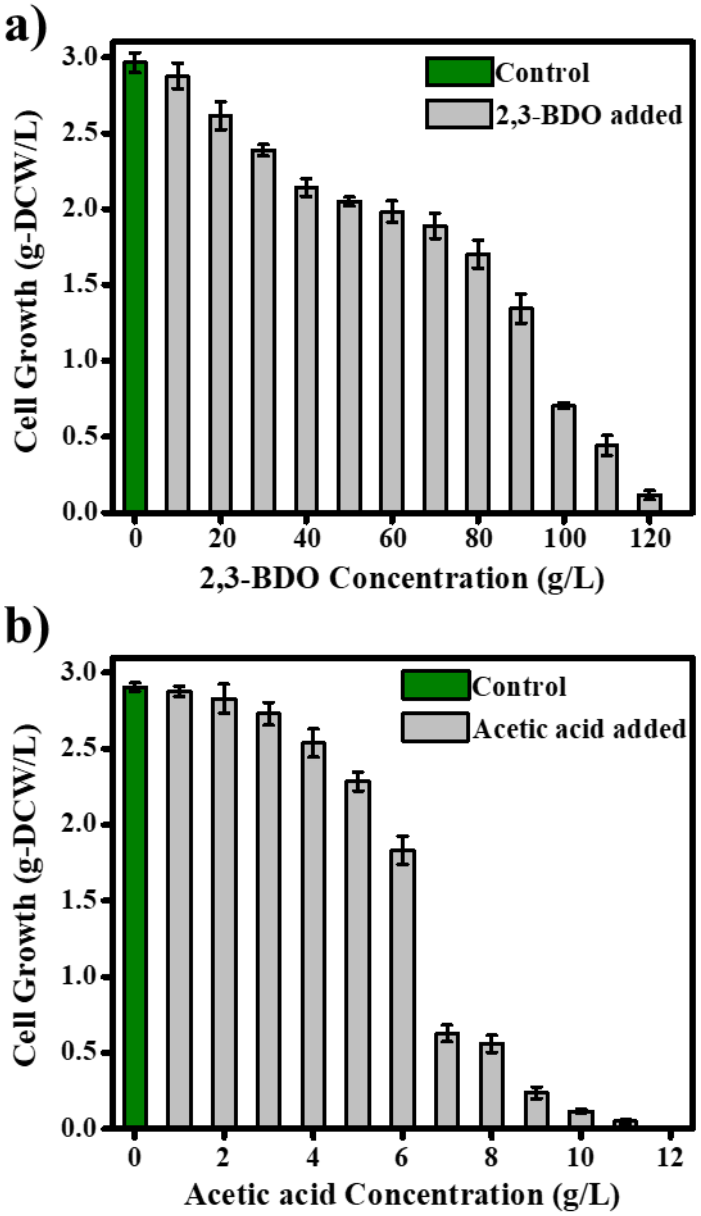
a)
Control
2,3-BDO added
Cell Growth (g-DCW/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
2,3-BDO Concentration (g/L)
b)
Control
Acetic acid added
Cell Growth (g-DCW/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
Acetic acid Concentration (g/L)

