High-Level Fermentative Production of Lactic acid from Bread Waste under Non-sterile Conditions with a Circular Biorefining Approach and Zero Waste Discharge

Rylan Cox [a*], Vivek Narisetty[b*], Sanjay Nagarajan[c], Deepti Agrawal[d], Vivek V. Ranade [c, e], Konstantinos Salonitis[a], Joachim Venus [f], Vinod Kumar[b]**

[a] School of Aerospace, Transport and Manufacturing, Cranfield University, Cranfield MK43 0AL, UK

[b]School of Water, Energy and Environment, Cranfield University, Cranfield MK43 0AL, UK

[c] Multiphase Flows, Reactors and Intensification Group, School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast BT9 5AG, United Kingdom

[d] Biochemistry and Biotechnology Area, Material Resource Efficiency Division, CSIR-Indian Institute of Petroleum, Mohkampur, Dehradun 248005, India

[e] Bernal Institute, University of Limerick, Limerick V94T9PX, Ireland

[f] Leibniz Institute for Agricultural Engineering and Bioeconomy, Max-Eyth-Allee 100, Potsdam, Germany

*Equal contribution

**Corresponding author

Phone: +44(0)1234754786

E-mail: Vinod.Kumar@cranfield.ac.uk

Abstract

Bread waste (BW) is a severe solid waste management problem in Europe. The current study demonstrates an environmentally friendly solution by valorising BW into lactic acid (LA) and the corresponding solid residues generated during hydrolysis and fermentation to biogas. To this end, BW was saccharified through acidic and enzymatic hydrolysis, and the hydrolysate obtained was used for LA fermentation under non-sterile conditions using thermophilic *Bacillus coagulans* DSM1. Maximum glucose concentration achieved during acid hydrolysis with 2% (v/v) acid loading and 20% (w/v) solid loading was 67.9 g/L glucose, with yield of 0.34 g/g BW. The LA accumulated with concentrated BW acid hydrolysate was 102.4 g/L with yield and productivity of 0.75 g/g and 1.42 g/L. h, respectively. For enzymatic hydrolysis, three commercial amylase preparations (Amyloglucosidase, Spirizyme, Dextrozyme) were employed, and the highest glucose release (98.6 g/L) and yield (0.49 g glucose/g bread) was attained with Dextrozyme from Novozymes. The fed-batch fermentation of *B. coagulans* was conducted using commercial glucose and glucose-rich BW hydrolysate from Dextrozyme. The LA titer, yield and productivity obtained with pure glucose were 222.6 g/L, 0.92 g/g and 1.86 g/L.h, respectively, whereas BW hydrolysate (BWH) resulted in 155.4 g/L LA, with a conversion yield and productivity of 0.85 g/g and 1.26 g/L. h, respectively. Further to the LA biosynthesis, the solid residues generated during hydrolysis and fermentation, were subjected to biogas generation, resulting in 553 mL CH_4/g volatile solids under batch mode. This massive LA titer amassed under non-sterile conditions and integrated biogas production using fermented residues demonstrates a high potential for an integrated biorefinery based on BW.

Keywords: Lactic acid; *Bacillus coagulans*; Bread waste; Acidic/Enzymatic hydrolysis; Non-sterile conditions; Biochemical methanation potential

1. Introduction

Renewable and sustainable are two explicit terms and essential components in constructing the path for the petroleum alternative. In this regard, biotransformation of organic and carbon-rich waste streams to fuels and chemicals via microbial route, offer an environment-friendly and lucrative opportunity. The waste-based production can spearhead the world towards sustainability with a reduced carbon footprint and simultaneously foster regional development via a newer generation of jobs, strengthening the rural economy and promoting self-reliance. The compound annual growth rate (CAGR) for biobased chemicals is estimated to be 14.5% between the year 2019 to 2028 (Global Bio-based Chemicals Market Forecast 2019-2028). The US Department of Energy identified twelve bio-based platform chemicals in 2004. The list was updated in 2010, and lactic acid (LA) was included among other chemical building blocks [1]. LA is an industrial chemical with numerous applications in diverse sectors, including pharmaceutical, cosmetics, food, speciality chemicals, textiles and leather. Currently, the majority of LA (~90%) is manufactured through the microbial fermentation of carbohydrates. The biological route has several advantages such as high enantiomeric purity, utilisation of inexpensive substrates and environmentally benign processes [2]. LA can be polymerised into polylactic acid (PLA), a biodegradable polymer and potential alternative to synthetic plastics. The global production of LA in 2016 was 1,220 kilotons, with an annual growth rate of 16.2% [3]. In 2014, an estimated 120 kilotons was used for PLA alone [4]. Furthermore, with the rising global demand for PLA, LA production is expected to reach 1,960 kilotons by 2025, with a global market of $9.8 billion [5].

The last decade has witnessed increasing public awareness towards waste reduction and its affordable management to promote environmental sustainability. Notably, the carbon-rich organic waste streams have drawn significant attention in terms of valorisation with a circular biorefining approach. A variety of substrates such as pure sugars, starchy feedstocks, molasses, and lignocellulosic biomass have been utilised in the past for LA fermentation [3,6,7]. In this work, food waste, specifically, bread waste (BW) was utilised as a substrate for LA fermentation. Bread is the staple food in Europe and one of the most wasted consumer materials. For instance, in Spain, 30% of all manufactured bread ends up as leftovers, which corresponds to >660,000ton/year of BW [8]. The bread wastage in Sweden is 80,410 ton/year [9]. Likewise, in

