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Fermentative production of 2,3-Butanediol using bread waste – A green approach for sustainable management of food waste

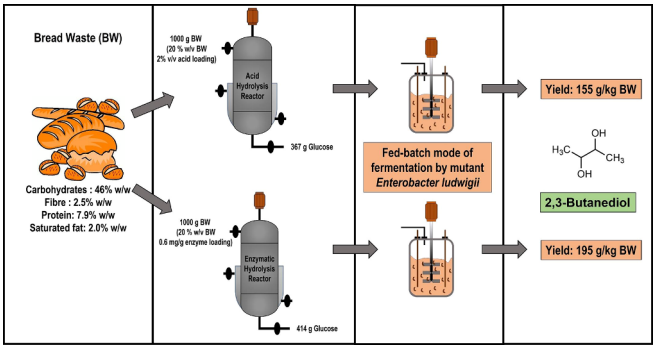
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HIGHLIGHTS

- Evaluation of bread waste (BW) as feedstock for 2,3-butanediol (BDO) production.
- Glucose yield of 330–530 g/kg BW was obtained by acid and enzymatic hydrolysis.
- BDO titer of 144.5 g/L and yield of 0.47 g/g was achieved on pure glucose.
- BDO titer of 135.4 g/L with a yield of 0.42 g/g was amassed from acid hydrolysed BW.
- Enzyme hydrolysed BW generated 138.8 g/L BDO with a yield of 0.48 g/g.

GRAPHICAL ABSTRACT



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ABSTRACT

Bread is Europe's most wasted food, and the second most wasted food after potatoes in UK. Bread waste (BW) is a clean source of high-quality fermentable sugars. In this study, the potential of *Enterobacter ludwigii* to accumulate 2,3-butanediol (BDO) from BW was evaluated. Initially, the optimal inoculum size and yeast extract concentration were determined, followed by extraction of sugars from BW using acid and enzymatic hydrolysis. A glucose yield of 330–530 g/kg BW was obtained, and the sugars released were utilised for BDO production by *E. ludwigii*. The fed-batch cultivation using pure glucose and glucose rich hydrolysates from acid and enzymatic hydrolysis resulted in BDO titres of 144.5, 135.4, and 138.8 g/L, after 96 h, with yield of 0.47, 0.42 and 0.48 g/g yield, respectively. The innovation of the work is valorisation of BW to BDO with a circular biorefining approach and thus, reducing BW disposal and associated environmental burden.

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1. Introduction

Mankind is witnessing an era of resource scarcity and the demand and cost of existing resources is increasing due to an exponentially increasing human population which combined with need for a clean environment is mounting massive pressure on natural reserves. Similarly, the staggering volume of food waste (FW) is matter of global concern. It has been estimated that about 1/3rd portion of the food produced is either thrown away or ends up in a landfill incurring a huge loss ($750 billion) to the global economy (Kumar and Longhurst, 2018). This has subjected food supplies to immense strain, which is compounded by the rapid increase in the global population. For example, every year 10–15 million metric ton of food is wasted across the supply chain in the UK and preventing it can save £15-20 billion and 27 million metric ton of greenhouse gases from entering the atmosphere (HM Government, 2018). FW is a valuable resource that needs to be utilised intelligently with a circular economy approach, instead of being dumped at landfill sites.

Currently, bio-based products such as ethanol, acetic acid (AA), lactic acid (LA), succinic acid (SA), and biodiesel are obtained from microbial conversion of edible or first-generation feedstocks. Considering the population explosion and global food shortage, the competing uses of edible substrates in food and feed industry represents a dilemma and has hastened the search for alternative non-competing feedstocks. Bread is staple food in Europe and North America with annual global production of 100 million metric ton and it is estimated that $>$ 10% of manufactured bread is wasted globally. Europe dominates the world market with share of $\sim$ 57% and at the same time, bread wastage is a serious problem in many of the EU countries ("World Bread Market and Trends [WWW Document], 2018). In UK, it is a severe problem with 20–25 million of bread slices goes to bin on daily basis. About 44% of manufactured bread is discarded, amounting to 292,000 metric ton per year that not only cause a massive loss of resources and revenue, but improper disposal like landfilling could result in CO_2 emissions around 584,000 metric ton (Narisetty et al., 2021). Currently, food/bread waste in UK is treated with anaerobic digestion (AD) which is not a suitable technology because of two reasons (Ackers, n.d.; Food surplus and waste in the UK – key facts [WWW Document], 2021). First, AD has low environmental savings (Brancoli et al., 2020) and secondly, methane produced from AD is a low-value product, whereas potential of BW as feedstock is much higher. BW contains 50–70% starch and is a clean source of high-quality fermentable sugars with consistent compositions that can be used as feedstock for bio-based production of chemical building blocks (Cox et al., 2022; Narisetty et al., 2021).

2,3-Butanediol (BDO) is a valuable established chemical and gateway molecule to a wide range of chemical derivatives. BDO exist in three isomeric forms (levo, dextro and meso) and different properties of these enantiomer enable its versatile applications. Being a versatile chemical feedstock and fuel due to its high heating value (27.2 kJ/g) and low vapor pressure (0.23 hPa), BDO finds extensive industrial (food, chemical, pharmaceutical, petrochemical, and aerospace) applications with vast commercial potential. It has been forecasted that BDO market would reach $300 million by 2030 (2,3-Butanediol Market, 2020; Amraoui et al., 2021; Maina et al., 2021a; b). In recent times, owing to the unsustainability of traditional fossil-based BDO production processes, associated environmental concerns, and increasing prices of fossil fuels, the BDO production from fermentative routes has become apparent as a potential alternative. The current market price of BDO is $\sim$ $3.23/kg and a low-cost production can lead to an economical bioprocess and enable wider applications of BDO (Tinôco et al., 2021). The cost of substrate is one of the several factors impacting the process economics and utilization of a cheap/renewable feedstock could result in a feasible process. To this end, the current study attempted use of BW for sustainable production of BDO by *Enterobacter ludwigii*. The work started with optimization of inoculum size and yeast extract concentration for BDO fermentation. BW was then saccharified using acid and enzymatic hydrolysis and the released sugars were used as substrate for BDO production in shake flasks. The data was scaled up in bioreactor and fed-batch cultivation was performed using glucose rich acidic/enzymatic hydrolysate and benchmarked against pure glucose.

2. Materials and methods

2.1. Materials, microorganism, and cultivation conditions

The analytical grade chemicals, media components, and other chemical standards used were purchased either from Sigma-Aldrich or Thermo Fisher Scientific. The expired bread was procured from the local supermarket (see supplementary material for composition, as provided by the manufacturer) and referred as bread waste (BW) in the following text. Dextrozyme Peak enzyme, kindly provided by Novozymes, was used for the enzymatic saccharification of BW. The BDO biosynthesis experiments were performed using a mutant *E. ludwigii* strain developed in previous study (Amraoui et al., 2022).

The strain was cultivated in sterile tryptic soy (TS) broth and incubated for 16 h. The glycerol stocks (50% v/v) were made using the harvested cells after the incubation and stored at −80 °C until further use (Amraoui et al., 2022). For the pre-inoculum preparation, sterile TS broth was inoculated with a *E. ludwigii* colony from a freshly subcultured plate and incubated for 12 h at 30 °C and 180 rpm. The details of the production medium used for the fermentative production of BDO have been described in detail in previous study (Amraoui et al., 2022).

2.2. Optimization of inoculum size and yeast extract concentration for improved BDO production

In previous work, the optimization of media components using the *E. ludwigii* wild type strain was carried work (Amraoui et al., 2022), however as the work progressed, the mutant strain was observed to be a potent strain for BDO production. To further evaluate the ability of the strain to improve substrate uptake and BDO production, the size of pre-inoculum (2, 5, 10, 15 and 20% v/v) and yeast extract (2.0, 5.0, 7.5 and 10.0 g/L) concentration were optimized. Prior to inoculation, the pH of the production medium was adjusted to 6.6. Since bacterial growth and BDO production are dependent on the pH, it was maintained within a range of 6.0 – 7.0 throughout the incubation period using 5 N NaOH (sodium hydroxide). All the shake flask cultivations in this study were performed in 500 mL Erlenmeyer flasks with 100 mL working volume in incubator shaker (Excella 24, New Brunswick) at 30 °C and 180 rpm.

2.3. Integrated hydrolysis of bread waste and BDO biosynthesis in shake flask experiments

A one pot integrated approach for hydrolysis of BW and BDO biosynthesis was carried out in shake flasks, wherein BW was hydrolysed using hydrochloric acid (HCl) and Dextrozyme Peak, respectively. Acidic hydrolysis of BW was carried out at different solid loadings (5 – 30% w/v) with 2% v/v HCl concentration and incubation at 121 °C for 15 min in an autoclave. After the hydrolysis, the solid–liquid suspension was cooled, media components at the required concentrations for BDO production were added to the suspension and the pH was adjusted to 6.6 using 5 N NaOH.

For enzymatic hydrolysis, the 500 mL Erlenmeyer flasks with different BW solid loadings (5 – 30% w/v) and suspension pH adjusted to 4.3 using HCl was subjected to gelatinization by incubating at 121 °C for 15 mins in an autoclave. After the gelatinization, the BW suspension was cooled to room temperature and incubated at 45 °C until the suspension attains the desired temperature followed by enzyme loading of 0.6 mg/g BW and further incubation for 48 h at 250 rpm. After enzymatic hydrolysis, the media components at the required concentrations for BDO production were added and the pH of suspension was adjusted to 6.6

using 5 N NaOH. As described in section 2.1 the freshly prepared pre-inoculum was added to the respective flasks containing glucose rich hydrolysate with media components and incubated in a shaker incubator (Excella 24, New Brunswick) at 30 °C and agitation rate of 180 rpm.

2.4. Bioreactor cultivation

For the bioreactor cultivation, the BW was saccharified using acid and enzyme in 2.0 L Erlenmeyer flasks with the procedure described in the section 2.3. The BW hydrolysates obtained were filtered using muslin cloth to filter the solid particles. Further, the glucose rich supernatant obtained was concentrated using vacuum rotavapor (BUCHI, UK) to a glucose concentration of about 200 g/L. The production media in the bioreactor was supplemented using the concentrated glucose solution as carbon source for BDO production.

Fed-batch mode of cultivation was carried out in a 2.5 L bench-top bioreactor (Electrolab, UK) with 1.0 L initial working volume using pure glucose and crude glucose rich acidic and enzymatic hydrolysates from BW. The operational parameters, temperature, agitation speed and aeration rate were maintained at 30 °C, 180 rpm and 1.0 vvm, respectively. The pH of production media was maintained at 6.6 using 5 N NaOH throughout the incubation period. Initially the fed-batch was initiated with the production media containing approximately 100 g/L glucose, further the bioreactor was supplemented using the concentrated solution of pure glucose or acidic/enzymatic glucose rich BW hydrolysates to maintain glucose concentration above 20 g/L for BDO production.

2.5. Analytical methods

The samples withdrawn at regular intervals from the experiments carried out in this study were analyzed for cell growth (OD_{600}), pH, concentration of residual glucose, BDO and by-products like acetoin, ethanol, LA, SA, and AA. Due to interference and high turbidity caused by the presence of solid particles in acid and enzymatic hydrolysate from BW, the OD_{600} could not be measured. Metabolite analysis was carried out using High-Pressure Liquid Chromatography (HPLC), where the samples withdrawn at regular intervals were centrifuged at 10,000 × g for 10 min, and further filtered through a 0.22 μm nylon membrane filters before loading the sample for analysis. Residual glucose, BDO, acetoin, and ethanol were analysed using Rezex ROA-Organic Acid H + (Phenomenex, USA) column connected to a Refractive Index Detector (RID) using 5 mM H_2SO_4 mobile phase with a flow rate of 0.4 mL/min. The organic acids like LA, SA, and AA were analysed using the same column connected with Diode Array Detector (DAD) and eluted using 5.0 mM H_2SO_4 as a mobile phase with a flow rate of 0.6 mL/min. All of the measurements were conducted in triplicate and the average values were determined. The standard deviation was not>10%.

3. Results and discussion

3.1. BDO production from glucose by E. Ludwigii at different inoculum levels

E. ludwigii is a Gram-negative bacterium and potent cell factory for accumulating large levels of BDO from a variety of carbon sources including monosaccharides (hexose and pentose) as well as disaccharides (Maina et al., 2019; Psaki et al., 2019). The fermentation process begins with the inoculation of culture medium, and the quality and size of the inoculum are important parameters influencing the success of fermentation. Inoculum size reflects the population of viable cells, and an appropriate size is essential for ensuring shorter lag phases, faster conversion of substrates, and increased productivity.

In shake flask experiments, different inoculum levels (2, 5, 10, 15 and 20% v/v) were tested to determine the optimal size. Fig. 1 shows the time-course profiles for glucose consumption, cell growth (OD_{600}), pH fluctuations and BDO and acetoin production for each tested inoculum size. BDO production is generally accompanied by a rapid drop in pH and the cells stop accumulating BDO once the pH drops below 5.0. This problem is often encountered in flask cultures with high sugar levels (>20 g/L), as a result of it, considerable amount of sugar is left unconsumed (Amraoui et al., 2021, 2022). The experiments in this study had initial glucose levels of 51–57 g/L and the pH was manually maintained between 6.0 and 7.0 to counteract the pH drop. The approach enabled a continuous increase in cell growth throughout the fermentation period. The cell growth (OD_{600}: 11–12) was similar at the 2% and 5% inoculum sizes, whereas the growth at inoculum size of 10–20% was considerably higher (OD_{600}: 14–19). The highest BDO titre obtained using 2, 5, 10, 15 and 20% v/v inoculum sizes were 19.0, 23.1, 22.1, 24.1 and 22.9 g/L, with a yield of 0.37, 0.44, 0.42, 0.43 and 0.41 g/g, respectively. The changes in the BDO titre and yield were insignificant at inoculum sizes > 5% v/v; therefore, the inoculum size of 5% v/v for further experiments. Acetoin, AA, LA, SA and ethanol were obtained as by-products. Acetoin was the major by-product obtained at concentrations of 1.6, 1.3, 1.7, 2.8 and 1.9 g/L, with inoculum sizes of 2, 5, 10, 15 and 20% v/v, respectively.

Enterobacter spp. such as *E. ludwigii*, *E. cloacae*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* are well-known for their ability to produce BDO. BDO production starts with the condensation of pyruvate, followed by decarboxylation and reduction. This mixed-acid fermentation also yields several by-products such as acetoin, AA, LA, SA, and ethanol. BDO, being a neutral metabolite, is produced by microbial cultures to counteract the acidification of broth (Maina et al., 2021a). Similar to the observations in this study, several studies have shown that certain inoculum sizes have a positive effect on the BDO production rate and yield (Nilegaonkar et al., 1992; Okonkwo et al., 2017; Song et al., 2019; Yu and Saddler, 1983). For example, Perego et al. (2003) found continuous improvements in BDO yield and productivity from 0.11 to 0.35 g/g and ~ 0.04 to 0.35 g/L/h by *Bacillus licheniformis* with increase in inoculum size from 0.5 to 10 g/L, respectively (Perego et al., 2003).

3.2. Impact of yeast extract on cell growth and BDO accumulation

Many bioprocesses for the fermentative production of chemicals rely on the use of complex nitrogen sources such as peptone, yeast extract and tryptone at high levels, which limit their implementation at the commercial scale. Microbial fermentation for BDO production requires the use of yeast extract, which contain various components such as essential amino acids, vitamins, minerals, and macro/micro-elements necessary for enhancing biomass formation and eventually, the BDO formation rate (Erian et al., 2018). However, yeast extract, when used at high concentrations, incurs extra costs to the bioprocess, complicates downstream processing and may limit the industrial feasibility of the bioprocess (Yu et al., 2022). Therefore, it is crucial to determine the optimal concentration of yeast extract to trade-off between its advantages and limitations. The *E. ludwigii* strain used in this study was cultivated on different concentrations of yeast extract (2.0, 5.0, 7.5 and 10.0 g/L) and its effects on substrate uptake, cell growth and product formation were examined (Fig. 2). At 2.0 g/L of yeast extract, the initial glucose concentration of 54.1 g/L was depleted in 28 h, leading to a cell growth of 12.2 (OD_{600}) and BDO accumulation of 22.1 g/L with a yield of 0.41 g/g. Increasing the yeast extract concentration from 2.0 to 5.0 g/L improved the glucose uptake rate, cell growth (OD_{600}:14.5) and BDO production. The fermentation lasted for 24 h and a final BDO titre of 25.4 g/L was obtained with a yield of 0.46 g/g. Further increments in the yeast extract concentration caused substantial enhancement in cell growth without any significant improvement in BDO accumulation. Therefore, the yeast extract concentration of 5.0 g/L was considered optimal for maximal BDO production. In contrast to the results in this study, the fermentative production of BDO has been shown to require a

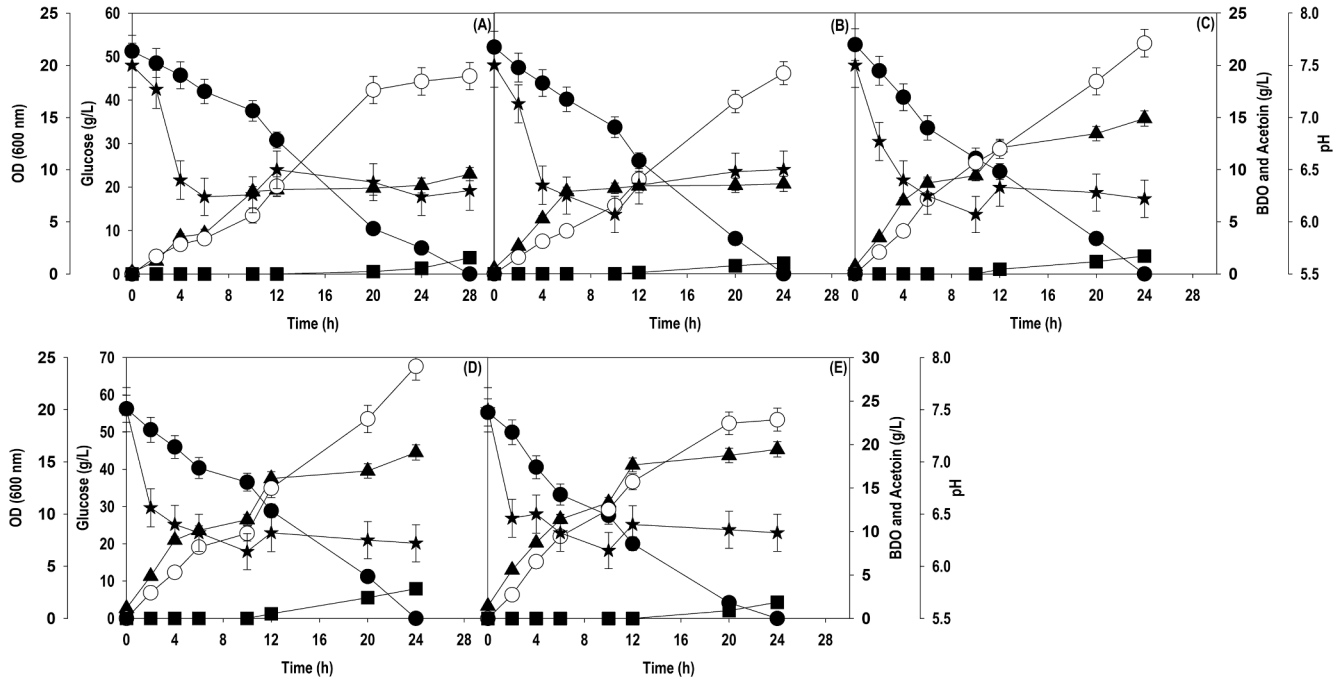


Fig. 1. Shake flask cultivation of *E. ludwigii* on glucose at different inoculum size (v/v): (A) 2%; (B) 5%; (C) 10%; (D) 15% and (E) 20%. Symbols: Glucose (filled circle), OD_{600} (filled triangle), BDO (empty circle), acetoin (filled square) and pH (filled star).

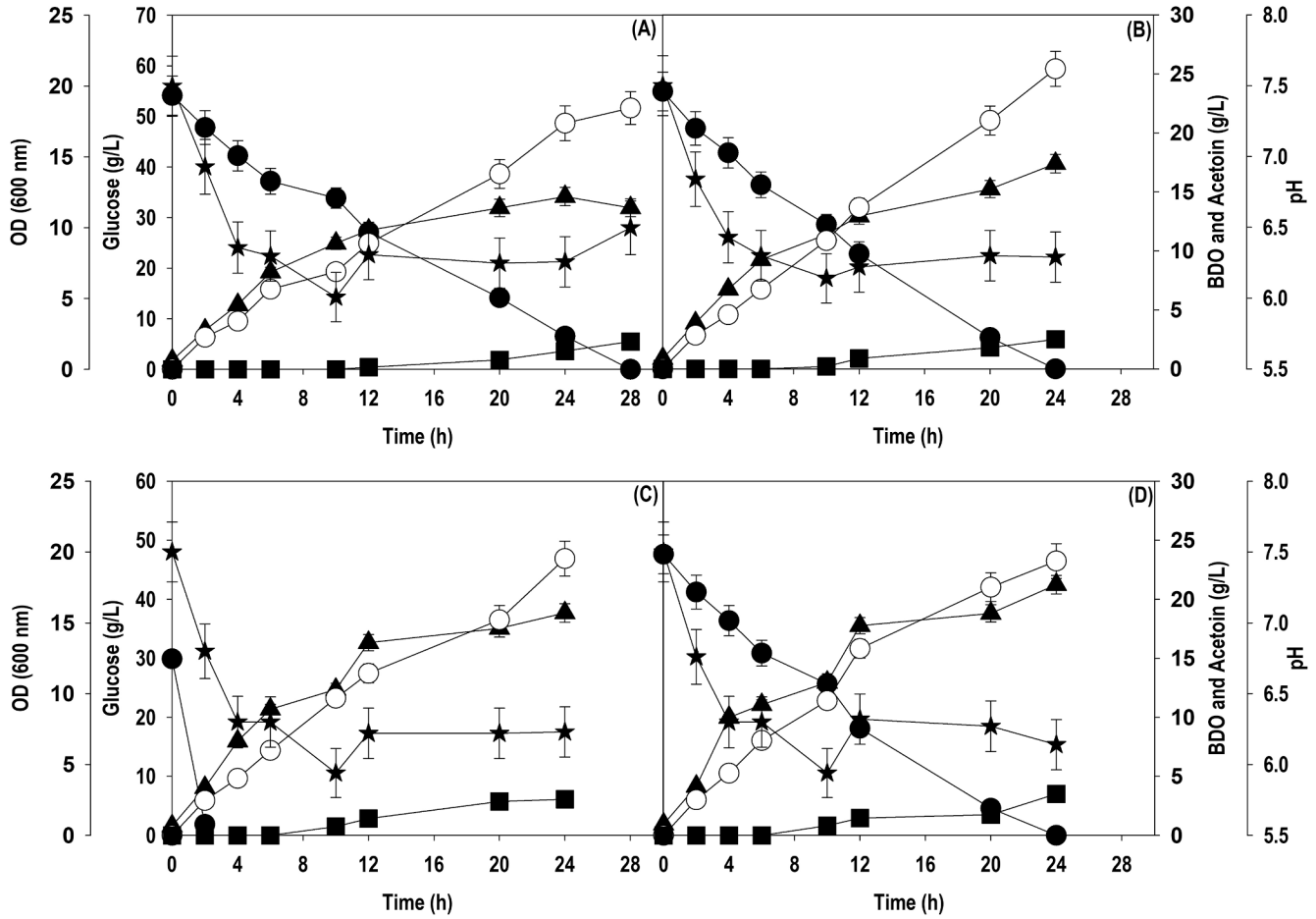


Fig. 2. Batch cultivation of *E. ludwigii* in shake flask supplemented with different levels of yeast extract (w/v): (A) 0.2%; (B) 0.5%; (C) 0.75% and (D) 1.0%. Symbols: Glucose (filled circle), OD_{600} (filled triangle), BDO (empty circle), acetoin (filled square) and pH (filled star).

high content of complex nitrogen sources for high productivity. Yu et al. (2022) achieved BDO production using *B. licheniformis* with an initial glucose concentration of 56 g/L and varying levels of yeast extract and peptone. They obtained a BDO titre of 17.2 g/L at 60 h using yeast extract and peptone at concentrations of 10 and 5 g/L, respectively. The fermentation slowed down, yielding a BDO titre of 11.5 g/L when the yeast extract and peptone were reduced to 5 and 2.5 g/L, respectively (Yu et al., 2022). Fermentation did not occur upon further decreasing the yeast extract and peptone concentrations to 2 and 1 g/L, respectively, indicating that an insufficient supply of complex nitrogen sources may even halt the bioprocess. Similarly, Tsigoriyna et al. (2021) also observed that the presence of yeast extract and peptone enhanced BDO production. They found the optimal yeast extract and tryptone concentrations to be 13.4 and 6.4 g/L, respectively and obtained a high BDO titre (>90 g/L) using these optimal values. Likewise, Häßler et al. (2012) achieved a BDO titre of 111 g/L in a fermentation medium containing massive amount of yeast extract (60 g/L). Compared with these reports, the *E. ludwigii* strain in the present study required a low yeast extract concentration of 2–5 g/L to obtain a high yield of BDO.

3.3. Integrated BDO manufacturing using glucose from acidic hydrolysate of bread waste

Glucose rich acid and enzymatic BW hydrolysates obtained after the saccharification of BW, was utilised as a source of fermentable sugar for BDO production. BW is a starchy material that requires pretreatment to release free glucose. Usually, enzymatic hydrolysis is used for the saccharification of starchy wastes such as BW. However, before enzymatic hydrolysis, acid hydrolysis was performed, which is not only more economical than enzymatic hydrolysis but also requires less time for saccharification. Acid hydrolysis was conducted at different solid loadings (5, 10, 15, 20, 25 and 30% w/v BW) with 2% v/v acid loading. The glucose concentration increased with an increase in solid loading, but the opposite trend was obtained for the glucose yield. The glucose titres obtained at solid loadings of 5, 10, 15, 20, 25 and 30% w/v were 25.0, 47.3, 62.9, 73.6, 87.2 and 99.8 g/L, respectively, which is equivalent to theoretical glucose yields of 97.9, 92.7, 82.1, 71.8, 68.3 and 65.2%, respectively. The BW hydrolysate obtained was used for BDO production, as mentioned in section 2.3. The trends in glucose assimilation, BDO and acetoin production and pH are presented in Fig. 3. The fermentation duration increased as the solid loading and glucose concentration increased. The BDO titres obtained at solid loadings of 5, 10, 15, 20, 25 and 30% w/v were 9.0, 21.7, 29.0, 30.9, 27.8 and 29.0 g/L at 10, 18, 28, 28, 32 and 32 h with yield of 0.36, 0.46, 0.47, 0.47, 0.40 and 0.40 g/g, respectively (Table 1). The BW hydrolysate using 2% v/v acid and 20% w/v solid loading resulted in highest BDO concentration and yield. Acetoin was obtained as major byproduct in all the cases and its concentration was under 5.0 g/L. However, glucose was not fully consumed at higher solid loading of 25 and 30% w/v where residual glucose concentration of 18.4 and 27.9 g/L was noticed even after 32 h.

In previous work, we noticed that release of furfural and 5-hydroxymethylfurfural (HMF) during acid hydrolysis of BW which linearly increased with increase in solid to liquid ratio. At 2% v/v acid concentration, furfural and HMF obtained with 15% w/v BW suspension were 0.49 and 1.79 g/L while in case of 20% w/v solid loading, their concentration was 0.65 and 2.34 g/L, respectively (Narisetty et al. 2022). Furfural and HMF are well known inhibitors for microbial systems. The

Table 1
Summary of glucose and BDO yields obtained from acid hydrolysis of BW at different solid loadings.

Solid loading (%w/v)	Glucose released (g/L)	Glucose yield (g/g)	Maximum achievable glucose (g/L)	Percentage of theoretical yield (%)	BDO Yield (g/g Glucose) *
5	25.0 ± 1.7	0.50	25.53	97.9	0.36
10	47.3 ± 2.8	0.47	51.06	92.7	0.46
15	62.9 ± 3.6	0.42	76.59	82.1	0.47
20	73.3 ± 4.7	0.37	102.12	71.8	0.47
25	87.2 ± 5.5	0.35	127.65	68.3	0.40
30	99.8 ± 5.4	0.33	153.18	65.2	0.40

*At solid loading beyond 15% w/v, glucose was not fully consumed and the yield was not calculated on the basis of total glucose supplied but glucose consumed till the end of fermentation period.

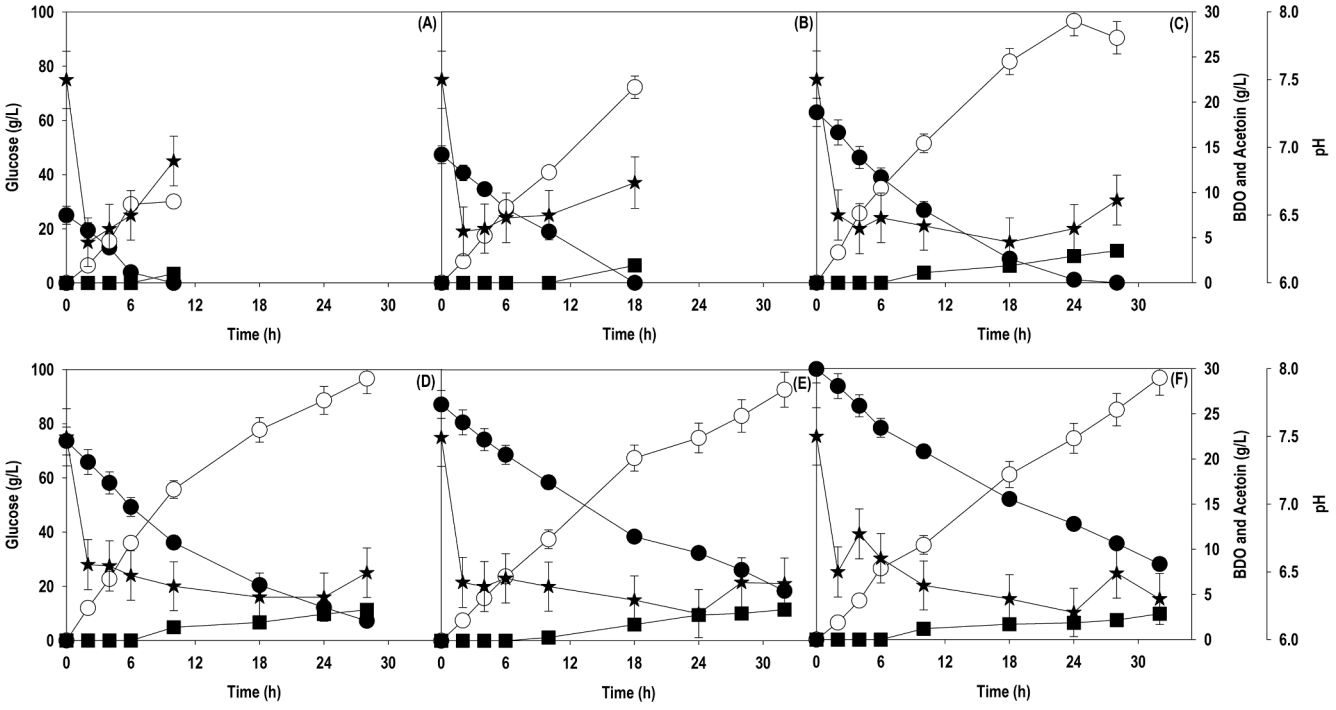


Fig. 3. Integrated acid hydrolysis of BW and BDO & acetoin production in shake flask at different solid loadings (w/v): (A) 5%; (B) 10%; (C) 15%; (D) 20%; (E) 25% and (F) 30%. Symbols: Glucose (filled circle), BDO (empty circle), acetoin (filled square) and pH (filled star).

other problem encountered with increment in solid to liquid ratio is that it impedes the mass and heat transfer, as the suspension becomes highly viscous. Pleissner and associates investigated lactic acid production from mixed restaurant starchy FW and witnessed a drop in lactic acid yield with increase in solid to liquid ratio of starchy FW suspension. They noticed the problem of mixing in suspension with solid loading above 20% w/v due to high viscosity which eventually slow down the mass transfer and negatively impact product formation (Pleissner et al., 2017). Similarly, Demichelis et al., 2017 made use of FW for bio-production of lactic acid and observed large drop in glucose yield with increase in the solid to liquid ratio of FW suspension beyond 12.5% w/v. In the current work, it is also speculated that the problem of high viscosity and mass transfer along with the presence of fermentation inhibitors at higher solid loadings (25 and 30% w/v) negatively impacted the release of glucose and cell metabolism. As a result of it, glucose assimilation and BDO formation were slowed down leading to significant amount of glucose unconsumed at the end of fermentation at solid loading $>$ 20% w/v. Thus, a better hydrolytic performance, mixing, mass transfer and BDO fermentation can be assumed at solid loading $<$ 20%.

There are few reports in last few years where acid hydrolysed BW has been used for fermentative production of chemicals. Torabi et al. (2020) made use of acid BW hydrolysates for ethanol production by *Saccharomyces cerevisiae*. They used solid loading of 16% w/v and achieved glucose yield of 69.8% at 1% v/v acid loading and the yield reduced to 48.1% when acid concentration was increased to 2.0% v/v. The ethanol yield obtained using hydrolysate with highest glucose yield was 248 g/kg bread residues. Cox et al. (2022) demonstrated LA production from glucose rich BW hydrolysate by *Bacillus coagulans* and for this purpose, BW was hydrolysed using acid as well enzymes. In case of acid hydrolysis, a glucose yield of 0.34 g/g BW was achieved at acid and solid loading of 2.0% v/v and 20% w/v, respectively. The obtained sugars were concentrated and used for LA production which resulted in LA concentration of 102.4 g/L with yield of 0.75 g/g. In a recently published report by Narisetty et al. (2022) utilised the BW hydrolysate obtained from 2% v/v acid and 20% w/v solid loading for ethanol production using a new *S. cerevisiae* KL17 strain. They achieved a glucose yield of 0.38 g/g BW and the fermentation of glucose rich hydrolysate resulted in ethanol concentration of 50.2 and 106.9 g/L with a yield of 0.50 and 0.47 g/g during batch and fed-batch cultivation, respectively (Narisetty et al., 2022). The glucose yield obtained in current work is comparable to values achieved by Cox et al. (2022) and Narisetty et al. (2022) but significantly higher than number reported by Torabi and associates even at solid loadings$>$15% w/v.

3.4. BDO accumulation from glucose rich enzymatic hydrolysate

After acid hydrolysis, Dextrozyme Peak from Novozymes was employed for saccharification of BW leading to glucose rich hydrolysates. Unlike acid hydrolysis, enzymatic one is free from any inhibitor such as furfural, HMF etc which otherwise could impede BDO application for food and pharmaceutical industries. The glucose rich hydrolysate obtained was supplemented with other nutrients and used as fermentation medium for BDO production. Fig. 4 depicts the glucose consumption, BDO and acetoin formation and change in pH during batch cultivation in flask culture. Like acid hydrolysis, efficiency of starch hydrolysis was affected with change in solid loading. Although the efficiency of saccharification diminished as solid loading was increased, the glucose titer and yield obtained with enzymatic hydrolysis were significantly higher than the numbers achieved for saccharification by acid and the gap widened as solid to liquid ratio was increased. The glucose titer achieved at 5, 10, 15, 20, 25 and 30% solid loading were 25.1, 51.0, 68.9, 82.9, 98.8 and 111.2 g/L with corresponding yields of 0.50, 0.51, 0.46, 0.41, 0.40 and 0.37 g/g BW, respectively. The glucose obtained was efficiently fermented into BDO and the BDO titer synthesized at solid to liquid ratio of 5, 10, 15, 20, 25 and 30% w/v were 10.4, 22.1, 28.1, 29.5, 36.8 and 42.1 g/L, respectively (Table 2). The interesting observation is that even at 25 and 30% solid loading, all the glucose was exhausted along with continuous BDO accumulation at the end of 36 h, unlike BDO fermentation based on glucose rich hydrolysate from acid hydrolysis. The difference in results could be attributed to the presence of inhibitors (furfural and HMF), especially at higher solid loadings where the amount was large enough to execute the deleterious effect. Acetoin was major byproduct but surprisingly the amount (3–11 g/L) was higher than fermentation based on acid hydrolysate. Similar to the acid hydrolysis, 20% w/v solid loading and an enzyme loading of 0.6 mg/g was observed to be optimum for enzymatic BW hydrolysis in terms of glucose release and BDO production. Comparable results have been obtained by others where BW was used as feedstock for biomanufacturing of chemicals. A thermotolerant *B. coagulans* strain was used for LA production using concentrated BW hydrolysate obtained from enzymatic hydrolysis using 20% w/v solid loading and an enzyme loading of 0.6 mg/g. The strain accumulated LA concentration of 155.4 g/L with yield of 0.85 g/g (Cox et al., 2022). Furthermore, Narisetty and associates employed enzymatic BW hydrolysate for ethanol production using *S. cerevisiae* KL17 strain resulting in 56.9 and 114.9 g/L ethanol with a yield of 0.50 and 0.49 g/g during batch and fed-batch cultivation, respectively (Narisetty et al., 2022). Leung et al., (2012) and Zhang et al., (2013) made use of BW and bakery wastes for fermentative production of succinic acid, respectively. They achieved a sugar yield of 0.47 g/g BW (104.8 g/L) from a solid loading of 30% w/v on dry basis with starch to glucose yield of 90.8%. The BW used for this purpose contained 59.8 g starch per 100 g BW on dry basis and enzymes for hydrolysis were produced by *Aspergillus awamori*. In a recent report, Maina et al. (2021b) employed BW (25% w/v on dry basis) for BDO production. The BW contained 53.6 g starch per 100 g was saccharified using α-amylase and glucoamylase and glucose yield obtained was 0.57 g/g BW (146 g/L) with starch to glucose yield of 96.3%. The sugar yield depends on several factors such as starch content of BW, solid to liquid ratio and whether BW has been used in dry form or with moisture. The BW used in the current study had a starch content of 46% w/w, which is lower than in reported in the abovementioned studies, and the BW was not dried to avoid an additional operation step and extra costs. Taking these factors into account, the high sugar yields obtained in this study reflect the efficiency of starch saccharification by the acid (HCl) and enzyme (Dextrozyme Peak, Novozymes) used for hydrolysis.

3.5. Fed-batch fermentation using pure glucose and glucose rich hydrolysates

Finally, fed-batch cultures were performed to assess the potential of BW hydrolysates for high level production of BDO. The results obtained with glucose rich hydrolysates from acid and enzymatic hydrolysis were compared with those obtained with commercial glucose. Fig. 5 presents the fed-batch kinetics for glucose uptake and metabolites production, where comparable results were quantified in all the three cases. The initial glucose concentration was in range of 90–100 g/L and 70–85% of supplied sugar was metabolized in 24 h with efficient conversion into BDO. Thereafter, the culture was fed at 24, 36, 48 and 60 h and glucose level reduced to zero at 96 h and the overall glucose consumption rate observed was 3.0–3.4 g/L. h. The final BDO titer amassed on pure glucose, glucose rich hydrolysates from acid and enzymatic hydrolysis at the end of 96 h was 144.5, 135.4 and 138.8 g/L, respectively, with a yield of 0.47, 0.42 and 0.48 g/g. The total amount of glucose consumed (323.9 g) in case of acid hydrolysate based BDO fermentation was obtained from 875.4 g BW which would translate to BDO yield of 154.7 g from 1 kg of BW. Similarly in case of BDO production using glucose rich hydrolysate from enzymatic hydrolysis, 291.9 g glucose generated from 710.0 g BW was assimilated during entire fermentation cycle, leading to BDO yield of 195.5 g /kg BW. Fig. 6 compares the theoretical and experimental yields of glucose and BDO that can be obtained by

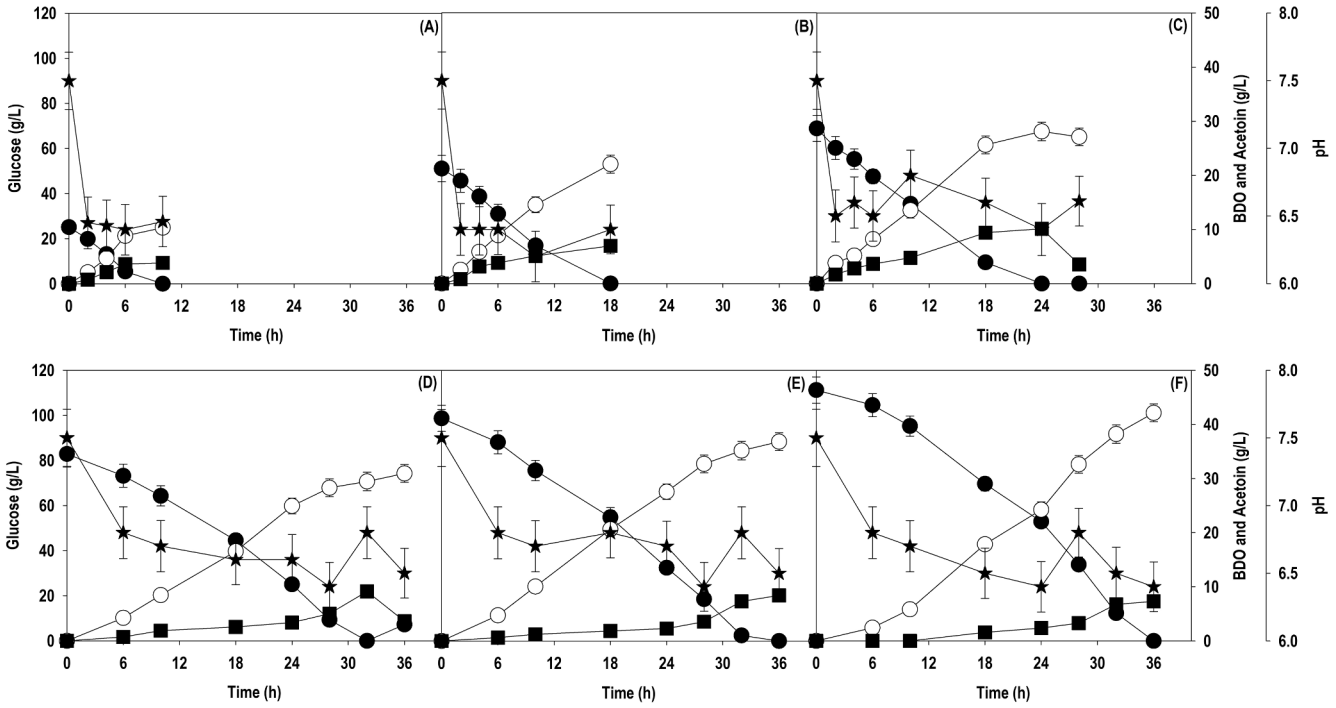


Fig. 4. Enzymatic hydrolysis of BW coupled with BDO & acetoin production at different solid loadings (w/v): (A) 5%; (B) 10%; (C) 15%; (D) 20%; (E) 25% and (F) 30%. Symbols: Glucose (filled circle), BDO (empty circle), acetoin (filled square) and pH (filled star).

Table 2
Summary of glucose and BDO yields obtained from enzymatic hydrolysis of BW at different solid loadings.

Solid loading (%w/v)	Glucose released (g/L)	Glucose yield (g/g)	Maximum achievable glucose (g/L)	Percentage of theoretical yield (%)	BDO Yield (g/g Glucose)
5	25.1 ± 0.9	0.50	25.53	98.3	0.41
10	51.0 ± 1.6	0.51	51.06	99.9	0.43
15	68.9 ± 2.2	0.46	76.59	89.9	0.41
20	82.9 ± 3.5	0.41	102.12	81.1	0.36
25	98.8 ± 3.9	0.40	127.65	77.4	0.37
30	111.2 ± 4.1	0.37	153.18	72.6	0.38

saccharification and fermentation of BW. Like, previous fermentations, BDO was accompanied with acetoin, LA, AA, SA, and ethanol. Acetoin emerged as major one in all the three cases with concentration in range of 19–22 g/L. The carbon calculation shows that carbon flux diverted towards BDO synthesis was 62.1, 55.8 and 63.4% in fermentation with pure glucose, sugar-rich hydrolysates from acid and enzymatic hydrolysis, respectively.

There have been several reports on BDO production from crude renewable sources, but the literature is scarce on BDO production from BW, which is a major solid waste in various parts of world. Only one study by Maina et al. (2021b) investigated acetoin and BDO production using bread and bakery waste by *B. amyloliquefaciens*. They varied the volumetric oxygen transfer (k_La) coefficient to divert bacterial metabolism towards acetoin or BDO. Acetoin (65.9 g/L) was main product at a higher k_La (200 h^{-1}), whereas BDO production (55.2 g/L) was favoured at a lower k_La (64 h^{-1}). Fed-batch cultivation at a k_La of 110 h^{-1} resulted in combined acetoin (~35%) and BDO (~65%) production of 103.9 g/L with a yield of 0.39 g/g and productivity of 0.87 g/L. h. The fermentation media used for the study contained 15 g/L yeast extract. On the contrary, the high BDO titer was attained in the current study with a yeast extract concentration of only 5.0 g/L. In another work by Yu et al., (2022) BDO was accumulated on a leftover food mixture containing bakery, vegetable and fruits waste rich in starch (23.9%), soluble sugars (7.7%) and proteins (7.8%). The bacterium *Bacillus licheniformis* produced 36.7 g/L BDO and a yield of 0.47 g/g using FW media. *Enterobacter* spp. have been documented as promising microorganisms for fermentative BDO production (Maina et al., 2021a). *E. ludwigii* used in current work has been able accumulate a stable high level of BDO from a variety of carbon sources reflecting the versatility of it. For example, in previous work, BDO titer of 118.5 and 65.3 g/L with product yield of 0.43 and 0.36 g/g from cellulosic and hemicellulosic fractions of brewer's spent grains and sugarcane bagasse, respectively, were achieved (Amraoui et al., 2022, 2021). Similarly, Maina et al., (2019) reported 86.8 g/L BDO using cane sugar from sugar mills with a yield of 0.37 g/g. The BDO titre and yield achieved using *E. ludwigii* in the current study are the highest till date. Moreover, the performances of acid (HCl) and enzyme (Dextrozyme Peak) in hydrolysing BW to produce a high level BDO production in this study are notable. This shows that use of mineral acids, which are much less expensive than enzymes, for BW saccharification can significantly reduce the manufacturing cost of BDO. The circular economy approach demonstrated in this study provides a solution for managing BW, which is a major solid waste in Europe and North America, while simultaneously producing high yields of BDO, a valuable chemical compound.

4. Conclusions

The present study highlights the immense potential of *E. ludwigii* as cell factory for BDO manufacturing from BW. The results obtained clearly shows that the BW can be efficiently integrated with BDO production (>100 g/L) and calculation shows that 150–200 g BDO can be manufactured from 1 kg of BW saccharified through acidic or enzymatic pretreatment. The outcome of this work will support establishing a BW-based biorefinery in alignment with circular economy approach. Future work will focus on techno-economic analysis of bioprocess to guide a more efficient and profitable BDO production which could be implemented at industrial scale.

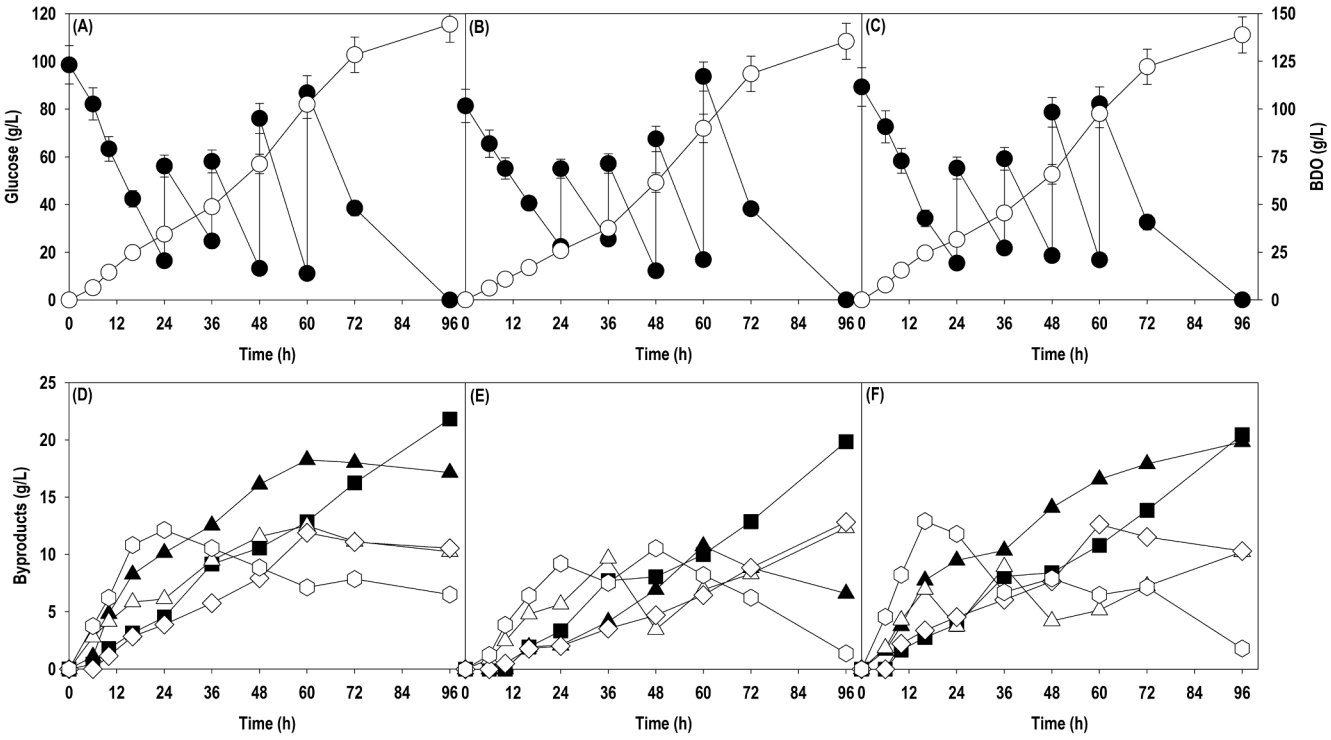


Fig. 5. Fed-batch kinetics of glucose assimilation and metabolite (BDO, acetoin, LA, AA, SA and ethanol) production using (A) & (D) commercial glucose; (B) & (E) acid-based glucose rich hydrolysate; (C) & (F) enzyme-based glucose rich hydrolysate. Symbols: Glucose (filled circle), BDO (empty circle), acetoin (filled square), pH (filled triangle), LA (filled triangle), AA (empty triangle), SA (empty diamond) and ethanol (empty hexagon).

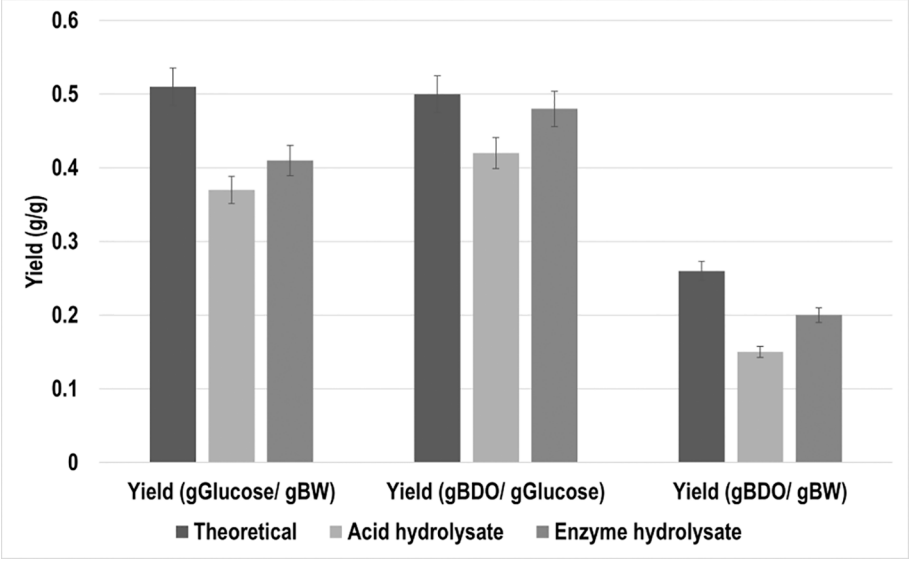


Fig. 6. Comparison of glucose and BDO yield achieved on acid and enzyme hydrolysed BW against theoretical yield.

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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Author Contributions

VN is responsible for the conceptualization, methodology, investigation, and validation of experimental data. VK analyzed the data, and involved in supervision, writing the original draft. LZ, JZ, CSKL, and AM

extended their supervision in conceptualization, and investigation. PLS, SKB, and YWT critically reviewed & edited the manuscript. All the authors read and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in the Manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127381.

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