Fig. 3

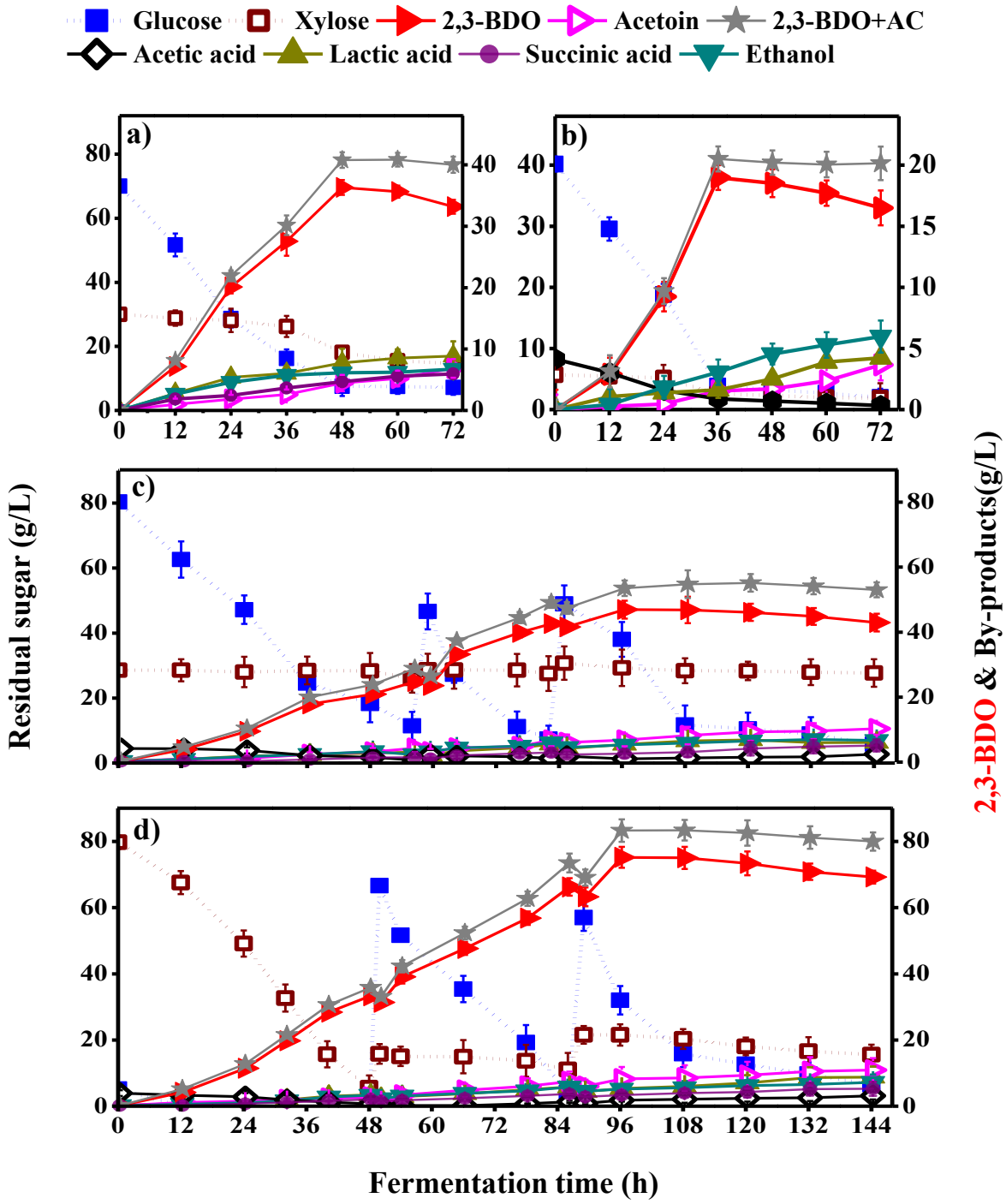
Glucose
Xylose
2,3-BDO
Acetoin
2,3-BDO+AC
Acetic acid
Lactic acid
Succinic acid
Ethanol
a)
b)
c)
d)
Residual sugar (g/L)
2,3-BDO & By-products(g/L)
Fermentation time (h)

Fig.4

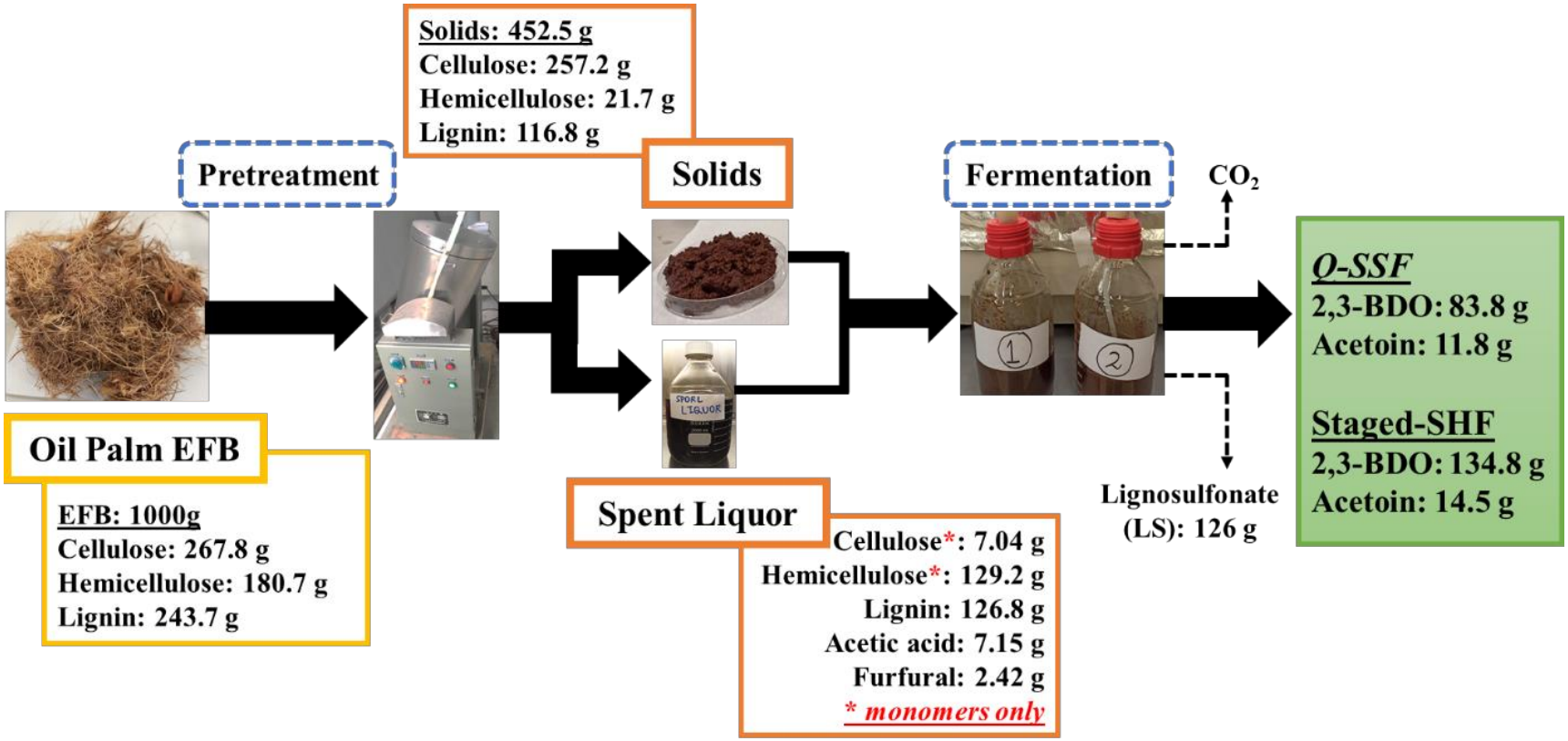
Solids: 452.5 g
Cellulose: 257.2 g
Hemicellulose: 21.7 g
Lignin: 116.8 g
Pretreatment
Solids
Fermentation
CO_2
Oil Palm EFB
EFB: 1000g
Cellulose: 267.8 g
Hemicellulose: 180.7 g
Lignin: 243.7 g
Spent Liquor
Cellulose*: 7.04 g
Hemicellulose*: 129.2 g
Lignin: 126.8 g
Acetic acid: 7.15 g
Furfural: 2.42 g
* monomers only
Lignosulfonate
(LS): 126 g
Q-SSF
2,3-BDO: 83.8 g
Acetoin: 11.8 g
Staged-SHF
2,3-BDO: 134.8 g
Acetoin: 14.5 g

