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Process optimization for recycling of bread waste into bioethanol and biomethane: A circular economy approach

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ABSTRACT

Bread is the second most wasted food in the UK with annual wastage of 292,000 tons. In the present work, bread waste (BW) was utilized for fermentative production of ethanol by *Saccharomyces cerevisiae* KL17. Acidic and enzymatic saccharification of BW was carried out resulting in the highest glucose release of 75 and 97.9 g/L which is 73.5 and 95.9% of theoretical yield, respectively. The obtained sugars were fermented into ethanol initially in shake flask followed by scale up in bioreactor in batch and fed-batch mode. In the fed-batch mode of cultivation, the maximum ethanol titers of 111.3, 106.9, and 114.9 g/L with conversion yield and productivity of 0.48, 0.47, and 0.49 g/g, and 3.1, 3.0, and 3.2 g/L.h was achieved from pure glucose, glucose-rich acidic and enzymatic hydrolysates, respectively. Further to improve the process economics, the solid residues after acidic (ABW) and enzymatic (EBW) hydrolysis of BW along with respective fermentation residues (FR) obtained after the ethanol production were pooled and subjected to anaerobic digestion. The solid residue from ABW + FR, and EBW + FR yielded a biochemical methanation potential (BMP) of 345 and 379 mL CH_4/g VS, respectively. Life cycle assessment of the process showed that the total emissions for ethanol production from BW were comparable to the emissions from more established feedstocks such as sugarcane and maize grain and much lower when compared to wheat and sweet potato. The current work demonstrates BW as promising feedstock for sustainable biofuel production with the aid of circular biorefining strategy. To the authors knowledge, this is the first time, such a sequential system has been investigated with BW for ethanol and biomethane production. Further work will be aimed at ethanol production at pilot scale and BMP will be accessed in a commercial anaerobic digester.

Abbreviations: AP, Acidification potential; AH, Glucose rich hydrolysate from acid hydrolysis of bread waste; BMP, Biomethane potential BW, Bread waste; ABW, Solid residues obtained after acid hydrolysis of bread waste; EBW, Solid residues obtained after enzymatic hydrolysis of bread waste; EH, Glucose rich hydrolysate from enzymatic hydrolysis of bread waste; EP, Eutrophication potential; FSU, Fossil energy use; GWP, Global warming potential; HTP, Human toxicity potential; PCOP, Photochemical oxidation potential.

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1. Introduction

The soaring oil prices and extreme consumption of non-renewable fossil fuels have created a massive environmental problem by increase in greenhouse gas emissions to a level where global community has become serious to find sustainable forms of energies as their substitute [1]. It has been estimated that with exponentially increasing human population, the global consumption of fossil fuels in 2013 was reported to be 89 million barrels per day and expected to increase up to115 million barrels per day by 2040. This rapid consumption will also cause depletion of fossil fuel reserves within the next 40–50 years [2,3]. The renewable energy sources like wind, solar and lignocellulosic biomass (agricultural residues) are viable and sustainable alternative to fossil fuels. Further, their use assist in keeping environment clean and contribute towards a carbon neutral society [4–7]. Bioethanol is a promising renewable energy source and has great potential to serve as the fuel additive and feedstock in chemical industries with annual global market of $58 billion [8]. In 2018, 108 billion litres of ethanol was produced and approximately 84% of it was used by the transportation sector either in pure form or blended with fossil fuels. The global consumption of ethanol is projected to 164 billion litres by 2030 with an anticipated annual growth of 5.07% [9]. Other than transportation sector, ethanol has application as feedstock in chemical synthesis of ethylene with a market demand of 140 million tons per year [10].

Bioethanol is the fermentative product obtained by biological assimilation of simple sugars obtained from various feedstocks. Bioethanol production from sugars/starch, wastes such as lignocellulosic biomass (LCB) and algae is considered as 1G (first generation), 2G (second generation) and 3G (third generation), respectively. However, these processes have several limitations including the socio-economic problems (1G), recalcitrant feedstocks (2G), and viability at pilot/commercial scales (3G). Therefore, in the current scenario waste streams rich in fermentable carbon where extraction of sugars is easy, have become highly valuable as feedstocks for biorefineries. For example, food, bakery, bread, and fruit wastes are the low hanging fruits in terms of fermentable sugars and produced in sufficient amounts to serve as feedstocks and fermentable sugars, obtained can be effectively converted to ethanol by *Saccharomyces cerevisiae* [1,2,6].

In European countries and most of the other developed nations, bread is a staple food that is frequently wasted. With the increase in population, and volatility in demand, the production and supply are far more than the demand resulting in huge amounts of BW [11,12]. Globally $>$ 100 million tons of bread is produced annually and approximately 10% of it is wasted during various steps of supply chain from manufacturer to the consumer. Europe dominates the bread supply chain with a market share of 53.6% where bread wastage is a serious problem. Bread is the second most wasted food in the UK with an annual wastage of 292,000 tons [13]. Bread consists of 50 – 70% of starch, 8 – 10 % protein and 1 – 5% fat. Being a starchy material, it is a rich source of fermentable sugars which can be easily and conveniently extracted. In the process of ethanol production from BW, the starchy feedstock should be saccharified by acidic or enzymatic pretreatment for production of simple sugars, that are fermented to bioethanol by ethanologenic yeast *S. cerevisiae*. The dilute acid hydrolysis of BW tends to break the glycosidic bonds between the glucose residues of amylose and amylopectin chains of starch to release free monomeric glucose units [14]. Though the acid hydrolysis of starchy feedstocks is well known, more environmentally friendly enzymatic hydrolysis involving amylases and glucoamylases generating clean sugar solution devoid of inhibitors is often considered. The process involves 1. Gelatinization (105 °C for 5 mins): formation of suspension of starch granules; 2. Liquefaction (95 °C for 2 hrs): partial hydrolysis using α-amylase, and 3. Saccharification (50 – 60 °C, 48 – 72 hrs): production of glucose by glucoamylases (The use of enzymes in starch hydrolysis (lsbu.ac.uk). The two commonly used modes of fermentation are: separate hydrolysis and fermentation (SHF); simultaneous saccharification and fermentation (SSF). SHF is a two-stage process wherein hydrolysis and fermentation take place in different reactors. The sugars released after saccharification in first reactor are fermented into ethanol into the second one. The process allows both saccharification and fermentation to operate at at their respective optimum conditions. However, continuous accumulation of sugars during saccharification inhibit the activity of hydrolytic enzymes and high sugar concentration achieved at the end exert osmotic stress on fermenting organism. On the other hand, SSF combines the saccharification fermentation in a single reactor and cut down the operational cost. Further, the sugars are readily fermented as soon as they are released, therefore, eliminate inhibition mediated through high sugar levels. The main limitation of SSF is difficulty in optimization of process parameters as optimal conditions for saccharification and fermentation are different [3,8].

S. cerevisiae KL17, a yeast strain efficient in galactose utilization for ethanol production was isolated from soil samples and identified through 18 s rRNA sequencing by Kim and associates [15]. In the current study, BW was used as feedstock for bioethanol production by *S. cerevisiae* KL17. The BW was saccharified using acidic and enzymatic hydrolysis and the process was optimized. The obtained sugars were fermented into ethanol initially in shake flask followed by scale up in bioreactor in batch and fed-batch mode. Further to improve the process economics, the residues from ethanol fermentation and hydrolysis of BW were pooled out and subjected to anaerobic digestion (AD) to investigate their biochemical methanation potential (BMP). Later, the study was focused on assessing the environmental sustainability through life cycle assessment (LCA) and identifying the impacts of the different sub-processes and key environmental hotspots that can guide future development and commercialization.

2. Materials and methods

2.1. Materials, microorganism, and cultivation conditions

All the chemicals and media components used in the study were purchased from Fisher Scientific and Sigma Aldrich (USA) and of analytical grade. Bread waste (BW) (out-dated bread) obtained from the local Co-op supermarket was used in this study. The BW slices were cut into tiny pieces, and moisture content was determined by drying the samples at 105 °C for 4 h [14]. The composition BW is as follows (% w/w): carbohydrates, 46.0; fibre, 2.5; protein, 7.9; saturated fat, 2.0; salt, 1.0; moisture, 35.8. Commercial enzyme Dextrozyme Peak, kindly provided by Novozymes was used for the enzymatic hydrolysis.

The yeast strain *Saccharomyces cerevisiae* KL17, was procured from South Korean culture collection. The strain was maintained on YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 18 g/L agar powder). The temperature was controlled to 30 °C while initial pH was 6.0 ± 0.5. The glycerol stocks of the culture were prepared using 20% w/v glycerol and stored at −80 °C. The pre-inoculum for the shake flask and bioreactor experiments were grown in Erlenmeyer flasks using sterile YPD medium and incubated for 16 h at 30 °C, with an agitation of 200 rpm on a rotary shaker incubator (Excella 24, New Brunswick).

The debris obtained from acidic and enzymatic hydrolysis of BW were referred as ABW and EBW, respectively. The BW, ABW/EBW and fermentation residues (FR) containing biomass and unspent nutrients were dried in an oven at 65 °C for 2 days. Upon drying, the solid biogenic residues were powdered (<600 μm) using a 120 W grinder (Cookworks, UK), and stored in airtight containers until further analysis and BMP experiments. The initial inoculum or the digestate was collected from an AD plant (Agri Food and Biosciences Institute (AFBI), Hillsborough, UK) and filtered (<1 cm). The filtered digestate was degassed at room temperature (20 ± 1 °C) for 15 days and then used as the inoculum for the BMP tests [16,17].

2.2. Submerged cultivations in shake flasks

The submerged shake flask cultivations were carried out in 500 mL Erlenmeyer flasks with 100 mL working volume. The complex medium used for the ethanol fermentation had the following composition (g/L): 7.5, yeast extract; 7.5 peptone; 6, $(NH_4)_2SO_4$; 3, KH_2PO_4; 5, $MgSO_4$ [15]. The medium was supplemented with different levels of pure glucose (50, 100, 150 and 200 g/L) to understand the effect of carbon source on cell growth and ethanol production. The pH of the production medium was adjusted to 6.0 ± 0.5 before sterilization using 5 M NaOH. The flasks were inoculated with 2% (v/v) pre-inoculum and incubated at 30 °C, with a constant agitation of 200 rpm on a rotary shaker incubator (Excella 24, New Brunswick).

2.3. Dilute acid and enzymatic hydrolysis of bread waste.

Dilute acid hydrolysis of BW was performed using hydrochloric acid (HCl). The experiments were carried out at different solid (5, 10, 15, and 20% w/v), and acid loadings (0.5, 1.0, 1.5, and 2.0% v/v) to understand the hydrolytic efficiency of acid with respect to the substrate level. The hydrolysis was carried out in an autoclave at 121 °C for 15 mins. After the hydrolysis, the supernatant consisting of the sugars was separated from the solid debris and the pH of the hydrolysate was adjusted to 6.0 using the sodium hydroxide (NaOH) pellets. The enzymatic hydrolysis of the BW was carried out in 2.0 L Erlenmeyer flasks with 1 L working volume at different solid loading (5, 10, 15, 20% w/v). The pH of BW suspension was adjusted to 4.3 using HCl followed by the autoclaving at 121 °C for 15 mins which eliminated the requirement of gelatinization and liquefaction steps. The pH 4.3 was chosen from the literature [14] as it has been described as an optimal pH for the maximum activity of amyloglucosidase, the enzyme required for the breakdown of starch into monomeric glucose units. After autoclaving, the suspension was allowed to cool down and further placed in an orbital shaker until a temperature of 60 °C was attained followed by addition of Dextrozyme Peak enzyme at 0.6 mg/g BW loading and incubated for 48 h in a rotary shaker at 250 rpm. After saccharification, the supernatant consisting of the sugars was separated from the enzymatic hydrolysed BW and the pH of the hydrolysate was adjusted to 6.0 using the sodium hydroxide (NaOH) pellets. The sugar rich supernatant obtained after acidic (AH)/enzymatic hydrolysis (EH) was concentrated using vacuum evaporation unit (BUCHI, UK) maintained at a pressure of 100 mbar and temperature 60 °C in water bath for 24 h to achieve a glucose concentration of 400 g/L and stored at 4 °C for further use. The saccharified broth was diluted to the desired concentration as per the requirement of the experiment. The solids obtained after the acidic (ABW) and enzymatic hydrolysis (EHW) were stored at 4 °C, and further mixed along with the fermentation residues (FR) including yeast cells, unconsumed nutrients and other solids obtained after the fermentation and referred as ABW + FR and EBW + FR, respectively. The mixtures ABW + FR and EBW + FR were subjected to AD for evaluating the BMP.

2.4. Bioreactor cultivation for ethanol production

Fermentation experiments using pure glucose and glucose-rich BW hydrolysates were performed in a Bench-top 2.5 L bioreactor (Electrolab, Bioreactors, UK) with 1.0L working volume in batch and fed-batch mode. The bioreactor was operated in controlled conditions of 30 °C temperature, 200 rpm agitation speed, 1.0 mL/min aeration rate and pH were maintained at 6.0 using 5 M NaOH. The initial glucose concentration was 100 g/L in batch mode, and it was initiated by addition of 2% (v/v) pre-inoculum. In case of fed-batch fermentation, the culture was fed using a concentrated glucose (400 g/L) solution when the substrate concentration dropped below 20 g/L.

2.5. Characterization of bread waste and fermented solids

Dried BW, ABW + FR, and EBW + FR were powdered and analysed for their elemental composition using a CHNS analyser (Series II Perkin Elmer PE 2400) and the oxygen content of the samples was determined by difference. The biomass samples and the inoculum were characterized for their total solids (TS), volatile solids (VS) and ash content [15,16]. The powder (0.2 g) and degassed inoculum (5 g) were first taken in separate crucibles in triplicates. The crucibles with the samples were then placed in an oven (Carbolite Gero) at 85 °C for 2 days. Once dry, the crucibles were cooled to room temperature and weighed. The weight loss was attributed to the moisture content and the remaining weight was accounted as dry matter (TS) of the sample. Sequentially, these samples were kept in a muffle furnace (SNOL 13/1100 LHM01) at 550 °C for 2 h. Upon combustion, the crucibles were allowed to cool down to room temperature and then weighed. The weight lost in this stage was determined as the VS content and the residue was quantified as ash.

2.6. Estimation of biomethane potential (BMP) of BW, ABW + FR and EBW + FR

The BMP test set up previously used and described by Nagarajan and Ranade (2019; 2020) was used in this work [18,19]. All the dried solid residues were subjected to batch BMP tests in a gas endeavour system (Bioprocess control, Sweden). The system was operated with four sets of bioreactors with each set operating in triplicates. The first triplicate bioreactors consisted of the inoculum only and no feedstock. This was termed as the control set. The second, third and fourth sets of bioreactors were fed with untreated dried powdered samples from BW, ABW + FR and EBW + FR, respectively. The required amount of inoculum was also added to the bioreactors in the ratio of 1:2 (VS basis, substrate: inoculum). All the reactors were made up to a final volume of 400 mL using tap water and the total VS content of the test bioreactors were 24 g VS/L. All the bioreactors were then fitted with the overhead stirrers, fastened and connected in series and placed in a water bath set to 41 °C. The stirrers were operated at 50 % of the maximal speed. The mixing cycle was set to a 10 s ON and 0 s OFF cycle where the contents were mixed alternatively in clockwise and counterclockwise directions. Gas generated was allowed to pass through 80 mL of 3 M NaOH and 0.4 % thymolphthalein solution to strip the CO_2 and H_2S in the biogas. This stripped gas was then passed through pre-calibrated counters connected to real time data logging using a dedicated PC. The volume of biomethane generated as raw data was normalised to standard temperature and pressure (STP, 0 °C and 1 atm). The tests were performed until a plateau in cumulative biomethane generation was observed. BMP of each feedstock was then calculated using Equation (1).

$$BMP = \frac{(V_{ft} - V_{it})}{VS_f} \quad (1)$$

where BMP is the biochemical methanation potential of the feedstock, mL CH_4/g VS, V_{ft} is the cumulative volume of biomethane (in mL) produced in the bioreactor at time t, V_{it} is the cumulative volume of biomethane (in mL) produced by the inoculum only in the control bioreactors at time t and VS_f is the weight of volatile solids (in g) present in the feedstock fed to the bioreactor, g VS. The average gas produced by the triplicates was reported unless mentioned otherwise and the corresponding standard deviation was used to calculate the error bars. A first order model reported by Nagarajan and Ranade (2020) was used to describe the BMP data as per Equation (2) and (3) [19].

$$G = G_{max}\left[1 - e^{-k(t-t_0)}\right] \quad for \quad t > t_0 \quad (2)$$

$$G = G_0\frac{t}{t_0} \quad for \quad t \leq t_0 \quad (3)$$

G: Biomethane (mL CH_4/g VS at t); G_{max}: BMP_{max} (mL CH_4/g VS from feedstock); k: rate of generation of biomethane; t_0: lag time; and G_0: gas produced during lag time, mL CH_4/g VS.

Upon plotting G vs t, a straight line was fitted through the first four data points. The x-axis intercept was determined and used as the lag time, t_0. G_0 is the respective biomethane generated during t_0. The non-linear regression tool in Microsoft Excel was then used to fit the data to the model to determine the two unknown parameters, G_{max} and k.

2.7. Analytical methods

The concentrations of glucose after the acid and enzymatic hydrolysis were quantified by High performance Liquid Chromatography (HPLC-A Agilent Technologies 1200 series, USA). The samples obtained after the hydrolysis and saccharification were subjected to centrifugation at 10,000 rpm for 10 min. The supernatant obtained was filtered using 0.22 μM nylon membrane (Sartorius, Germany), further analyzed in HPLC with Rezex ROA-Organic Acid H^+ (Phenomenex, USA) column, connected with Refractive Index Detector (RID). For the inhibitor analysis, supernatant obtained after filtering through 0.22 μM nylon membrane (Sartorius, Germany), was injected into HPLC with Rezex RNM-Carbohydrate Na^+ (Phenomenex, USA) column, connected with Photodiode-Array Detector (PDA). During the shake flask, and bioreactor cultivations, samples taken at regular intervals were processed and quantified for cell growth (optical density), residual glucose and ethanol. The optical density of the sample collected was measured at wavelength of 600 nm in a 1 mm-path-length cuvette using a double beam spectrophotometer (Jenway 6310, UK). Because the samples from the experiments conducted using BW hydrolysate were highly turbid, the optical density measurement was not possible. The supernatant was processed similar to the hydrolysates provided above. The samples were eluted through RID detector as mentioned above for the sugars and ethanol. The mobile phase used for elution of was 5 mM H_2SO_4, at a flow rate of 0.4 mL/min while in case of inhibitors, it was Millipore water at a flow rate of 0.4 mL/min. The glucose yields obtained from acid and enzymatic hydrolysis of BW were calculated based on the maximum theoretical glucose obtained after complete hydrolysis of starch based on the formula:

$$Glucose\ yield\ \left(\frac{g}{g}\right) = \frac{Glucose\ produced\ (g)}{Waste\ bread\ (g) \times Starch\ content \times 1.11}$$

where 1.11 is the polymerisation factor of starch to glucose [14].

2.8. Life cycle assessment supporting the bread waste as a sustainable feedstock for production of ethanol

LCA is carried out in four phases: (i) goal and scope definition; (ii) inventory analysis (iii) impact assessment and (iv) interpretation, in line with the International Organization of Standardization (ISO 14040–44) guidelines. The goal of this study was to estimate the potential environmental costs and benefits of bioethanol production via fermentation of BW. The functional unit is 1 kg of ethanol, produced from BW in the UK. The scope of the study is cradle-to-gate, i.e. spanning from the extraction of raw materials for biorefinery to its operation for the production of ethanol (99.7%). The system boundary, as described in Fig. 1, includes BW transportation, pre-treatment, hydrolysis, fermentation, and downstream recovery of ethanol. The solid residue after centrifugation operation is forwarded to an anaerobic digestor and the generated biogas is used to produce steam and electricity, which are utilised in the process. In addition to the solid waste, 100 kg/day of BW is also used in the AD to fulfil the total steam requirement in the distillation processes.

The inventory data used in this work is based on the material and energy balances obtained for the ethanol production process described in this work, which is assumed to be scaled up to a biorefinery pilot plant processing 1 ton BW /day with 330 operating days annually. Based on this information, the inventory data for producing 1 kg ethanol is calculated and presented in Table 1. Ecoinvent LCI database version 3.6 as available in Simapro (version 7.2), was used to extract majority of the background life cycle inventory data. BW is assumed as a post-consumer waste and thus the environmental impact associated with the generation of the BW is excluded from this analysis. For transportation of feedstock, a maximum distance of 500 km was assumed (in the UK scope). In this study, the life cycle impact assessment was conducted using CML-IA baseline V3.06 / EU25 and single-issue LCIA method Cumulative

Table 1
Life cycle inventory data for producing 1 kg ethanol via fermentation of bread waste.

Inputs	Amount	Unit	Data Source
Enzyme	0.0019	kg	Enzyme (Glucoamylases), Dextrozyme Peak (Novozymes)/kg/RER
Yeast extract	0.1202	kg	Adom and Dunn, 2015
Process water	40.170	kg	Tap water (Europe without Switzerland)
Inoculum	0.3205	L	This work
Ammonium sulphate	0.0962	kg	Ammonium sulphate (RER)
Magnesium sulphate	0.0801	kg	Magnesium sulphate (GLO)
Potassium phosphate monobasic	0.0481	kg	Monoammonium phosphate (RER)
Peptone	0.1202	kg	Ramgiri et al., 2021
Granular activated carbon	0.0000	kg	Activated carbon, granular (GLO)
Electricity	2.5417	kWh	Electricity, medium voltage (GB)
Transportation	0.2518	tkm	Transport, Freight. Lorry 3.5 – 7.5 metric ton, Euro6 (RER)
Outputs			
Ethanol (99.7%)	1	kg	
Emissions			
Carbon dioxide	1.0417	kg	Ecoinvent data on emissions to air
Nitrogen	13.022	kg	
Oxygen	3.9519	kg	

*RER: Europe; GLO: Global; GB: Great Britain.

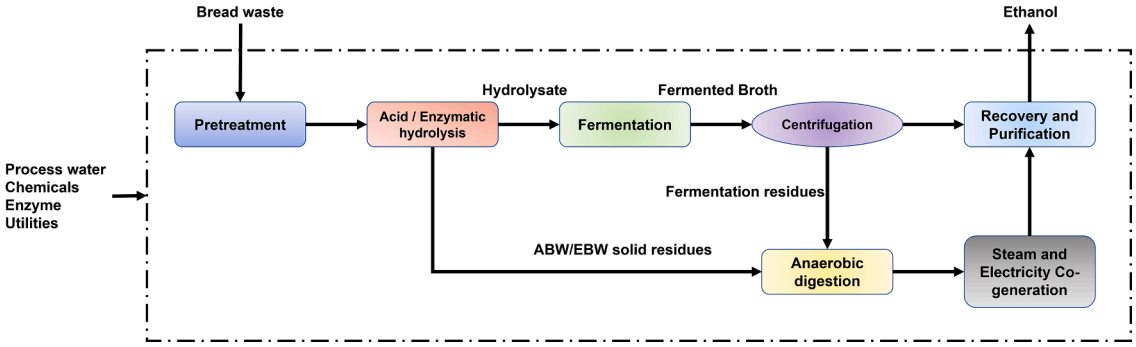


Fig. 1. Life cycle assessment (LCA) system boundary.

Energy Demand (CED) (v 1.11). The environmental performances were evaluated using the following impact categories: global warming potential (GWP), human toxicity potential (HTP), photochemical oxidation potential (PCOP), acidification potential (AP), eutrophication potential (EP) and fossil energy use (FSU).

3. Results and discussion

3.1. Effect of initial substrate concentration on growth and ethanol production by S. cerevisiae KL17 strain in shake flask experiments

In the current study, we made use of *S. cerevisiae* KL17 for fermentative production of ethanol. The strain was isolated by Kim and associates and observed to be identical to *S. cerevisiae* Z75578 with 99.5% homology [15]. The yeast strain was cultured in shake flask and ethanol fermentation performance of the strain at various initial glucose concentrations (50, 100, 150 and 200 g/L) was evaluated. The time course profiles for glucose consumption, cell growth (OD_{600}) and the ethanol production are summarised in Fig. 2 (A-D).

At 50 and 100 g/L, ~45% glucose utilisation was observed in first 6 h of the cultivation, and the remaining carbon was completely exhausted within 24 h, and the behaviour was concomitant with cell growth and ethanol production. In case of 50 g/L, the highest OD_{600}, ethanol titer, yield and productivity achieved were 47.7, 24.9 g/L, 0.49 g/g and 1.04 g/L. h, respectively. At 100 g/L, a marginal increment in OD_{600} (48.9) was observed and ethanol yield (0.50 g/g) was almost unaffected, while titer (50.2 g/L) and productivity (2.09 g/L. h) were almost doubled. No visible inhibition of growth or ethanol production was noticed with further increase in glucose concentration to 150 and 200 g/L. In fact, there was continuous improvement in cell growth, ethanol concentration and productivity with complete depletion of glucose within 36 h while the yields were close to maximum theoretical value. The maximum OD_{600}, ethanol titers, yield and productivity obtained were at: 150 g/L – 69.6, 72.9 g/L, 0.49 g/g, 2.02 g/L. h; at 200 g/L – 72.7, 106.7 g/L, 0.50 g/g, 2.96 g/L. h, respectively. Similar results in terms of substrate utilization and product formation pattern were reported by Kim et al. (2014) when *S. cerevisiae* KL17 was cultivated in shake flask on glucose and galactose. For example, 100 g/L galactose was completely assimilated in 20 h leading to ethanol production of 41.5 g/L with conversion yield and productivity of 0.41 g/g and 2.07 g/L. h, respectively [15]. As these experiments were carried out in the Erlenmeyer flasks, the limitations like lack of aeration, mass transfer, and nutrients due to batch mode of cultivation, could impact on the cell growth and metabolism. Therefore, further in this study, the submerged fermentations were carried out in the bioreactor wherein the controlled to increase the product formation.

3.2. Dilute acid hydrolysis of bread waste: Effect of solid and acid loading

Higher starchy content of BW makes it easily digestible either through dilute acid or enzymatic treatment for extraction of fermentable sugars. Most of the literature reports make use of enzymes for extraction of sugars from starch feedstocks [11,20,21], but enzymes are expensive and in comparison, mineral acids are much cheaper and can make the process economically viable. The solid loading and the concentration of acid as the catalyst for the breakdown of the starch molecules can be considered as the influential parameters for the increment in the glucose concentrations. Initially, the BW was subjected to dilute acid pretreatment, and the process was performed at different range of acid (0.5, 1.0, 1.5, 2.0 % v/v) and solid (5, 10, 15, 20% w/v) loadings to find out the optimal combination.

Fig. 3 represents the glucose amount and yield, HMF and furfural (g/L) obtained in the hydrolysates obtained from BW at different acid and solid loadings. At all solid loadings, there was linear increment in amount of glucose released as concentration of acid was enhanced. The highest glucose concentration of 75.0 g/L was obtained at acid and solid loading of 2% v/v and 20% w/v, respectively, with glucose yield of 0.38 g/g which is 73.5% of theoretical yield, whereas maximum glucose yield of 0.45 g/g was recorded at same acid loading but with solid loading of 15% w/v. The yield of glucose per kg of bread was 375 g/kg and lower

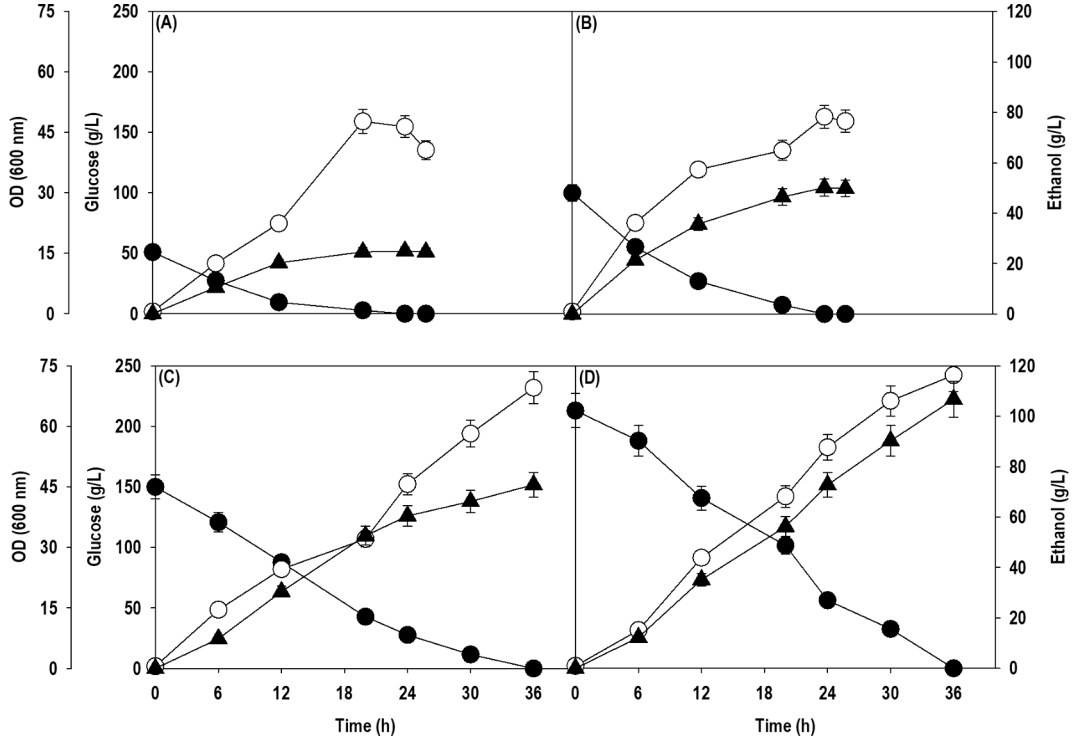


Fig. 2. Time course profiles of residual glucose, cell growth (OD_{600}) and ethanol production by *S. cerevisiae* KL17 in shake flask at different initial glucose concentrations: (A) 50 g/L; (B) 100 g/L; (C) 150 g/L; and (D) 200 g/L. Symbols: Glucose (filled circle); OD_{600} (empty circle); Ethanol (filled triangle).

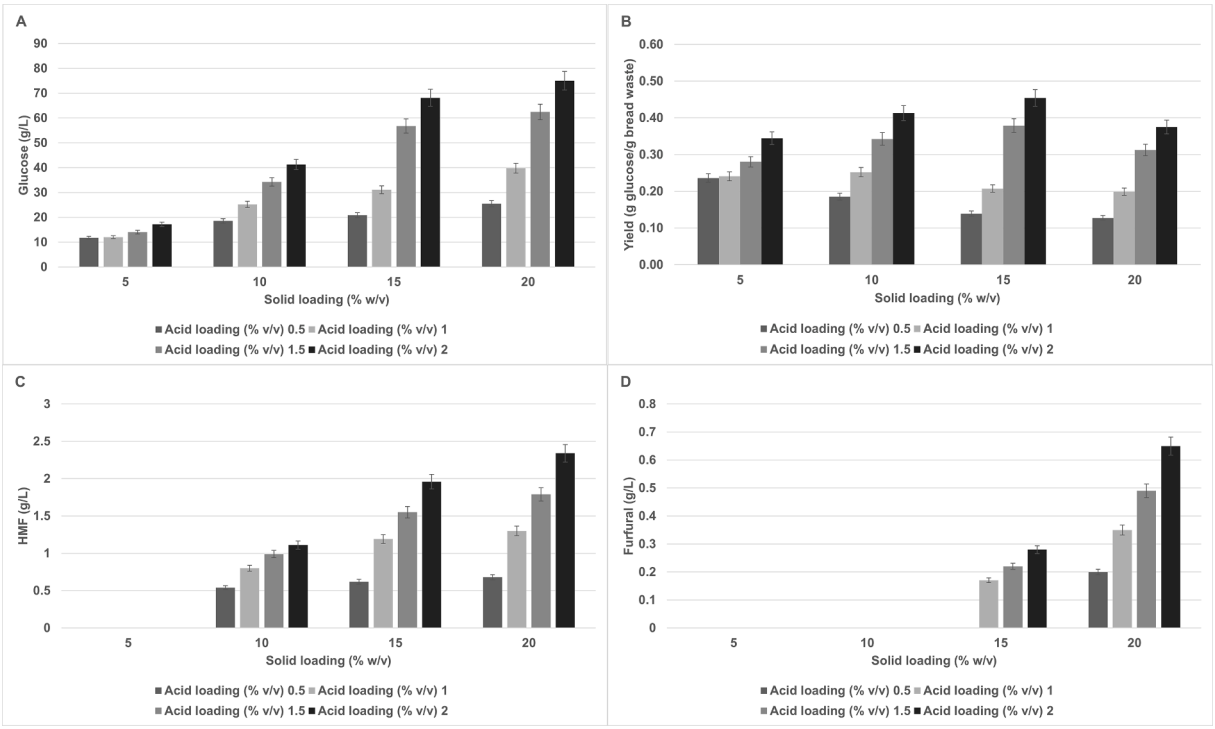


Fig. 3. Bar graphs showing (A) glucose release; (B) glucose yield; (C) HMF; and (D) furfural obtained during dilute acid hydrolysis of BW at different acid and solid loadings.

than yield achieved by Torabi et al. (2020). In addition to acid and solid loading, they also optimized incubation time and optimal values leading to sugar yield of 450 g glucose/kg BW. The optimal values of acid concentration, solid loading and incubation time were 1% v/v HCl, 20% w/v dry bread residues, and 20 mins, respectively, at 121 °C. The difference could also be attributed to variation in substrate used. We made use of bread as such whereas Torabi et al. (2020) employed dry bread residues causing enhancement in glucose yield. Polysaccharide hydrolysis kinetics include the first reaction of pretreatment involving the conversion of polymeric structure into monomeric glucose units and upon prolonging the incubation, the second reaction involves the conversion of glucose into furfurals, hydroxymethylfurfural (HMF), levulinic acid, formic acid, etc [14]. Besides glucose, HMF and furfural were also released during saccharification. HMF was obtained at acid loadings of 1, 1.5 and 2.0% v/v while furfural production was observed at only 1.5 and 2.0%. Like glucose, the amount of HMF and furfural continuously enhanced with increase in solid loading at above acid concentrations and their highest levels (HMF – 2.34 g/L; furfural – 0.65 g/L) were obtained where maximum glucose concentration (75.0 g/L) was achieved. HMF and furfural are considered as fermentation inhibitors and known to inhibit the growth of microorganisms. The concentrations of these two compounds in lignocellulosic biomass hydrolysates is usually higher than the concentrations observed in this study [3]. Taherzadeh and associates observed the physiological behaviour of *S. cerevisiae* strains in the presence of furfural and HMF and noticed no significant inhibition on cell growth at HMF and furfural concentrations of 2 g/L. Whereas increase in HMF concentration to 4.0 g/L reduced biomass yield, specfic growth rate and specific ethanol production rate by approximately by 29% (83 vs 59 mg/g), 71% (0.45 vs 0.13 h^{-1}) and 41% (1.6 vs 0.95 g/g. h), respectively. The suitation further deterioarted with addition of 2 g/L + 2 g/L furfural [22]. Therefore, it would be interesting to see how this new isolate *S. cerevisiae* KL17 would perform in presence of HMF and furfural.

3.3. Enzymatic hydrolysis of the BW using Dextrozyme peak

Unlike acidic hydrolysis, enzymatic hydrolysis is performed under milder conditions resulting in hydrolysates free from inhibitors. After acidic hydrolysis, the enzymatic hydrolysis of BW was performed at different solid loadings (5, 10, 15, 20% w/v) with an enzyme loading of 0.6 mg enzyme/g BW. The commercial enzyme Dextrozyme Peak (Novozymes, EU) containing amylase and amyloglucosidase activity was employed for this purpose and the glucose released was recorded at 24 and 48 h. The process of starch hydrolysis into simple sugars is of utmost importance as the increase in hydrolysis efficiency improves the total sugar accumulated in the hydrolysate, which eventually improves process efficiency through translation into product. For calculation of starch to glucose conversion, stoichiometric coefficient of 1.11 has been used. Fig. 4 shows the concentration of glucose obtained at 24 and 48 h, based on different solid loadings. The locked glucose released at 24 h in case of 5 and 10% solid loading was 70–80% of theoretical value while at 15 and 20%, it was 50–60%. The final value obtained at 5, 10, 15 and 20% solid loadings after 48 h were 97.4, 94.4, 93.8 and 96% of theoretical value, respectively. The final glucose concentration achieved at 5, 10, 15 and 20% solid loadings were 24.8, 48.2, 71.9 and 97.9 g/L with yield of 0.50, 0.48, 0.48 and 0.49 g/g bread. These results indicates that 450 – 495 g of glucose, which is high quality food grade sugar, can be obtained from one kg of BW.

The perusal of literature shows that different approaches have been employed for enzymatic saccharification of BW. There are various studies where, different cocktails of in-house enzymes are used to decrease the cost of the biocatalyst. For example, Leung et al. (2012) treated the bread suspension with fungal strains, *Aspergillus awamori* and *Aspergillus oryzae* producing amylolytic and proteolytic enzymes for extraction of glucose and amino acids from BW [23]. The results obtained were similar to ours and glucose yield recorded was 0.47 g/g BW with 90.8% starch conversion to glucose at 30% (w/v) solid loading. In a study conducted by Mihajlovski and associates, an enzyme isolated from *Hymenobacter* sp., was used for saccharification resulting in maximum reducing sugar concentrations of 19.9 g/L with 20% (w/v) solid loading

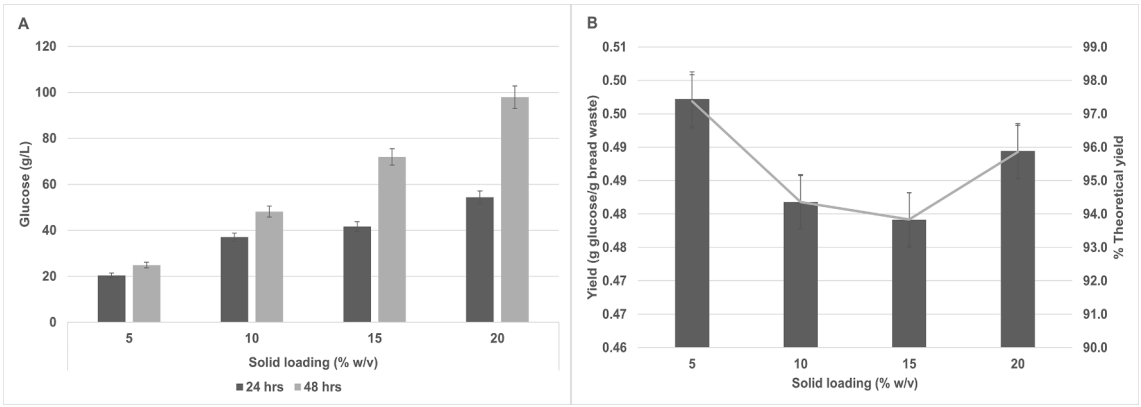


Fig. 4. Bar graphs showing (A) glucose release during enzymatic hydrolysis of BW using Dextrozyme Peak; (B) corresponding yield on BW and % theoretical yield.

[21]. Hudeckova et al., 2017 quantified the glucose concentration of 70.3 g/L from saccharification of 15% (w/v) BW using the commercially known enzymes like α-amylase (BAN 240L) and glucoamylase (AMG 300L) [24]. While in this study we have performed the saccharification with 15 and 20% w/v solid loadings and the concentration of glucose obtained was 71.8 and 97.9 g/L, respectively, which were significantly higher. Torabi and associates optimized sugar extraction from BW and hydrolysis consisted of liquefaction followed by saccharification using α-amylase and glucoamylase. They reported a maximum glucose concentration of 58 g/L with 10% solid loading and incubation time of 48 h [14] and similar to our observations, glucose yield achieved were near to the theoretical maximum. Similarly, Ebrahimi et al. (2008) obtained a glucose yield of 0.80 g/g bread from 35% w/w suspension using α-amylase and glucoamylase [25].

3.4. Batch cultivation in bioreactor for ethanol production

After saccharification, the potential of BW hydrolysates for ethanol production was investigated in batch cultures. The glucose-rich hydrolysate from acid and enzymatic hydrolysis were referred as AH and EH, respectively. The AH and EH were fermented to ethanol by *S. cerevisiae* KL17 in bioreactor and the results were compared with ethanol accumulated on pure glucose. The saccharified BW hydrolysates for this purpose were appropriately diluted to provide the initial concentration in range of 100–115 g/L.

Fig. 5 presents the time course profiles for glucose consumption and ethanol production. Similar results were obtained in all the three cases. The glucose uptake was fast from the beginning and ~ 70–80% of substrate was assimilated within 12 h and complete depletion of glucose was observed at 24 h. The ethanol production was concomitant with glucose consumption and was first noticed at 6 h. The most active phase for ethanol biosynthesis was in the initial 12 h where volumetric productivity was at peak. The final ethanol concentration achieved at the end of 24 h from pure glucose, AH and EH were 52.2, 50.2 and 56.9 g/L, respectively, with same ethanol yield of 0.50 g/g glucose, near to theoretical yield of 0.51 g/g. The corresponding overall productivities were 2.2, 2.1 and 2.4 g/L. h, respectively. These results indicate high osmo-tolerance of strain as no inhibition was observed at glucose levels of 10–11% w/v. Further, the presence of furfural and HMF in AH did not have any impact on performance of strain showing its robustness against fermentation inhibitors. Literature review reveals that there are handful of reports on fermentative ethanol production from BW via acid and enzyme hydrolysis. The results obtained during batch cultivation in current work are better in terms of titer, yield, and productivity than following studies. For example, Ebrahimi et al. (2008) obtained ethanol titer of 100 g/L from saccharified bread solution containing 250 g/L glucose in 10 h using dry yeast concentration of 20 g/L at time of inoculation leading to a very high productivity [25]. Similarly, Torabi et al. (2020) carried out shake flask fermentation using AH and EH accumulated 44.3 and 33.9 g/L ethanol after 96 h, with 86.9% (0.44 g/g) and 83.0% (0.34 g/g) of theoretical yield, respectively [14].

3.5. Fed-batch culture for ethanol production using pure glucose and bread hydrolysates

To maximize the titers of end product is the common requisite for the commercial scale production, which can be achieved through fed-batch mode of fermentation. In the fed-batch mode, higher substrate concentrations can be supplemented to the biocatalyst by avoiding the substrate mediated inhibition. Similar to batch, fed-batch culture was performed using pure glucose, AH and EH. The intermittent feeding was carried out using the concentrated glucose rich BW hydrolysates (400 g/L) when glucose concentration dropped below 20 g/L. Fig. 6 depicts the fermentation profiles for glucose consumption, feeding, and ethanol formation. The culture feeding started at 12th h when 80–85% supplied glucose was exhausted, and next feeding was done at 24th h. The total glucose supplied (225–235 g/L) in span of 24 h was completely exhausted within 36 h leading to the maximum ethanol titers of 111.3, 106.9, and 114.9 g/L with a conversion yield and productivity of 0.48, 0.47, and 0.49 g/g, and 3.1, 3.0, and 3.2 g/L. h from pure glucose, AH and EH, respectively. Although the conversion yields obtained in the batch and fed-batch fermentations are quite similar, the titers and productivities have improved significantly during fed-batch cultivation. The maximum amount of ethanol recoverable from 1 kg of BW used in current study is 260 g. As per these results, the amount of ethanol which can be obtained from 1 kg of BW saccharified via acidic and enzymatic hydrolysis are 176 g/kg and 240 g/kg, which is 67.7 and 92.1% of theoretical yield, respectively.

The summary of ethanol production from various starchy food wastes reported in literature is provided in Table 2. The TYP (Titer, Yield, and Productivity) metrics achieved in current work are comparable to or better than many of the reported work. Further, the similar results obtained with AH and EH are highly encouraging as enzymes are the major cost contributor to the process. The yield of ethanol on BW obtained in current work is lower than Torabi et al. (2020) who achieved yields of 248 and 313 g/kg BW using saccharified dilute acid and enzymatic hydrolysates, respectively. Similarly, 350–355 g ethanol per kilogram of BW was achieved by Ebrahimi et al., 2008 [25]. The product yield depends on several factors and lower ethanol yield in present work could be due to difference in type and starch content of bread. Further, ethanol yield is also dependent on sugar yield which depends on many factors including solid loading. For example, Emrahimi et al. (2008) used a solid loading of 35% which led a high glucose yield of 0.80 g/g BW. Similarly, Torabi et al. (2020) made use of dried bread residues resulting in higher glucose concentrations which eventually results in higher product yield. It is worthwhile to mention here the ethanol

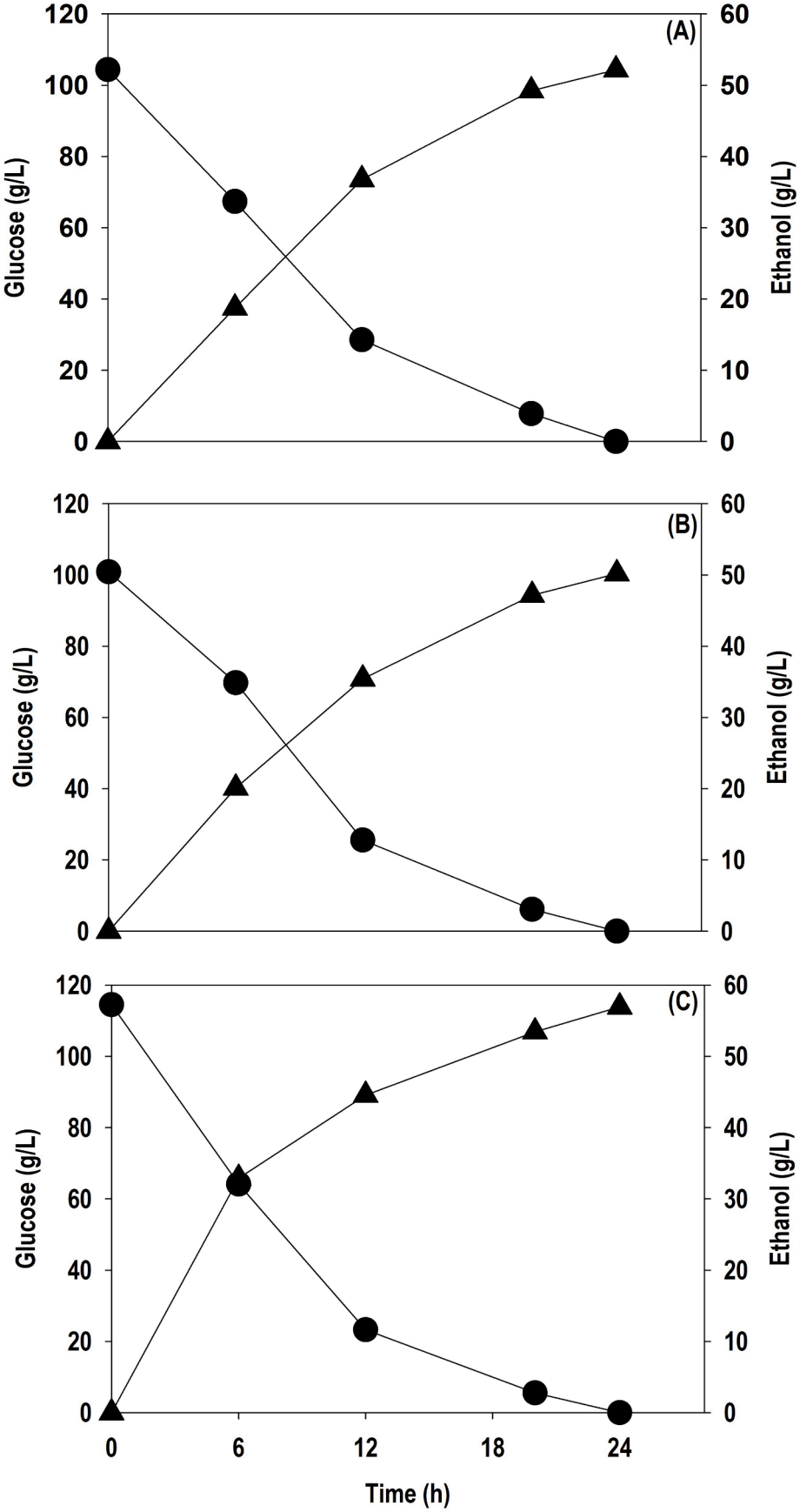


Fig. 5. Batch cultivation of *S. cerevisiae* KL17 in bioreactor on (A) pure glucose; (B) AH; (C) EH. Symbol: Glucose (filled circle); Ethanol (filled triangle).

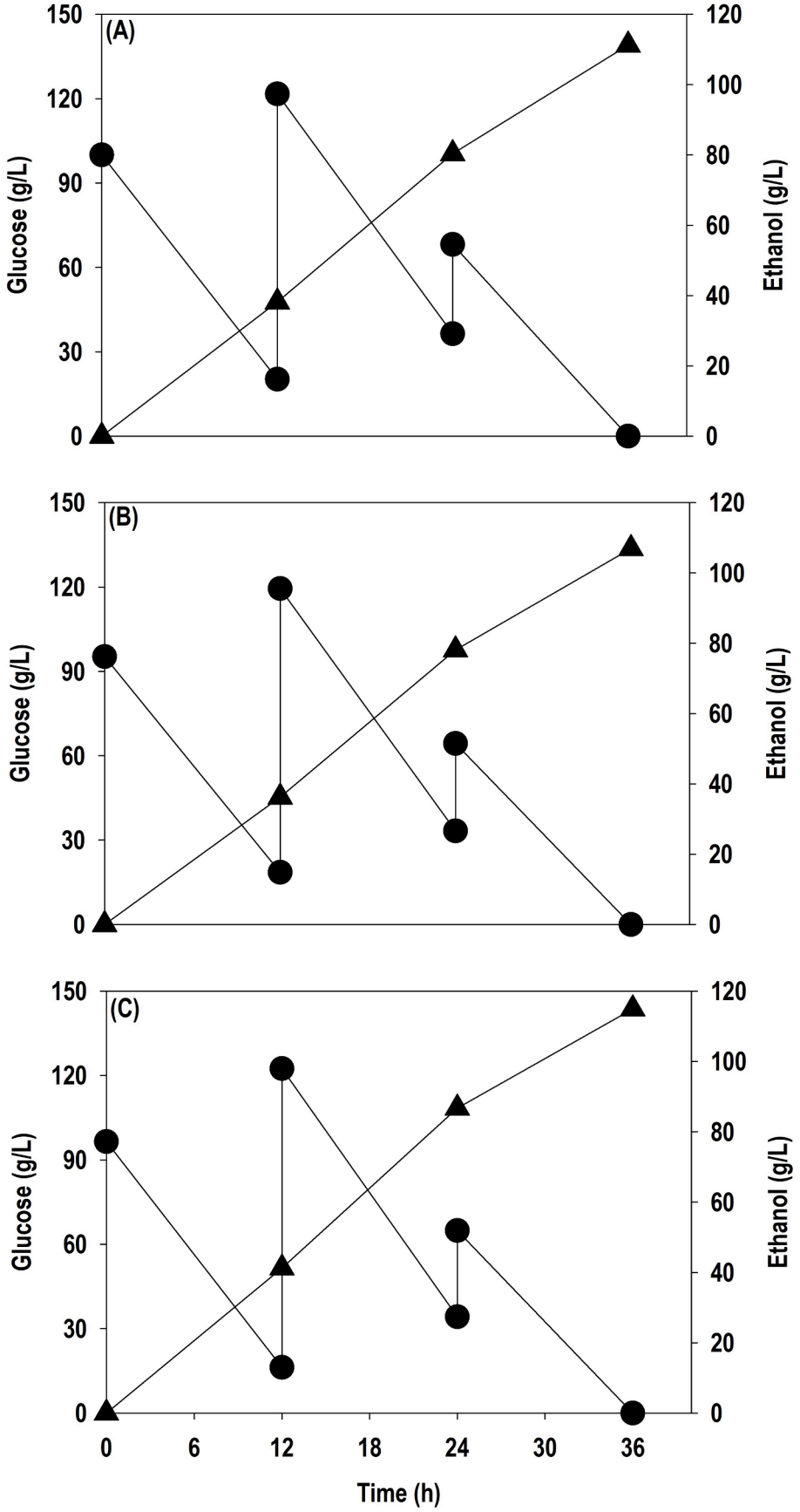


Fig. 6. Time course profiles of glucose consumption and ethanol production by *S. cerevisiae* KL17 during fed-batch bioreactor cultivation using (A) pure glucose; (B) AH; (C) EH. Symbol: Glucose (filled circle); Ethanol (filled triangle).

Table 2
Summary of the ethanol production from starchy feedstocks.

Microorganism	Feedstock	Pretreatment	Mode of Fermentation*	Titers (g/L)	Yield		Productivity (g/L. h)	Reference
					(g/g sugar)	(g/kg feedstock)		
S. cerevisiae	Waste wheat bread	Acid pretreatment	SHF	35.2	0.44	248.0	0.36	[14]
S. cerevisiae	Waste wheat bread	α-Amylase + glucoamylase	SHF	42.3	0.42	313.0	0.44	[14]
S. cerevisiae	Bread residues	α-Amylase + glucoamylase	SHF	100.0	0.40	350.0	10.0	[25]
S. cerevisiae	Wheat-rye bread	α-Amylase + glucoamylase + protease	SSF	128.0	0.49	425.0**	2.70	[26]
S. cerevisiae	Waste Cake	α-Amylase	SHF	46.6	1.13	1120.0**	1.17	[27]
S. cerevisiae	Potato waste	Glucoamylase from fungal strains	SSF	37.9	0.45	410.3***	0.53	[28]
S. cerevisiae ATCC 24,859	Pie waste	α-Amylase + γ-amylase + pectinase	SHF	111.7	0.48	372.3	2.33	[29]
S. cerevisiae ATCC 4124	Waste pizza	α-Amylase	SHF	27.6	NA	292.0	0.46	[30]
Zymomonas mobilis (ZMA7-2)	Food waste	Glucoamylase	SHF	99.8	0.50	-	2.27	[31]
Mucor indicus CCUG 22,424	Organic fraction of MSW	Acid pretreatment	SHF	19.2	0.45	173.4	0.27	[32]
S. cerevisiae KL17	Bread waste	Acid pretreatment	SHF	106.9	0.47	176.0	2.97	This study
S. cerevisiae KL17	Bread waste	Dextrozyme Peak	SHF	114.9	0.49	240.0	3.19	This study

* SHF - Separate hydrolysis and fermentation; SSF - Simultaneous saccharification and fermentation; MSW: Municipal solid waste; **Yield on dry substrate; ***Yield on starch.

production from galactose using the same *S. cerevisiae* KL17 strain by Kim et al. (2014). They achieved 95 g/L with yield and productivity of 0.39 g/g and 3.03 g/L. h, respectively [15]. Glucose and galactose are two major sugars present in all types of food waste and a strain like *S. cerevisiae* KL17 which can efficiently manufacture ethanol from these two sugars can be highly promising biocatalyst for industrial production from waste streams rich in these two sugars.

3.6. Estimation of biomethane potential (BMP) using solid residues generated in the process

The production of multiple energy vectors within the circular economy framework have been reported to be beneficial from an exergy perspective [5]. Though bioethanol generation from BW was the primary goal of the work, the biomethane production was identified as the allied valorisation route to utilise the residues from hydrolytic and fermentation process to assess the possibilities of zero waste discharge along the lines of circular economy approach. Therefore, the residue and debris, originally deemed as waste from the process were subjected to BMP tests to improve carbon conversion and energy recovery. To determine the theoretical BMP, firstly, the dried and finely powdered BW, ABW + FR, and EBW + FR were subjected to elemental analysis. The empirical formula of BW was found to be $CH_{1.9}N_{0.045}S_{0.005}O_{0.8}$, whereas that of the ABW and EBW were found to be $CH_{1.9}N_{0.168}S_{0.014}O_{0.5}$ and $CH_{1.8}N_{0.125}S_{0.006}O_{0.5}$, respectively. Since the FR contained microbial cells in addition to the unconsumed nutrients, the N content is significantly higher in case of ABW + FR, and EBW + FR in comparison to BW. The Buswell-Muller-Boyle equation was used to quantify the BMP potential of the feedstocks [33] and found to be 423, 499 and 494 mL CH_4/g VS respectively. These values are theoretical maximum BMP that could be produced with assumption of 100 % conversion of VS to biogas which is not possible practically and cannot be achieved. This is because, a small percentage of the feedstock is either recalcitrant or will be utilized to meet the microbial energetic demands.

Unlike lignocellulosic biomass, BW is not recalcitrant and contains starch that is a branched homopolymer of glucose where monomeric units in linear amylose and branched amylopectin chains are connected by α1-4 and α1-6 linkages, respectively. This bonding variation of the glucose monomers in starch make it partially soluble in water as compared to insoluble cellulose. Therefore, starch is more bioavailable than cellulose and can be hydrolysed relatively easily compared to crystalline cellulose. BW has been used in the past as feedstock for generating biogas. For instance, Dubrovskis and Plume reported a BMP of 427 mL CH_4/g VS with BW [34]. In the present work, BW yielded a similar BMP of 421 mL CH_4/g VS Fig. 7.

This corresponds to a near complete utilisation of the VS content of BW during BMP based on the theoretical calculations as presented earlier based on the empirical elemental formula of BW. The solid residue from ABW + FR, and EBW + FR yielded a BMP of 345 and 379 mL CH_4/g VS, respectively. Similar sequential waste utilisation has also been reported with pretreated oat straw [5], paper sludge [35], corn stover [36], and municipal solid waste [37] where the ethanol fermentation residue or the whole stillage was subjected to BMP. In some cases, biomethane production followed by ethanol fermentation from the digestate [38] has also been performed. To the authors knowledge, this is the first time, such a sequential system has been investigated with BW for ethanol and biogas production.

Comparing the BMP of all the feedstocks, it is evident that the rate of biomethane generation and the extent of VS conversion are higher with the BW. While with the ABW + FR, and EBW + FR, VS conversion of 69 and 77 % were achieved, respectively. The higher N content of ABW + FR, and EBW + FR in comparison to BW led to a C/N ratio of 6 and 8, respectively, whereas for BW, it was 22. It has been reported that the higher conversion of the feedstock to biogas would occur within a C/N range of 20–30 [39,40]. This could be a possible reason for a lower VS conversion observed with the ABW + FR, and EBW + FR. Furthermore, the residue from the AH had a higher S content which would have also triggered the competition of substrates between sulphate reducing bacteria and methanogens, further affecting the VS conversion to biogas. No lag time in gas generation was observed with any of the residues, however the rates of generation varied. The fitted G_{max}, along with the rate of biomethane generation for all the three substrates are summarised in Table 3. While the residue obtained after fermentation was dried and powdered prior to BMP estimation, whole stillage could also use to further recover more biomethane as reported by other researchers [36].

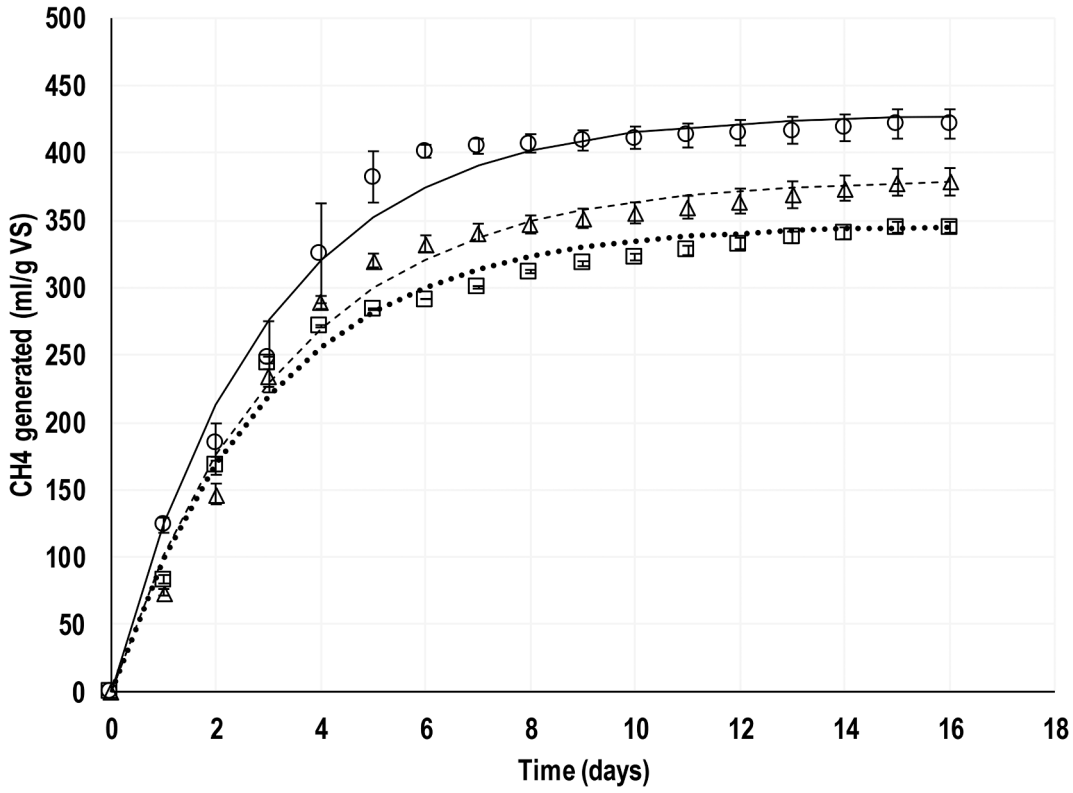


Fig. 7. Experimental biomethane generation (markers) and fitted data (lines) from BW, ABW + FR, and EBW + FR. Symbol: BW (empty circle); ABW + FR (empty square); EBW + FR (empty triangle).

Table 3
Parameters used in the model to describe the BMP of BW, ABW + FR, and EBW + FR.