the UK,~ 20 million bread slices are thrown daily [10], leading to an annual wastage of 328,000 tonnes. This waste implies huge economic losses and has a substantial environmental and social impact from a life cycle assessment perspective. Valorisation of these wastes as a mitigation strategy can prove to be a game-changer by contributing towards the circular economy. Currently employed food waste management practices globally include anaerobic digestion (AD), incineration, composting and/or landfilling. For example, in the UK, the majority of the food waste was disposed into landfills earlier. However, new rules aimed at reducing greenhouse gas emissions, and zero-carbon wastage, rerouted it to AD and a further increase in the awareness in the society could assist in the complete elimination of food wastes into landfills. Due to the acceptance of AD for waste management and biogas production, the total number of AD plants in UK has been steadily increasing from 63 to 420 plants being fed by food or farm wastes [11]. However, according to biorefinery principle (Task 42), Circular Economy EU policies, and Sustainable Development Goals of Agenda 2030, it has been recommended that the production of platform chemicals should be prioritised over the formation of compost and bioenergy (Task-42-Biobased-Chemicals-value-added-products-from-biorefineries.pdf (ieabioenergy.com)). Bread contains 47-59.8% starch and 8-10% proteins and has a consistent and homogeneous composition [12–14]. Furthermore, it is a clean source of fermentable sugars unlike lignocellulosic feedstocks where harsh physical or chemical pre-treatments are employed, and sugars are often accompanied with microbial inhibitors. On the contrary, the soluble fermentable sugars can be obtained from bread with low energy and cheap enzymatic hydrolysis with no inhibitors [14,15]. Thus, BW shows the potential to serve as excellent feedstock for a viable biorefinery manufacturing chemical building blocks via microbial cell factories.

Bacillus coagulans is a well-known LA accumulating bacterium with several advantages over other LA producing microorganisms such as *Lactobacillus sp., Escherichia coli, Corynebacterium glutamicum, Rhizopus sp.,* etc., due to its ability to metabolise a variety of carbon source, grow and synthesise optically pure LA, an essential prerequisite for PLA synthesis, at higher temperatures (50-52 °C). With its high production rates, low or no by-products formation, contamination resistance, tolerance to nutrient deprivation and high yields for optically pure L(+) LA, it remains one of the most preferred commercially viable strains [3,16,17]. BW is starch

and protein-rich feedstock; its hydrolysate would provide a suitable medium for fermentative LA production. The current study initially investigated the optimization of temperature and substrate concentration favouring maximum accumulation of LA by thermophilic *B. coagulans* during fermentation under non-sterile conditions. Glucose-rich hydrolysate from BW was produced using either acid or enzymatic hydrolysis. The enzymatic hydrolysis was performed using three commercial amylase preparations (Amyloglucosidase, Spirizyme, Dextrozyme), and the results were compared. In the present study, the amylase loading was 0.6 mg / g BW with hydrolysis conducted at 60°C for 48 h, based on the process conditions optimised during our previous study (currently unpublished pending review in a separate article). Using the BW hydrolysate (BWH), LA production by *B. coagulans* was carried out in a bioreactor. Later, fed-batch cultivation using sugars from enzymatic hydrolysis was conducted in a bioreactor and compared with pure glucose. Further, the solid waste generated during enzymatic BW hydrolysis and the fermentation residues obtained at the end of fermentation were pooled, characterised, stored at 4°C and referred to FR+EBW. The FR+EBW was further subjected to anaerobic digestion (AD), and biochemical methanation potential (BMP) was determined to explore the possibilities of a BW-based viable biorefinery.

2. Materials and Methods

2.1 Materials, microorganism, and cultivation maintenance

Chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich and Fisher Scientific, unless stated otherwise. The out-dated bread procured from local Co-op supermarket (See supplementary data for composition given by supplier) was used in this study. The BW samples were cut into small pieces and dried at 105°C for 4 hours to measure the moisture content. The BW sample moisture content was 35.8%, and carbohydrate and protein contents were 46% and 7.9% w/w, respectively, as provided by the supplier. For the acid and enzymatic hydrolysis, out-dated bread as such was used without any drying. The acid hydrolysis was carried out using two different acid (1% and 2% v/v) loadings. The enzymatic hydrolysis was carried out using three different glucoamylase preparations, laboratory-grade enzyme Amyloglucosidase from *Aspergillus niger* (260U/ml) (Sigma-Aldrich) and commercial enzymes Spirizyme fuel HS and Dextrozyme peak, kindly gifted by Novozymes (Bagsvaerd, Denmark). The bacterial culture *Bacillus coagulans* DSM1 strain was

procured from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). It was cultured on Tryptone Soya (TS) [1.7% (w/v) pancreatic digest of casein, 0.3% (w/v) soybean meal, 0.5% (w/v) NaCl, 0.25% (w/v), KH_2PO_4, 0.25% (w/v), glucose] medium at pH 7.0, 50°C, and maintained in 30% w/v glycerol at −80°C. The seed culture was grown in 250 mL Erlenmeyer flasks with 50 mL TS medium and incubated for 16 h at 50°C on a rotary shaker (Excella 24, New Brunswick) at an agitation speed of 250 RPM.

2.2 Effect of temperature and initial glucose levels on growth and lactic acid production

To investigate the impact of temperature and initial glucose levels on cell growth and LA accumulation, *B. coagulans* was cultured at different temperatures (45°C, 50°C, 55°C, and 60°C) and glucose concentrations (50, 100, 150 and 200 g/L). The fermentation was conducted by maintaining pH-stat conditions at 6.0 ± 0.2 using 5M NaOH as an optimal value observed by Zhou and associates [18].

2.3 Acidic and enzymatic hydrolysis of bread waste

Acid hydrolysis of the BW was performed at 20% (w/v) solid loading (wet weight) using two different HCl concentrations (1% and 2% v/v) at 121°C for 15 minutes in the autoclave, as this is the simplest method for the starch ready for hydrolysis (Unpublished work). After the hydrolysis, the pH of bread waste hydrolysate (BWH) was adjusted to 6.0 using 10M NaOH, and concentrated using rotavapor (BUCHI, UK) at 100 mbar pressure and water bath temperature of 60°C for 24 h to obtain a glucose concentration of 200 g/L, stored at 4°C until further use. The hydrolysate was diluted according to the required concentrations during LA fermentation. In the hydrolysate, no colouration was observed, which is evident that the glucose was not degraded, and there is a linear increase in the concentration to the volume ratio.