Fig. 5

Table 1. Chemical compositions of the biomass and substrates

Components (wt%, dry basis)	**Raw EFB**	**Sulfite-pretreated EFB**	
		Pretreated Solids	**Spent Liquor**
Cellulose	26.78 ± 0.29	56.83 ±0.37	0.64 ±0.03
Hemicellulose	18.07 ±0.83	4.81 ±0.16	11.75 ±0.14
Lignin	24.37 ±0.02	25.82 ±1.11	11.53 ±0.23
Ash	2.15 ±0.05	1.86 ±0.11	-
Acetic acid	-	-	0.65 ±0.12
Furfural	-	-	0.22 ± 0.05

Table 2. 2,3-Butanediol production by different microorganisms (wild type) from lignocellulosic biomass substrates

Microorganism	Substrate (Pure/Crude)	Pretreatment method	Culture mode	Fermentation performance				Reference
				C_{BDO}[b] (g/L)	$S_{add.}$[c] (g/L)	Y_{BDO}[d] (g/g)	Q_{BDO}[e] (g/L/h)	
K. pneumoniae CGMCC 1.9131	Sugarcane bagasse	Alkali/peracetic acid	Batch SSF	9.2	34	0.35	0.07	(Zhao et al., 2011)
Klebsiella sp. Zmd30	Bagasse hydrolysate	Alkaline	Batch SHF	8.3	150	0.18	0.66	(Wong et al., 2012)
	Rice straw hydrolysate			24.6	150	0.31	2.41	
K. pneumoniae PM2	Oil palm empty fruit bunches	Sulfite (SPORL[a])	Batch SSF	19.0	46	0.48	0.52	**This work**
			Fed-batch SSF	**51.9**	192	0.41	0.54	**This work**
			Fed-batch SHF	**75.0**	225	0.43	0.78	**This work**
K. pneumoniae	Soybean hull hydrolysate	Dilute acid	Batch SHF	21.9	62	0.40	0.30	(Cortivo et al., 2019)
Pantoea agglomerans				20.5	52	0.44	0.55	
E. aerogenes NCIMB 10102	Corn starch hydrolysate	Industrial by-product	Batch SHF	8.8	20	0.44	0.06	(Perego et al., 2000)
E. cloacae SG1	Oil palm frond	Alkaline	Batch-SSF	30.7	120	0.47	0.32	(Hazeena et al., 2019)
K. oxytoca CICC 22912	Jatropha hull hydrolysate	Detergent washing & acid hydrolysis	Batch SHF	31.4	80	0.40	0.79	(Jiang et al., 2012)
P. polymyxa DSM 365	Wheat straw hydrolysate	Dilute acid	Batch SHF	32.5	110	0.33	0.45	(Okonkwo et al., 2021)

[a] Sulfite pretreatment to overcome recalcitrance of lignocellulose; [b] 2,3-BDO concentration in the broth; [c] Total sugar added; [d] 2,3-BDO yield (g/g-sugar consumed); and [e] Productivity (at which maximum 2,3-BDO titer was observed).

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: