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Brewer's spent grain as a self-sufficient feedstock for homofermentative production of optically pure L-lactic acid using *Lactobacillus rhamnosus*

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ABSTRACT

Brewer's spent grain (BSG), accounting for 85% of the total by-product from breweries, was used as a feedstock for L-lactic acid production in the present study. BSG was enzymatically hydrolysed and promoted to be a self-sufficient feedstock for L-lactic acid production by using it as a sole source of carbon, protein and minerals. Process parameters like glucose concentration (10–20 g/L), glucose-to-protein source ratio (1:1 – 5:1), source of protein and inoculum concentration (3 – 10% v/v) were selected to divert the carbon source and enhance L-lactic acid concentration, which would otherwise result in bacterial biomass production. Yeast extract and whey permeate resulted in high bacterial growth, whereas self-sustained protein (SSP) in BSG resulted in higher L-lactic acid production. Further, the glucose-to-protein source ratio was maintained at its lowest level for better glucose conversion to L-lactic acid. Glucose concentration strongly influenced L-lactic acid production. Therefore glucose concentration in the batch fermentation process was further increased from 60 to 120 g/L. A maximum L-lactic acid concentration of 114.4 g/L and productivity of 5.14 g/L·h was achieved with an initial glucose concentration of 120 g/L, and the rest of the process parameters such as glucose to protein source ratio of 1:1, inoculum concentration of 10% v/v and SSP in BSG were maintained at its optimum level. Finally, L-lactic acid in the fermentation broth was purified and analysed for its similarity with commercially available L-lactic acid using proton-NMR and FTIR spectroscopy. Thus, the present study valorised BSG by producing L-lactic acid under a biorefinery approach.

Abbreviations: ATR, Attenuated total reflectance; DOE –, Design of Experiments; FTIR, Peroxidase, Fourier transform infraredGOD-POD – Glucose oxidase; LAB, Lactic acid bacteria; MRS, deMan, Rogosa and Sharpe; NMR, Nuclear magnetic resonance; BSG, Brewer's spent grain; SSP, Self-sustained protein; WP, Whey permeate; YE, Yeast extract.

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1. Introduction

Lactic acid is one of the twelve essential platform chemicals listed by the department of energy (DOE) that can be produced from biobased feedstocks (Nagarajan et al., 2022). Lactic acid is highly appealing as it is a precursor for producing green solvents, fine chemicals, food preservatives (Sørensen et al., 2022, Sørensen et al., 2023a) and biodegradable polymers (Dusselier et al., 2013, Freeland et al., 2022). Lactic acid naturally exists as D-(−)- and L-(+)- isomers commonly outlined as dextro-lactic acid and levo-lactic acid, respectively. (Tong et al., 2022). Fermentative production of optically pure D-(−)- or L-(+)- lactic acid is desirable and currently practised on an industrial scale (Djukić-Vuković et al., 2016; Nagarajan et al., 2022). In fermentation, *Lactobacillus sp.* is aseptically grown under oxygen-limited conditions to convert sugars like glucose and sucrose to lactic acid. For instance, fermentation of sucrose and glucose using *Lactobacillus sp.* produced an elevated L-lactic acid concentration of 175.8 and 108.1 g/L, respectively (Liu et al., 2022). Further, the gram-positive, non-spore-forming, acid and aero-tolerant nature of lactic acid bacteria (LAB) make it more appealing for its use in lactic acid biosynthesis (Radosavljević et al., 2018).

The economics of lactic acid production is greatly limited by the feedstock cost, i.e. cost of carbohydrate and protein sources used in the fermentation (Liu et al., 2022). For instance, feedstock alone can ramp up over 70% of the overall lactic acid production cost (Radosavljević et al., 2021). Thus, replacing pure sugars and YE with various industrial refuse is often researched and is a matter of great interest (Tong et al., 2022; Wang et al., 2015). Brewer's spent grain (BSG) is a major by-product generated during the beer production process, and nearly 20 Kg of BSG is generated for every hectolitre of beer produced in the breweries (Djukić-Vuković et al., 2016). Thus, resulting in 40 million tons of BSG produced worldwide and 8 million tons only in Europe (Naibaho et al., 2022). The inexpensive nature (0.1 USD/kg) of BSG and its constituents enriched with 50% fibre and up to 30% protein content makes it more amenable to L-lactic acid production (Djukić-Vuković et al., 2016).

In that regard, various studies were reported for the production L-lactic acid using *Lactobacillus rhamnosus*. For instance, a maximum L-lactic acid concentration of 16.09 ± 0.07 g/L was produced using BSG hydrolysate when supplemented with 0.5% of YE (Pejin et al., 2015a). Further, BSG was hydrolysed using phosphoric acid and used for *L. rhamnosus* cultivation, resulting in maximal lactic acid productivity of 0.48 g/L·h (Djukić-Vuković et al., 2016). Encapsulation of *L. rhamnosus* in polyvinyl alcohol was carried out to ferment the enzymatically hydrolysed BSG and malt rootlets mixture in the presence of YE (Radosavljević et al., 2021). A maximum L-(+)-concentration of 48.5 and 50.1 g/L and volumetric productivity of 1.35 and 2.09 g/L·h was reported by fermentation carried out under batch and repeated batch, respectively (Radosavljević et al., 2021). A mixture of BSG, brewer's yeast, malt rootlets and soy lecithin were enzymatically hydrolysed and fermented under batch and fed-batch to get maximum productivity of 1.09 and 1.22 g/L·h, respectively. As visualised in all the studies above, BSG is a lignocellulosic material and demands a pre-treatment step to isolate glucose and protein sources from the raw biomass (Wang et al., 2015). Thus, acid hydrolysis using phosphoric acid (Pejin et al., 2015b) and enzymatic hydrolysis (Djukić-Vuković et al., 2016) using cellulase were widely used to hydrolyse the BSG before fermentation. Enzymatic hydrolysis was preferred over acid hydrolysis as the pre-treatment using acid hydrolysis results in the formation of microbial inhibitors (Balakrishnan et al., 2018a; Liu et al., 2012).

Additionally, the presence of lignin in BSG makes the feedstock recalcitrant by liberating the phenolic compounds, which would negatively affect microbial growth and product formation (Manikandan et al., 2020). Thus, enzymatic hydrolysis is preferential, but enzymatic hydrolysis involving cellulolytic and proteolytic enzymes for BSG to prepare fermentation broth for L-lactic acid production is still lacking in the literature. This is particularly intriguing as the protein source in the BSG can fill the protein or nitrogen source requirement by *Lactobacillus sp.* In addition to the requirement for carbohydrates and protein sources, usage of entire BSG is demanded in a biorefinery and circular economy context as they ensure complete valorisation and prevent waste generation (Tong et al., 2022). The mineral requirements for *Lactobacillus* growth are conventionally supplied by adding DeMan- Rogosa-Sharpe (MRS) medium (Pejin et al., 2015b). However, in the present study, the inherent presence of minerals in BSG was used without adding any mineral salt medium in the batch bioreactor experiments. Finally, 50 – 70% moisture content in the BSG reduces the requirement of freshwater usage in enzymatic hydrolysis and lactic acid fermentation.

Thus, the present study aims to uniquely tap the carbohydrate, proteins, minerals and water source from the BSG, thereby promoting it as a self-sufficient feedstock for lactic acid production. In the past the group has implemented digital-twins (Sørensen et al., 2023b) and feed control strategies to optimise the product outputs (Kottelat et al., 2021, Brignoli et al., 2020, Donnelly et al., 2022). However, this study outlines a method to utilise BSG as a cost-effective feedstock for lactic acid production. The present study also screens the fermentation process parameters using Taguchi experimental design to better utilise the feedstock for lactic acid production, which would otherwise result in generating *Lactobacillus* biomass. Batch kinetic experiments were carried out in a bioreactor to achieve maximum lactic acid concentration and productivity. Finally, as-produced L-lactic acid was purified, and its similarity with commercially available L-lactic acid was evaluated using proton- nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy.

2. Materials and methods

2.1. Feedstock and chemicals

BSG was produced in-house using the Microbial Bioprocessing brewing facilities (50 L Speidel Braumeister) in Dublin City University (Dublin, Ireland). Since BSG consisted of 50 wt% moisture, the BSG biomass was frozen (– 20°C) to avoid microbial growth. The enzymes used for hydrolysis for example, amylase (min. 137 U/mL), amyloglucosidase (300 U/mL), cellulase (glucanase) and protease

(NDB3 papain) were procured from Murphy & Son Limited (Nottingham, UK). Additional enzymes namely endoglucanase (500 U/mL), cellobiohydrolase (90 U/mL) and protease (6 U/mg) were procured from Megazyme® (Wicklow, Ireland). *Lactobacillus rhamnosus* was cultured in MRS medium obtained from Thermoscientific™ (Hampshire, UK). Glycerol stocks with a 15% v/v concentration mix were prepared and stored at −80°C for further use.

Ammonium hydroxide, hydrochloric acid, sulfuric acid, sodium hydroxide and ethyl acetate used in this study were procured from Fisher Chemical® (Loughborough, UK). Steam-activated and acid-washed Norit GSX® activated carbon was purchased from Thermo Scientific® (Klazienaveen, Netherlands). Tri-ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, and polysorbate 80 were procured from Merck® (Darmstadt, Germany) or Fisher Chemical® (Kandel, Germany). Milli-Q water with an average conductivity of 1 $\mu\Omega\ cm^{-1}$ was used throughout the study.

2.2. Enzymatic hydrolysis

Simultaneous saccharification and proteolysis were performed in screw-capped glass bottles with a working volume of 100 mL. 15 wt% of blended BSG (dry basis) with respective enzymes added. BSG being a lignocellulosic material, contains majorly cellulose and some residual starch left during the mashing process. Therefore, enzymes like cellulase, amylase and amyloglucosidase were used for enzymatic hydrolysis of BSG. Best enzyme and its combination were chosen based on the high glucose concentration observed with respective enzymes and its combination (g/L). Prior to simultaneous saccharification and proteolysis, the BSG biomass was blended in its wet state using a blender (Kenwood, Kenwood Ltd, UK) for 5 minutes with the additional water required to compensate 15 wt%. Process conditions were selected based on the technical data sheets provided by the enzyme suppliers and from the previous literature. For instance, the solution pH was corrected to 5.5 ± 0.5 using 0.1 M NaOH or H_2SO_4, and the hydrolysis was carried out in a shaking water bath (Isotemp™, Fisherbrand™, UK) operated at 60° C and an agitation rate of 120 rpm (Ariaeenejad et al., 2020).

Four different hydrolysis experiments were carried out in triplicate with the following combination of enzymes: Exp - A (amyloglucosidase and protease), Exp - B (glucanase, amylase, amyloglucosidase and protease), Exp - C (glucanase, cellobiohydrolase and protease), Exp – D (glucanase, cellobiohydrolase, amyloglucosidase and protease). Samples were collected every 2.5 h up to 48 h and analysed for glucose concentration following GOD-POD assay as portrayed by Megazyme® (Wicklow, Ireland). In all the experiments, 0.1% v/v of each enzyme was added for enzymatic hydrolysis for BSG. Apart from the simultaneous saccharification and proteolysis of BSG with the aforementioned enzymes, additional proteolysis treatment was carried out only with 0.1% v/v of the papain enzyme at 7 ± 0.5 pH and 60° C temperature. The protein extract from BSG was centrifuged at 10,000 rpm for 10 minutes (Mikro 120, Hettich, Westphalia, Germany) and the supernatant was frozen overnight at – 80° C and freeze-dried to get self-sustained protein. The additional protein extract produced from BSG was utilized to supplement and control the C/N ratio in the Taguchi experimental design portrayed in Section 2.3.

2.3. Screening of process parameters using Taguchi experimental design

Process parameters such as glucose concentration (10, 15 and 20 g/L), type of protein source (SSP – Self-sustained protein, WP – Whey permeate and YE – Yeast extract), glucose to protein source ratio (1, 2 and 5), and inoculum concentration (3, 5 and 10% v/v) were screened using Taguchi orthogonal experimental design. Nine experimental runs were carried out in triplicates using glass bottles with a total volume of 250 mL and working volume of 100 mL (Table 1).

All the screening experiments were conducted in static flask mode with an operating temperature of 37 °C (Balakrishnan et al., 2018b). Minerals were supplemented in all the experiments with the composition and concentration described in MRS medium (Balakrishnan et al., 2018b). However, the experiment on effect of oxygen on biomass concentration and L-lactic acid production was carried out by culturing *Lactobacillus rhamnosus* under aerobic, microaerophilic and anaerobic condition. For aerobic cultivation, vented and baffled Erlenmeyer flask agitated at 250 rpm was used for *Lactobacillus rhamnosus* cultivation. Microaerophilic conditions were maintained by carrying out the experiment in a closed bottle without any agitation. And anaerobic cultivation was achieved by displacing dissolved oxygen through nitrogen gas sparging for 15 minutes and the experiment was once again carried out in a closed glass bottle without any agitation. The significance of the various data sets obtained by aerobic, microaerophilic and anaerobic

Table 1
Taguchi orthogonal experimental design showing a total of nine experimental runs with different combinations and varying levels of process parameters along with their responses (SSP – Self-sustained protein, WP – Whey permeate and YE – Yeast extract).

Run	Glucose concentration (g/l)	Protein source	C/N ratio	Inoculum (% v/v)	Biomass concentration (g/L)	L-lactic acid concentration (g/L)
1	10	SSP	1	3	0.8 ± 0.1	9.3± 0.3
2	10	WP	2	5	1.2 ± 0.2	10 ± 0.3
3	10	YE	5	10	1.6 ± 0.1	10.4 ± 0.1
4	15	SSP	2	10	1.4 ± 0.1	15 ± 1.2
5	15	WP	5	3	1 ± 0.1	13.6 ± 0.2
6	15	YE	1	5	2.5 ± 0.1	14.6 ± 0.1
7	20	SSP	5	5	1.3 ± 0.1	19.4 ± 0.2
8	20	WP	1	10	2.2 ± 0.1	19.8 ± 0.8
9	20	YE	2	3	3.1 ± 0.2	18.3 ± 0.9

condition was statistically analysed by following Tukey's test through OriginPro 2018 software (v. 9.5.95, Northampton, USA).

The solution pH was buffered by adding 20% w/w of calcium carbonate with respect to the amount of glucose contained in the respective bottles (Balakrishnan et al., 2018b). Experiments were carried out for 24 h, and the samples were collected after every 4 h and analysed for the residual glucose concentration, biomass and lactic acid production. Although glucose, biomass and lactic acid concentration were recorded, only biomass and lactic acid concentration were used as a response for the Taguchi analysis. Both biomass and lactic acid production was analysed under the "Largest is the best" strategy (Manikandan et al., 2020; Manikandan and Lens, 2022). The signal to noise (S/N) ratio for each process parameter for lactic acid and biomass production was calculated using Eq. (1) (Manikandan and Lens, 2022; Sinharoy et al., 2015):

$$\frac{S}{N} = -10\left[\log\left(\frac{1/Y^2}{n}\right)\right] \tag{1}$$

Where Y is lactic acid or biomass concentration (g/L) and n is the number of experimental runs. Mean and S/N ratio analysis for the current Taguchi experimental design was carried out using Minitab® (version 16, PA, USA) statistical software.

2.4. Batch bioreactor experiments

Batch bioreactor studies were conducted to enhance the lactic acid concentration and exploit the microbe's potential for complete conversion of BSG hydrolysate to lactic acid. All the batch bioreactor experiments were carried out in a 2.5 L bioreactor (RALF®, Bioengineering, Switzerland) having a 1 L working volume. The optimal process parameters obtained by the Taguchi experimental design, for instance, the protein source (SSP from BSG) and glucose-to-protein source ratio of 1 were used to operate the bioreactor. The concentration of glucose was increased by vacuum distillation using Buchi® rotavap. As mentioned earlier, the maximum feedstock potential of BSG was realised by carrying out the fermentation without any addition of minerals, and 50 wt% of aqueous medium used for hydrolysis was sourced from the moisture content present in BSG. Prior to starting the batch experiments, the bioreactor was autoclaved at 121 °C for 15 minutes (Balakrishnan et al., 2020), and the fermentation broth was pumped thereafter and pasteurised at 63.5 °C for 30 minutes as carried out in the literature by (Rotta et al., 2020). Finally, *L. rhamnosus* was axenically transferred to the bioreactor (RALF®, Bioengineering, Switzerland) at an inoculum concentration of 10% v/v.

The mesophilic condition required for *L. rhamnosus* growth was maintained by nitrogen sparging with an average flow rate of 2.5 nL/h. The bioreactor was agitated at 250 rpm, and the solution pH in the bioreactor was controlled to 6 ± 0.5 by adding 25% w/v of ammonium hydroxide or 0.4 M HCl (Balakrishnan et al., 2020). Glucose consumption, biomass and lactic acid were measured over 24 h with a regular interval of 4 h. In batch bioreactor experiments, L-lactic acid was measured continuously, while D-lactic acid concentration was measured only at the end of the batch experiments to measure the optical purity.