All enzymatic hydrolysis of BW was performed in 2.0 L Erlenmeyer flasks with pH of bread suspension with 20% w/v solid loading (unevenly cut BW) was adjusted to 4.3 using hydrochloric acid (HCl) followed by autoclaving for 15 minutes at 121°C. A pH of 4.3 was chosen as it was found to be optimal for the enzyme to maximise glucose output and not possible to adjust post autoclaving. Before the enzyme was added, the suspension was allowed to cool and incubated at 60°C. After attaining the desired temperature, the suspension was supplemented with respective enzyme solution at a loading of 0.6mg/g BW and incubated for

48 h in a rotary shaker at 250 RPM. The enzymatic hydrolysis of BW was performed using three commercial enzyme preparations: Amyloglucosidase (Sigma), Dextrozyme Peak, and Spirizyme fuel HS (Novozymes). The samples were taken at regular intervals to monitor the glucose concentration. The BW hydrolysate was filtered using a muslin cloth to remove the solid BW residues or enzymatically hydrolysed BW (EBW), which were stored at 4°C for AD assessment. Later, the clarified supernatant solution was concentrated using rotavapor (BUCHI, UK) at 100 mbar pressure, with water bath being set at 60°C for 24 h to obtain a glucose concentration of ~200g/L. The fermentation medium was supplemented with this concentrated glucose- rich solution as sole carbon source for LA fermentation.

2.4 Bioreactor cultivation

Since LA production by *B. coagulans* is sensitive to pH and oxygen level, all the LA fermentations in this study were carried out in a 2.5 L bioreactor (Electrolab Bioreactors, UK) to ensure maintenance of favourable pH and oxygen-limited conditions for LA production. The culture was not aerated, and pH was controlled at 6.0 using 5M NaOH. The temperature and agitation speed were controlled at 50°C and 100RPM, respectively, unless stated otherwise. An overnight grown culture of *B. coagulans* was used for inoculating the bioreactor at an initial OD_{600} of 0.1. Fermentation medium contained (g/L): 1.97, $(NH_4)_2SO_4$; 0.26, KH_2PO_4; 0.025, $CaCl_2$; 0.2, $MgCl_2$; 2.17, $Na_2PO_4.6H_2O$ and 2.0, yeast extract. For fed-batch fermentation, the residual glucose concentration was maintained at or above 10 g/L with concentrated feed containing 500 g/L glucose + 5 g/L yeast extract for pure sugars and 200 g/L glucose and 5 g/L yeast extract for BWH. All LA fermentations were conducted under non-sterile conditions, while BW mass was autoclaved for saccharification via acidic and enzymatic hydrolysis. After the batch fermentations, the microbial cells and other insoluble residues in the fermented broth were separated by centrifugation for 10 min at 2000 RPM. The collected solid residue or fermented residues (FR) combined with EBW was dried at 65°C in an oven for two days until a constant mass was achieved. The dried samples obtained were used for AD, and BW was used as control.

2.5 Analytical methods

The efficacy of two different methods namely acid and enzymatic hydrolysis, was evaluated by measuring the glucose released from BW using High Performance Liquid Chromatography (HPLC-Agilent Technologies 1200

series, USA). Starch content within the bread was assumed equal to the carbohydrate content given in the bread contents, (See supplementary data for composition given by supplier). Glucose yields were calculated based on the maximum theoretical glucose obtained after complete hydrolysis of starch according to the following 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.

During the bioreactor experiments, the samples were withdrawn at regular time intervals to determine optical density, residual glucose, and LA concentration. Cell growth was quantified by measuring the optical density at 600nm wavelength in a 1 mm path-length cuvette using a double beam spectrophotometer (Jenway 6310, UK). Cell growth quantification could be done only in case of pure glucose (as positive control), but when the glucose source shifted to BWH, the measurement was not possible, as it was highly turbid owing to suspended bread particles. The concentrations of glucose and LA were measured by HPLC (HPLC - Agilent Technologies 1200 series, USA). The supernatants, obtained by centrifugation of the culture samples at 10,000 rpm for 10 min, were filtered through a 0.22 µm polyvinylidene fluoride (PVDF) membrane (Sartorius, Germany) and eluted through Rezex ROA-Organic Acid H + (Phenomenex, USA) column, connected with Refractive Index Detector (RID) for sugars and Diode Array Detector (DAD) for LA and inhibitors. With DAD detector, the acids were quantified at 210 nm and inhibitors at 254 nm wavelength. The mobile phase and flow rate were 5 mM H_2SO_4 and 0.4 mL/min and 0.6mL/min for sugars and acids, respectively.

2.6 Kinetics of biochemical methanation potential (BMP) analysis

For the BMP analysis, both BW and FR+EBW were first dried in an oven at 65°C for two days and then ground to a powder (<600 µm) using a 120 W Cookworks coffee and herb grinder and stored in airtight containers prior to characterisation and further experiments. Primary digestate of an anaerobic digester, digesting cow slurry and grass silage in Agri Food and Biosciences Institute (AFBI), Hillsborough, Northern Ireland, United Kingdom, was collected, filtered through a 1 cm sieve, degassed at room temperature (20 ± 1°C) for 15 days and used as an inoculum for biogas generation. Dried BW and FR+EBW powder were subjected to elemental analysis using a Series II Perkin Elmer PE2400 CHNS analyser, and oxygen content

was determined by difference. BW, FR+EBW, and the degassed inoculum were characterised for their total solids (TS), volatile solids (VS) and ash content based on methods adapted from reported literature [19,20]. About 0.2 g of BW/FR and 5.0 g of degassed inoculum were taken separately in crucibles for characterisation. The crucibles were then kept in a Carbolite Gero oven at 85°C for 2 days. The weight lost due to drying was calculated as the moisture content and the remaining weight was determined to calculate the TS content of the sample. The dried samples were then placed in a SNOL 13/1100 LHM01 muffle furnace and held at 550 °C for 2 h. The weight lost due to combustion was noted as the VS content and the weight of the remaining sample was accounted as the ash content of the sample. All measurements were conducted in triplicates and the values were averaged. The standard deviation between replicates for any composition was < 1%.

The batch BMP tests with BW and FR+EBW were set up based on prior literature [19,21]. A gas endeavour system (Bioprocess control, Sweden) was used to perform the tests. A schematic and photograph of the set up used is shown in the supplementary information. The endeavour system was operated with three sets of bioreactors with each set operating in triplicates (Table S1). Further, information on the BMP set up can be found in the supplementary information (title 'BMP bioreactor set up and quantification'). The BMP data can be effectively described using an empirical first order equation (1) as described previously [19,21]:

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

$$G = G_0 \frac{t}{t_0}\ for\ t \leq t_0 \qquad (2)$$

where G is the biomethane produced (mL CH_4/g VS) at time t, G_{max} is the maximum BMP (mL CH_4/g VS) that could be produced from the feedstock, k is the rate of biomethane generation, (day^{-1}),t_0 is the lag time and G_0 is the gas produced (mL CH_4/g VS) during lag time. The gas generated during the first four days was fitted using Equation (2) for estimating t_0 and G_0. Using these two parameters, the other two parameters, G_{max} and k were estimated using the rest of the experimental data and non-linear regression with the solver tool in Microsoft Excel.

3. Results

3.1 Impact of temperature on cell growth and LA production

Temperature has a significant influential impact on the performance of a bioprocess. Thermophilic strains are always preferred in bioprocesses as risk of contamination is low and operation can be performed under non-sterile conditions that would significantly cut down the operational cost [22]. The optimal temperature for maximal saccharification efficiency of hydrolytic enzymes is above 40°C and therefore, fermentation at high temperature can enable simultaneous saccharification and fermentation [22]. To evaluate the effect of temperature and identify the optimal temperature for cell growth and LA formation, *B. coagulans* was cultured at four different temperatures: 45, 50, 55 and 60°C. Figure 1 compares time course profiles for cell growth and LA at these temperatures. At 45°C, the initial glucose concentration of 50 g/L was dropped to 20.3 ± 1.3 g/L in first 24 h with OD_{600} and LA accumulation of 4.7 ± 0.2 and 23.7 ± 1.6 g/L, respectively. In next 24 h, metabolism almost came to halt, cell growth declined and merely 5 g/L of glucose was assimilated with marginal improvement in LA titer from 23.7 to 27.3 ± 1.8 g/L (Figure 1A). Even after 48 h, 15.3 g/L glucose was left consumed. In comparison, fermentation rate was higher at 50°C; cell growth and LA production started without any evident lag phase. About 95% of supplied glucose was exhausted in 24 h leading to cell OD_{600} and LA production of 8.7 ± 0.45 and 42.4 ± 2.6 g/L, respectively. The final LA titer achieved at the end of 48 h was 47.0 ± 2.9 g/L with conversion yield of 0.94 g/g (Figure 1B). Further increase in temperature to 55°C deteriorated the performance with diminished growth (OD_{600}: 2.4 ± 0.16), LA production (27.9 ± 1.72 g/L) and residual glucose concentration of 11.9 ± 0.78 g/L at 48 h (Figure 1C). A large variance on either side of 50°C indicates temperature sensitiveness of *B. coagulans* DSM1 strain. We also attempted to cultivate the strain at 60°C but fermentation did not progress as no growth and LA synthesis was observed. This experiment concluded with the fact that 50°C was not only the ideal temperature for the growth of the bacterium but promoted maximum lactic acid accumulation and hence was used in all the future experiments.

3.2 Influence of substrate concentration on growth and LA production

An efficient LA production at high substrate concentrations is always desirable for an economical process. Therefore, the influence of initial glucose concentration ranging from 50 to 200 g/L on substrate assimilation, cell growth and product formation with *B. coagulans* was investigated. The batch fermentation was carried out in bioreactor maintained at 50°C and the time course profiles of glucose consumption, OD, and LA production

at various concentrations (50, 100, 150 and 200 g/L) of glucose are shown in Figure 2. At 50 g/L, the fermentation continued for 48 h and glucose was completely exhausted in 36 h. The maximum cell OD_{600} obtained was 7.25 ± 0.46 with LA titer and yield of 44.2 ± 3.2 g/L and 0.88 g/g, respectively (Figure 2A). When the glucose level was increased from 50 to 100 g/L, the fermentation time nearly doubled, glucose level was depleted to 1.2 g/L after 72 h concomitant with highest LA accumulation of 74.2 ± 5.2 g/L (Figure 2B) and conversion yield of 0.75 g/g. At 150 g/L, the fermentation prolonged to 120 h where LA concentration of 127.3 ± 8.8 g/L (Figure 2C) was achieved at the end of fermentation and the conversion yield was 0.88 g/g. The highest cell growth was noticed at 50 g/L glucose whereas at 100 and 150 g/L, growth was similar (OD_{600}: 4.0-6.0) and substantially lower than at 50 g/L. The substrate inhibition was evident at 200 g/L as shown by a long lag phase coupled with minimal cell growth without any further increase. During the entire fermentation period, ~20 g/L glucose was assimilated with LA production of 5.5 ± 0.38 g/L at 48 h and thereafter, remained almost constant (Figure 2D).

3.3 LA production from glucose released through acid hydrolysis of bread waste

BW contains 50-70% starch, a homo-polymer of α-D- glucose, which can easily be released when BW was subjected to acid hydrolysis. The glucose concentration obtained after hydrolysis of BW suspension (20% w/v) were 57.3± 4.3 and 67.9 ± 5.5g/L with glucose yields of 0.28 and 0.33 g/g BW at acid loadings of 1.0 and 2.0% (v/v), respectively (Table 1).The theoretical conversion of starch to glucose is 1.11 g/g [24,25], as the BW used in this study has 46% w/v starch, the theoretical maximum glucose yield achievable is 0.51 g/g. The glucose obtained with 1 and 2% w/v acid loading were 70.7and 82.9% of the theoretical yield. Besides glucose, the hydrolysate contained inhibitors namely HMF (1.1 and 2.3 g/L) and furfural (0.1 and 0.65 g/L). The concentrated BWH was used for the LA fermentation. The pH of BWH obtained was brought up to 6.0 using sodium hydroxide pellets and supplemented with other nutrients before inoculation. The final volume was made to one litre and used for LA fermentation in the bioreactor.

When LA fermentation studies were conducted with the concentrated BWH obtained from two acid loadings namely 1.0 and 2.0% (v/v), they exhibited similar profiles and the initial glucose concentration was completely exhausted within 72 h. The cell OD_{600} could not be measured due to interference caused by bread

particles in suspension. Figure 3 shows time-course profiles for glucose uptake and LA formation using sugars released from acid hydrolysis. The LA production achieved with 2.0% acid loading was 102.4 ± 8.2 g/L with a productivity of 1.42 g/L. h while at 1.0%, the highest LA titer and productivity achieved was 91.8 ± 6.6 g/L and1.53 g/L. h. The LA yield in terms of glucose and BW were as follows: 1.0% - 0.89 g LA/g glucose, 0.23 g LA/g BW; 2.0% - 0.75 g LA/g glucose, 0.26 g LA/g BW. These results show the presence of HMF, and furfural had no significant impact on LA production.

3.4 Integrated LA production with enzymatic hydrolysis of bread waste

Unlike acid hydrolysis, enzymatic hydrolysis was performed under mild conditions and generated cleaner hydrolysates devoid of inhibitors and such preparations are often preferred for manufacturing products having applications in food and pharmaceutical industries. Three commercial glucoamylase enzymes, Amyloglucosidase (Sigma), Spirizyme fuel HS, and Dextrozyme peak (Novozymes) were employed for saccharification of BW. Enzymatic hydrolysis of bread requires careful process control of pH, temperature, solid and enzyme loading to maximise the glucose release. It was found in our previous study, the optimal values were pH – 4.3, 60°C, 20% (w/v) solid loading, and 0.6 mg enzyme/ g BW, respectively (unpublished data). These optimal parameters were used for glucose release from BW. Figure 4A shows the time course profiles for glucose release from the enzymatic hydrolysis of BW. The saccharification was relatively rapid in first 24 h, releasing more than 70% of total sugars, while it was slow in next 24 h. The final glucose concentrations accumulated at the end of 48 h with Amyloglucosidase, Spirizyme fuel HS and Dextrozyme peak enzymes were 94.5 ± 5.6, 93.7± 7.4, and 98.6 ± 7.9 g/L with a glucose yield of 0.47, 0.46 and 0.48 g/g BW, respectively (Table 1). Comparatively higher performance of the Dextrozyme Peak could be attributed to extra pullulanase activity exhibited by the enzyme preparation, an important debranching enzyme that acts on α-1,6-glycosidic bonds, that aided in robust saccharification and release of oligomeric glucose units. Since best results were obtained with Dextrozyme peak, it was used for later hydrolysis experiments. The glucose solution obtained using Dextrozyme was concentrated and used for LA fermentation in a batch mode. The initial glucose concentration used in this experiment was 156 ± 12.8 g/L. The strain rapidly metabolised glucose, and almost 100 g/L was assimilated in the initial 72 h while it took the next 48 h to consume the

311 remaining sugar (Figure 4B). LA formation was fairly quick at the beginning, where 43.8 ± 3.3 g/L LA was
312 synthesised in the first 24 h (1.83 g/L. h). Thereafter, productivity dropped (1.1 – 1.4 g/L. h) but remained
313 almost constant throughout the fermentation. The final LA titer achieved at the end of fermentation was 129.3
314 ± 9.7 g/L with a productivity of 1.08 g/L. h and yield of 0.83 g/g glucose and 0.40 g/g BW. Although the
315 productivity is lower, the results obtained are far superior to LA accumulated-on bread hydrolysate from acid
316 hydrolysis.

317 **3.5 Fed-batch cultivation**

318 Successful implementation of industrial bioprocessing is often met by employing fed-batch strategy during
319 microbial fermentation that successfully overcomes the substrate inhibition. After batch fermentation, fed-batch
320 tests were conducted to further improve the fermentation efficiency. Two parallel runs were performed using
321 commercial glucose and glucose rich concentrated BW hydrolysate obtained from enzymatic hydrolysis with
322 an initial glucose level of 45-50 g/L. In case of pure glucose, nearly 43-52 g/L glucose was consumed, and 43-
323 48 g/L LA was formed in every 24 h and this productivity (1.7 – 1.9 g/L.h) was consistent throughout the
324 fermentation that is unusual as productivity decline in later stage of fermentations (Figure 5 and Table 2). The
325 maximum LA amassed at 120 h was 222.6 ± 18.9 g/L. To the best of our knowledge this is one of the highest
326 reported titres of LA within the literature domain using B. *coagulans* under non-sterile conditions. This
327 corresponded with yield and productivity of 0.92 g/g and 1.86 g/L. h, respectively. The glucose consumption
328 profile using enzymatic BWH was similar to pure glucose, however, glucose uptake was not translated into LA
329 formation except in 0-24h. As a result of it, final LA titer achieved was less in comparison to pure glucose. The
330 final LA titer, overall yield and productivity with enzymatic BWH were 155.4 ± 13.29 g/L, 0.85 g/g and 1.26 g/L.
331 h, respectively. According to these results, 155.4 g LA was produced by 182.6 g glucose which was obtained
332 from 320 g BW. It means that 412.4 g LA can be generated from 1 kg of BW. The authors anticipate that
333 further research on different feeding strategies to improve the glucose utilisation rate could result in increased
334 LA titers, yield and productivity.

335 **3.6 Kinetics of biochemical methanation potential of bread waste and fermented residues**

According to Bastidas-Oyanedel and Schmidt, 2018, LA fermentation followed by AD leads to a revenue of $94/ton compared to $19/ton food waste with AD alone [26]. The residue remaining after LA fermentation may be further valorised by deriving biogas/ biomethane from it via anaerobic digestion. Such a step will not only lead to more revenue because of generated biogas (electricity generation) or biomethane (transportation fuel) but will also promote full utilisation of BW and promote circular economy. In order to evaluate valorisation potential of FR+EBW, biomethane potential (BMP) of FR+EBW was measured. For providing a reference, BMP of BW was also measured. Firstly, elemental analysis of BW and FR+EBW was carried out which revealed their empirical formula to be $CH_{1.9}N_{0.045}S_{0.005}O_{0.8}$ and $CH_{1.9}N_{0.112}S_{0.006}O_{0.2}$ respectively. The outdated bread as reported in literature had a similar (calculated) empirical formula to BW ($CH_{0.14}N_{0.048}S_{0.03}O_{0.94}$) [27]. Striking differences can be observed in the N and O contents of BW and FR. The presence of *Bacillus coagulans* DSM1 cells in FR+EBW contributed to the excess N content whereas the LA fermentation affected the O content. The ultimate BMP of each of these feedstocks were then determined using the Buswell-Muller-Boyle equation [28] and found to be 423 and 765 mL CH_4/g VS, respectively. The ultimate BMP is the theoretical maximum that could be produced from the feedstock assuming a 100 % conversion of the organics to biogas. Practically the theoretical maximum is not achieved since some fraction of the feedstock is recalcitrant (not digestible) and some fraction is used to fulfil the energetic needs of the anaerobic microbial consortia (cell growth). BMP of BW and FR+EBW were determined experimentally via the batch tests as described in section 2.6. The corresponding BMP profiles and the fitted parameters are shown in **Error! Reference source not found.**6 and **Error! Reference source not found.**3, respectively. No lag time for gas generation was observed with either of the feedstocks indicating their bioavailability for biogas generation. Almost 100 % of the theoretical BMP was achieved with BW (421 ± 10 mL CH_4/g VS). A similar BMP of 427 ± 3 mL CH_4/g VS [29] has been reported for BW. For FR+EBW, the BMP of 553 ± 57 mL CH_4/g VS (72% of ultimate BMP) was achieved. This high value of BMP indicates excellent potential for further conversion of FR+EBW to biogas.

4. Discussion

LA is a platform chemical with huge commercial potential and market size of $2.64 billion due to its versatile applications. Although LA production has been well documented in literature, enhanced production parameters leading to reduced production costs are always of interest for industrial applications [3,30].One of the major applications of LA in current time is its polymerisation to biodegradable polylactic acid (PLA). A low-cost LA can enable wider applications of PLA, a potential alternative for synthetic plastics that has attracted unprecedented global attention. For PLA to compete with petrochemical-based plastics, the cost of LA must be reduced by 50% [16].The commercial applications of LA require robust microbial cell factories which can accumulate LA at high titer (>100 g/L), yield (>80 %) and productivity (>2.5 g/L. h) from cost-effective feedstocks [16]. If the chassis strain can accumulate LA as sole fermentation product, it can further minimise the purification and overall production cost. The biorefinery and the circular bioeconomy concept emphasises towards the utilisation of renewable, sustainable, and cost-effective feedstocks, usually wastes which not only aims to cut manufacturing price but also implement better eco-friendly disposal and management systems.

Bread wastage is a serious solid waste management problem in Europe. While AD is increasingly being used to manage food wastes, it does not harness the full potential of the feedstock. For example, AD generates low-value products, namely biogas, and compost. The current study proposes a solution for BW by first valorising it into LA, the commercially viable chemical and using the FR+EBW for further conversion to biogas. *B. coagulans*, a homo-fermentative thermophilic bacterium was employed for manufacturing LA from BW. *Bacillus* strains have several advantages over LAB such as simple nutritional requirements and spore forming capacity [31]. The thermophilic behaviour of bacterium confers several advantages such as potential for effective simultaneous saccharification and fermentation, reduced viscosity, increased diffusion rates, improved substrate solubility, and reduced risk of contamination [32,33]. Cultivation conditions (e.g., Temperature) are vital parameters and profoundly impact cell growth and product formation. Hence, optimisation through single parameter approach was performed to evaluate the strain efficiency at four different temperatures, 45, 50, 55 and 60°C. The best results in terms of cell growth and LA accumulation were achieved at 50°C which agrees with most of the reports carrying out LA fermentation at 50 or 52°C. Earlier Aulitto and associates isolated a thermophilic and cellulolytic *B. coagulans* MA-13 strain which

fermented lignocellulosic-derived sugars into LA. To determine the optimal growth temperature, the strain was cultured 37, 50, 55 and 60°C and highest cell OD and maximum specific growth temperature was observed at 55°C and best LA fermentation ability at 50°C [34].

Batch fermentation is a preferred method for industrial LA production due to ease of operation and minimal cost, although it suffers from substrate inhibition and end-product toxicity. Therefore, it is important to determine the critical substrate concentration beyond which the outcome of fermentation is affected drastically. In our study, we observed that increasing glucose levels from 50 to 150 g/L resulted in a constant increase in LA titer, with a narrow range of yield and productivity. Glucose-based LA fermentation by *B. coagulans* has been shown to be inhibited by high substrate concentration and a glucose level ≥100 g/L reduce growth rate and prolong the fermentation time [31]. Wang and associates also made the same observation during LA production by *B. coagulans* IPE22 with various initial glucose concentrations (60, 80, 100, 115 and 145 g/L)[22]. The cell growth was largely unaffected. The LA accumulation improved from 56.1 to 80.1 g/L and productivity from 2.34 to 3.34 g/L. h with enhancement in initial glucose levels. While yield dropped substantially beyond glucose concentration of 80 g/L and at 145 g/L, a significant amount of residual glucose (34 g/L) was observed at the end of fermentation. They speculated that reduced glucose utilisation and low yield at 145 g/L might be due to combined effect of high substrate concentration and end-product toxicity. Michelson and associates performed a batch and fed-batch fermentation by *B. coagulans* with an equal amount of total glucose. The batch fermentation was performed with 126 g/L glucose while in case of fed-batch, it was divided into two equal lots, 63 g/L each. The results obtained in both the fermentations for LA production were quite similar: batch – 91.5 g/L, 0.97 g/g, 4.0 g/L. h; fed-batch - 91.6 g/L, 0.97 g/g, 4.3 g/L. h. Further, a batch fermentation with 144 g/L glucose yielded similar results: 101.7 g/L, 0.95 g/g, 3.1 g/L. h [34]. These results also confirm marginal impact of enhancement in glucose level up to 150 g/L on LA accumulation. A recent study by Zhang and associates examined the effect of temperature and glucose concentration on cell growth and LA synthesis by *B. coagulans* H-2. Consistent with our results, they observed that best results were obtained at 50 and 52°C with LA accumulation of 186.9 and 193.5 g/L, respectively, whereas glucose uptake rate, cell growth and LA accumulation declined significantly during fermentation at 55

413 and 57°C [35]. Similar performance of strain at glucose concentration in range of 160-220 g/L indicated the
414 weaker impact of high substrate levels with marginal fluctuations in OD_{600}, LA titer and yield which is not in
415 accordance with our findings where clear substrate inhibition was observed at 200 g/L with cell OD <1.0 and
416 LA titer < 10 g/L. In a study conducted by Zhou and associates using a newly isolated high-glucose tolerant
417 thermophilic *Bacillus coagulans* WCP10-4, a maximum LA titers of 210.5 g/L with a conversion yield of 0.95
418 g/g and 3.5 g/L. h productivity was observed, with no effect on cell growth or the yield due to high glucose
419 concentrations [18]. Thus, the present study further reaffirms that the osmotic tolerance limit of Bacillus sp.
420 owing to initially high sugar concentrations as well as its response to low water activity and end-product
421 inhibition varies from species to species.

422 Hydrolysis is an important step to unlock the glucose present in bread waste. Acid, alkali, or enzymatic
423 methods are commonly used for saccharification of crude renewable sources. Most of the literature reports
424 have made use of enzymatic route for hydrolysis of food, bakery, and BW. In the current study, we performed
425 acid as well as enzymatic hydrolysis of BW and compare the two processes for glucose release (Table 1) and
426 subsequent LA fermentations. Bread suspension was hydrolysed using two different acid loadings (1 and 2%
427 v/v) which resulted in a glucose rich solution with little amount of HMF (<2.0 g/L) and furfural (<1 g/L). But no
428 noticeable impact of these inhibitors on performance of LA fermentation was noticed as *B. coagulans* has
429 been reported to have high resistance to fermentation inhibitors. The amount of glucose released from 20%
430 w/v BW was higher with 2% v/v (67.9 g/L) acid loading than with 1% v/v (57.2 g/L), which was also reflected in
431 the LA accumulation (102.4, and 91.8 g/L, respectively). The enzymatic hydrolysis was conducted with three
432 commercial preparations, with Dextrozyme (98.6 g/L) releasing the most glucose, followed by
433 Amyloglucosidase (94.5 g/L) and Spirizyme (93.7g/L). Looking at the glucose yield from acid or enzymatic
434 hydrolysis (Table 1), 350-500 g of glucose can be produced from 1 kg of BW. The glucose-rich solution
435 obtained by Dextrozyme was concentrated, used for LA fermentation and 129.3 g/L LA was produced from
436 156 g/L glucose. The comparison of acid and enzymatic hydrolysis indicates similar performance in terms of
437 LA yield. The higher LA titer and lower productivity in case of enzymatic hydrolysis than acid, may be due to
438 high initial glucose concentration. If we look at cost economics, the acid hydrolysis approach is much cheaper

than enzymatic method. Acid hydrolysis just requires 15-20 min autoclaving with a small amount of acid, while enzymatic hydrolysis necessitates the use of expensive enzymes and long duration of saccharification (24-48 h) at high temperatures (45°C) adding extra capital and operation cost to the bioprocess. The downside of acid hydrolysis is the generation of inhibitors during the hydrolysis which may be troublesome for achieving a high purity LA. Majority of work on fermentative production of chemicals from BW has been mediated through enzymatic hydrolysis. Some examples includes production of succinic acid, lactic acid, industrial enzymes, xanthan gum, hydrogen, and baker's yeast [12,14,36,37]. The perusal of literature reveals that reports on acid hydrolysis of BW are scarce, though there have been few studies on starchy food wastes. But it is possible to compare our results with the study performed by Torabi and associates where they hydrolysed BW via acid and enzymatic hydrolysis and released sugars was fermented into ethanol [24]. The maximum glucose release of 69.8% (80 g/L) was obtained with 160 g/L solid loading, 1% acid loading and 20 min hydrolysis time whereas glucose yield (48.1%) was lower with 2% acid loading. In case of enzymatic hydrolysis, highest glucose yield of 93% (84 g/L) was recorded with solid loading of 125 g/L with a productivity of 1.5 g/L. h. The glucose titer (57 – 97 g/L), yield (0.28 – 0.48 g/g BW) and productivity (1.4 – 2.0 g/L. h) achieved from saccharification of BW in the present study are better than the results of Torabi and associates [24].

To eliminate the effect of higher substrate concentration and further improve LA production, fed-batch cultivation was performed using pure glucose and BWH as carbon source and massive LA titer of 222.6 and 155.4 g/L, respectively, was amassed. The dynamics of process was analysed by dividing fermentation period into five phases, each with duration of 24 h (Table 2). The amount of glucose assimilated and LA accumulated (titer, yield and productivity) was similar in with pure glucose and BWH in initial batch mode for 24h. During the fed-batch mode, lower LA yields and productivities were observed with BWH compared to pure glucose. The said difference in the results might be due to high viscosity of BWH and its heterogeneous nature, difference in glucose levels and the dilution caused by feeding. There are a handful of studies on fermentative manufacturing of LA from BW. In a recent study by Sadaf and associates, LA was produced from BW by LAB strains with simultaneous addition of amyloglucosidase. They employed simultaneous saccharification and fermentation (SSF) and solid-state fermentation for LA production from BW by three LAB strains, SKL-9

(*Lactobacillus paracasei*), SKL-11 (*Lactobacillus paracasei*), and SKL-21 (*Lactobacillus paracasei*) strains. The LA accumulated by SKL-9, SKL-11 and SKL-21 through SSF were 26.4, 28 and 27 g/L, respectively [23]. The LA yield by SKL-9, SKL-11, and SKL-21 via solid state fermentation were 212, 223, and 250 mg/g bread. The results obtained are much lesser than LA titer (155.4 g/L) and yield (412.4 mg/ g BW) obtained in current study.

Table 4 compare fermentative LA production from starchy food waste which have been as good as pure sugars as feedstocks for LA production from different strains. Kwan and associates investigated potential of mixed food and bakery waste for LA production by *Lactobacillus casei* Shirota [38]. The sugar and amino acids were released from wastes through fungal hydrolysis by *Aspergillus awamori* and *Aspergillus oryzae*, respectively. They reported LA concentration of 94.0 and 82.6 g/L from mixed food and bakery waste hydrolysate supplemented with yeast extract (10 g/L) respectively, with a yield (0.27 – 0.31 g/g) and similar productivity (2.50-2.61 g/L. h). In another study by Olszewska-Widdratand associates, various substrate were examined for LA accumulation by *B. coagulans* and one of them was sugar bread which was saccharified using commercial enzymes [39]. The subsequent fermentation using the released sugars enriched with yeast extract (15 g/L) resulted in LA titer, yield and productivity of 80.0 g/L, 0.85 g/g and 2.67 g/L. h, respectively. The SSF of raw sweet potato with exogenous addition of enzymes (α-amylase & amyloglucosidase), yeast extract (3 g/L) and peptone (5 g/L) by *Lactobacillus paracasei* generated LA concentration of 198.3 g/L with conversion yield of 0.90 g/g and productivity of 3.81 g/L. h [40]. The LA titer achieved in the current work is one of the highest, however, yield is comparable, and productivity is low and need to be improved. The LA titer achieved in the current work is one of the highest, however, yield is comparable, and productivity is low and need to be improved. The interesting feature of current work is that this high titer was accumulated on just 2.0 g/L yeast extract. In contrast, many reports in Table 4 made use of a high concentration of yeast extract (10-15 g/L). The presence of a large amount of yeast extract not only make the upstream expensive but also complicates the downstream processing. The bacterium did not produce any by-product other than LA which is an advantageous feature for an efficient product recovery. Further, the process was performed under non-

490 sterile conditions which would cut the cost of LA production at commercial level as medium sterilization
491 consumes a lot of time and energy.

492 After the LA fermentation with a maximum yield of 412.4 mg LA/g BW, significant FR+EBW remains.
493 The FR+EBW has a significant BMP and therefore can be used for biogas generation. The sequential
494 production of LA from BW followed by biomethane from FR+EBW offers multiple benefits; reduction of waste
495 and contribution towards development of zero waste biorefineries; reducing the LA production energy
496 requirements by utilising the energy produced from the generated biogas; enhancement in overall profitability
497 resulting in low-cost LA production. A simple representation of a BW based biorefinery is shown in Figure 7.
498 When extrapolated out, the data from batch experiments using acid pre-treated and enzyme (Dextrozyme)
499 hydrolysed BWH presented in this work indicates that one tonne BW will produce 256 and 401.5 kg LA,
500 respectively. The yield of LA in fed batch system is even higher (412.4 kg LA/ tonne of BW). The BMP
501 measured in this work indicates that the FR+EBW remaining after bio-based LA production from BW has a
502 potential to produce 87 kg biogas/ tonne of FR (63% volumetric CH_4 in biogas). This potential for biogas
503 generation from FR+EBW in reality will be higher than the reported values since in the present work BMP was
504 measured after drying FR+EBW. In real biorefinery, drying step is not needed and FR+EBW in liquid/ slurry
505 form may directly be digested to produce biogas. In that case, since as the liquid phase might have unused
506 soluble sugars, residual LA and other organics, the potential of FR+EBW to produce biogas will be significantly
507 higher than 87 kg/ tonne of FR+EBW. Assuming a 40% efficient electrical energy conversion and the calorific
508 value of methane to be 10 kWh/m^3, the electricity generated from this biogas would be187 kWh/tonne
509 FR+EBW. Considering that UK produces 328 metric tonnes BW, it proves to be an excellent feedstock for the
510 production of LA and biogas. The FR+EBW residue or slurry, rich in nutrients and residual organics can also
511 be used as a co-digestion feedstock to supplement the nutrient needs of otherwise recalcitrant AD systems,
512 such as agri-residue based AD. This excess energy that can be generated alongside LA from BW in the UK
513 equates to 61 GWh electricity which is >0.5 % monthly electricity consumed in the UK for domestic purposes.
514 With an average UK electricity price of 17 p/kWh, the revenue from biogas alone will be ~£30/tonne BW. The
515 excess energy recovered as biogas could either then be used to offset the energy costs or production costs of

LA. Furthermore, to boost revenue, digestate can be sold as a potential fertiliser, however this aspect of the biorefinery was not investigated in this work. The experimental results presented in this work will provide a basis for further detailed techno-economic analysis which is outside the scope of this manuscript. The approach of valorising BW by combination of LA and biogas (along with fertiliser) appears to be quite attractive and is aligned with circular bioeconomy approach.

5. Conclusions

The concept of circular economy is based on best out of waste. Therefore, the utilisation of biogenic residues for the bioproduction of chemical building blocks has become a priority in the bioprocessing industry. The current study evaluates the potential of BW as feedstock for LA production with a circular biorefining approach. The thermophilic *B. coagulans* strain was able to accumulate high LA titers from commercial glucose as well as BW with minimal fortification of yeast extract, and without any by-product formation. The results demonstrate the efficient bioconversion of BW and the feasibility of commercial manufacturing of LA under non-sterile conditions. Furthermore, the biogas production from waste residues generated during bread hydrolysis and fermentation would contribute to development of zero-waste biorefineries. The work also provides a solution of solid waste management problem of BW, a severe problem in UK/Europe. Further work on improving the LA productivity and techno-economic analysis will be conducted to economic viability of bioprocess.

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Contributions

RC, VN and SN carried out all the experimental work. RC, VN, SN, VVR and VK analyzed the data and wrote the Manuscript. DA, KS and JV were involved in proofreading the Manuscript and revised it critically. All authors read and approved the final Manuscript.

Corresponding author

Correspondence to Vinod Kumar.

Ethics declarations

Competing interests

The authors declare that they have no competing interests.

Availability of Data and Materials

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

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