Parameter	BW	ABW + FR	EBW + FR
Experimental G_{max} (mL CH_4/g VS)	421 ± 10	345 ± 4	379 ± 9
Fitted G_{max} (mL CH_4/g VS)	429	346	381
Fitted k (day^{-1})	0.344	0.335	0.308
Correlation coefficient (R^2)	>0.99	>0.99	>0.99
% Theoretical yield	99%	69%	77%

3.7. Life cycle assessment supporting the bread waste as a sustainable feedstock for ethanol production

The environmental impact of the integrated process including the ethanol fermentation and bio-methanation potential of the solid residues was evaluated through life-cycle assessment (LCA) analysis. Table 4 shows the LCA results obtained for the different impact categories for bioethanol produced using the BW as feedstock as described in this work, which is compared with the conventional fossil-based production of ethanol from ethylene. As can be seen, the GWP in terms of kg CO_2 eq for the ethanol produced from BW is only slightly higher (7%) than the more established fossil-based process. GHG emissions can be further reduced by effective optimization and identification of hotspots. Among the other categories, BW-based ethanol production results in >50% reduction in fossil energy use, about 85% reduction in photochemical oxidation and slight reduction in eutrophication (11%), when compared to the fossil-based ethanol. However, the BW-based process results in about 30% increase in both human toxicity and acidification.

Table 4
Environmental impact potentials of 1 kg bioethanol production from fermentation of BW.

Impact category	BW-based ethanol	Fossil-based ethanol	Unit
GWP	1.27	1.2	kg CO_2 eq.
HTP	0.71	0.54	kg 1,4-DB eq.
PCOP	0.00020	0.00142	kg C_2H_4 eq.
AP	0.0047	0.0036	kg SO_2 eq.
EP	0.00156	0.00175	kg PO_4^{3-} eq.
FSU	20.49	44.18	MJ

Brancoli et al. (2020) also performed LCA of ethanol production using BW. However, they defined the functional unit based on 1 kg of bread and not on the basis of the product. Their analysis showed that use of bread for ethanol production could result in net savings of −0.56 kg CO_2 eq per kg of bread. This is obtained after assuming that ethanol produced from this process can be directly substituted as vehicle fuel and that the process also results in co-production of dried distiller's grains with soluble (DDGS), which can replace soybean meal and barley as animal feed [41]. Both these assumptions are farfetched and need further validation before implementation in LCA. In the current work if we assume that the ethanol produced from fermentation of BW can replace the fossil-based ethanol, then this study predicts GWP of 0.018 kg CO_2 eq per kg of BW.

LCA studies for ethanol production using various other biomass-based feedstocks are available, however, many of these studies are based on different assumptions with regards to co-production and use different assessment methods, which makes direct comparison quite difficult. However, several studies have reported the impact of specific greenhouse gases (GHG) to the GWP of CO_2 over a 100-year period. When comparing with other bio-based feedstocks, GWP from the current process per kg of ethanol (1.267 kg CO_2 eq) is comparable to those reported for sugarcane from north east Brazil (1.06 kg CO_2 eq) and maize grain, US (1.05 kg CO_2 eq) and lower when compared to wheat, France (1.5 kg CO_2 eq), switchgrass, Uruguay (2.43 kg CO2 eq), sweet Potato (1.87 kg CO_2 eq) or wheat Straw (4.2 kg CO_2 eq) [6,42–44]. These results are encouraging, considering that the BW fermentation has only been evaluated at laboratory scale with further scope of optimization. GHG emissions from the process were further evaluated using a

contribution analysis. Fig. 8A shows the GWP contribution of important inputs to the process, such as chemicals, electricity, transportation, and water. As can be seen, energy used for electricity production is one of the major contributors to the GHG emissions, followed by chemicals, transportation, and water. The electricity usage in the five main stages of the production process is calculated to identify the hotspots leading to the high consumption of electricity. As can be seen from Fig. 8B, among the different stages, fermentation and DSP consume most of the electricity, with individual contribution of 67% and 29% each. Improvement of process yield such that more products can be recovered from each fermentation cycle, can not only help in reducing the overall electricity requirement in fermentation per kg of product but also reduce the energy demand for DSP. Also, if the current process plant is part of a larger biorefinery, and electricity can be sourced from cleaner renewable/waste resources from other parts of the plant, this can further help in reducing the GWP and making the process more sustainable.

4. Conclusions

The waste starchy materials which are cheap, rich and clean source

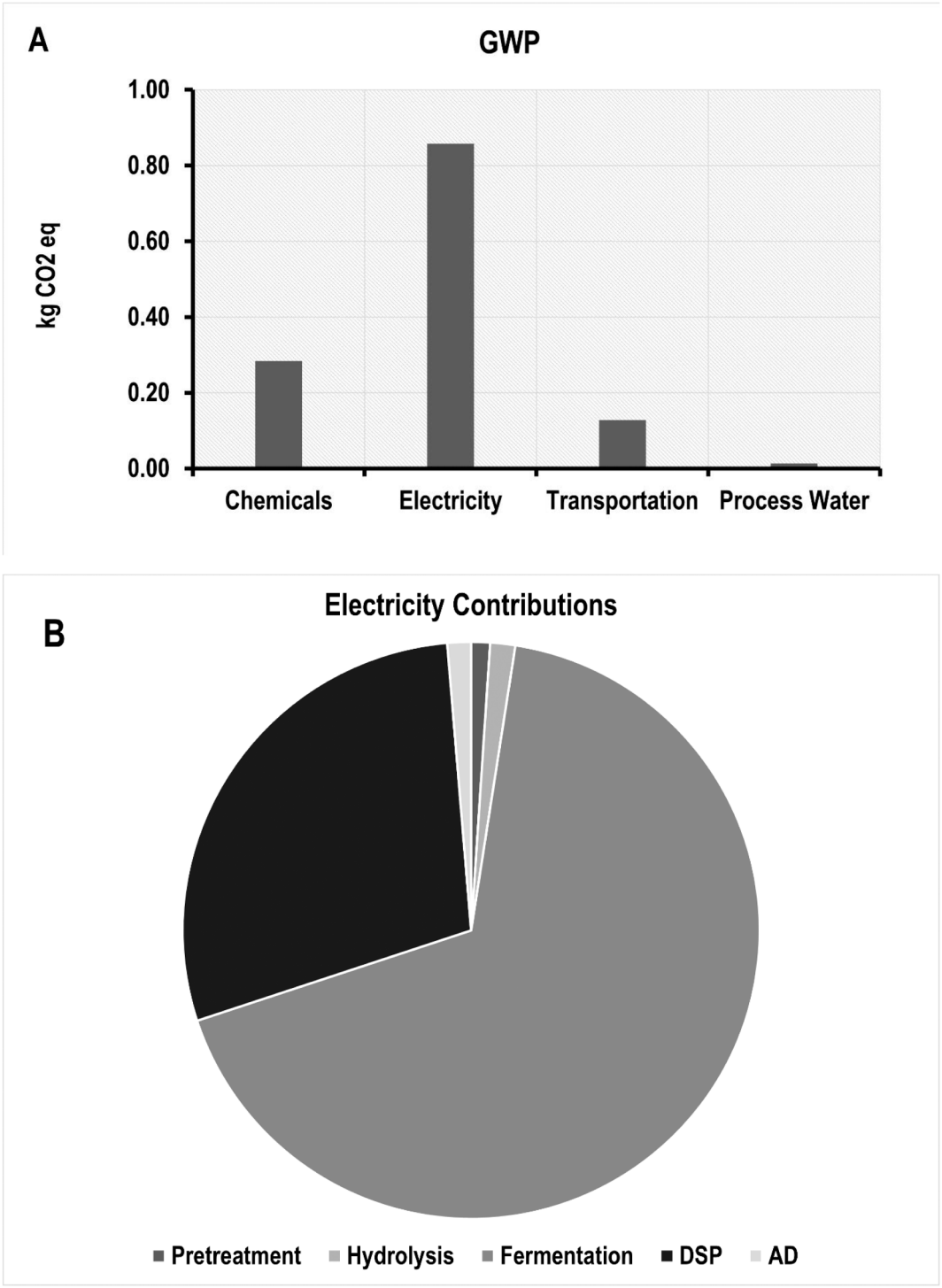


Fig. 8. (A) Individual contribution of different inputs towards GWP; (B) contributions of the different sub-processes towards total electricity consumption during fermentative ethanol production from BW.

of fermentable sugars are quite lucrative as feedstock and their usage also assist in cleaning the environment. Bread wastage is a severe problem in UK and Europe. The current study demonstrates the feasibility of using BW as feedstock for sustainable production of two biofuels, ethanol, and methane, using a circular biorefining approach, simultaneously addressing the problem of its disposal. The accumulation of high levels of ethanol from BW and yields close to theoretical values along with high production rates and ability to cope with fermentation inhibitors make *S. cerevisiae* KL17 a highly promising for industrial manufacturing. Moreover, comparable TYP metrics with acid hydrolysed BW, a cheap and rapid method for obtaining fermentable sugars, gives higher hopes for a cost-effective bioprocess. The process efficacy was also demonstrated by analysing the environmental benefits by performing LCA analysis. Future work will be directed at techno-economic analysis to evaluate the process economics for large scale production. Although ethanol was targeted in present work, several other high value products including platform chemicals could be generated from BW.

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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Contributions

Conceptualization, Investigation, Methodology was carried out by VN, SN, and SG. VN, SN, SG and VK were involved in data curation, formal analysis, and writing original draft. VVR, JZ, and KP were involved in conceptualization, and methodology. AB, MKA, and AP were involved in writing – review & editing the manuscript. All authors read and approved the final manuscript.

Availability of Data and Materials

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

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