The biomass concentration kinetic profile was used to estimate the specific growth rate, and D or L-lactic acid concentration was used to measure the optical purity (%). The equation used to estimate specific growth rate (μ) and optical purity can be found in Eqs. (2) and (3), and the same is as follows (Gali et al., 2021):

$$\mu = \frac{1}{x} \times \frac{dx}{dt} \tag{2}$$

$$\text{Optical purity}(\%) = \left(\frac{C_L}{C_L + C_D}\right) \times 100 \tag{3}$$

Where μ and x are the specific growth rate (h^{-1}) and biomass concentrations (g/L), respectively, C_L and C_D are L-lactic and D-lactic acid concentrations (g/L).

2.5. Downstream processing and characterisation of lactic acid

Downstream lactic acid processing from the fermentation broth was initiated by removing the bacterial biomass and other debris by spinning at 14,000 rpm for 30 minutes in a centrifuge (Lynx, Thermoscientific™, UK). The cell-free fermentation broth was acidified with concentrated sulfuric acid (98%) to isolate the ammonium as ammonium sulfate and to release the free lactic acid (Ahmad et al., 2021a). Acidification was carried out using a magnetic stirrer in a 2 L glass bottle having a 1 L working volume, and the reaction was monitored using a pH meter. The addition of sulfuric acid was stopped as the pH meter displayed 1. After acidification, decolourisation was carried out for 5 h using 100 g/L of activated carbon.

At the end of 5 h, the activated carbon was first removed by centrifugation at 14,000 rpm for 30 minutes. The supernatant was filtered using Whatman®-1 filter to ensure the complete removal of finer carbon particles (Ahmad et al., 2021b). Secondly, during liquid-liquid extraction ammonium sulfate remains in aqueous phase and the lactic acid migrates to the organic phase. Thus, the ammonium sulfate was separated from the aqueous phase by distillation using Buchi® rotavap (R-200, Switzerland) operated at 60 °C and 35 mbar.

Liquid-liquid extraction was carried out using ethyl acetate as the solvent. The extraction was done with a 1:3 ratio of decolourised fermentation broth and ethyl acetate. Liquid-liquid extraction was carried out in a magnetic stirrer, which lasted 90 minutes. The extraction was repeated three times with fresh ethyl acetate for complete lactic acid recovery. Finally, the organic phase comprising lactic acid was distilled to separate lactic acid using Buchi® rotavap (R-200, Switzerland) operated at 60 °C. However, the organic

phase was distilled at 150 mbar, and the aqueous phase at 35 mbar.

Characterisation of ammonium sulfate and lactic acid was carried out using FTIR analysis under attenuated total reflectance (ATR) mode (Nicolet 6700 FTIR, Thermo Scientific, UK). An average of four scans with 2 cm^{-1} resolution was carried out over a wavelength range of 400 – 4000 cm-1 (Manikandan and Lens, 2022). Comparison of as-produced ammonium sulfate and lactic acid was compared with that of the commercially available pure compounds. Similarly, comparison of lactic acid produced using BSG was compared to that of commercially available pure L-lactic acid using 600 MHz proton-NMR analysis (Bruker Avance Ultrashield 600).

2.6. Analysis

Protein analysis was carried out using Lowry's protocol, as described by Manikandan and Lens, (2022). Glucose analysis followed the GOD-POD assay, and biomass estimation was carried out by the spectrophotometric analysis using a UV-Vis spectrophotometer (Spectronics 200, ThermoFisher, UK) (Gali et al., 2021). The L-lactic acid assay was carried out by the kit procured from Megazyme® (Wicklow, Ireland), and all the analyses were carried out per the manufacturer's protocol in a plate reader (Spark, Multimode Plate Reader, Tecan, Switzerland) (Balakrishnan et al., 2018b; Gali et al., 2021). Before glucose and lactic acid analysis, all the samples were centrifuged at 10,000 rpm for 10 minutes.

Biomass concentration was measured by gravimetric analysis. For this analysis, 2 mL of samples were spun at 10,000 rpm for 10 minutes in a centrifuge. The supernatant was decanted carefully, while the bacterial pellet was dried at 70 °C until it reached a constant weight (Manikandan and Lens, 2022). Finally, the dried samples were measured using high precision weighing scale to get the biomass concentration.

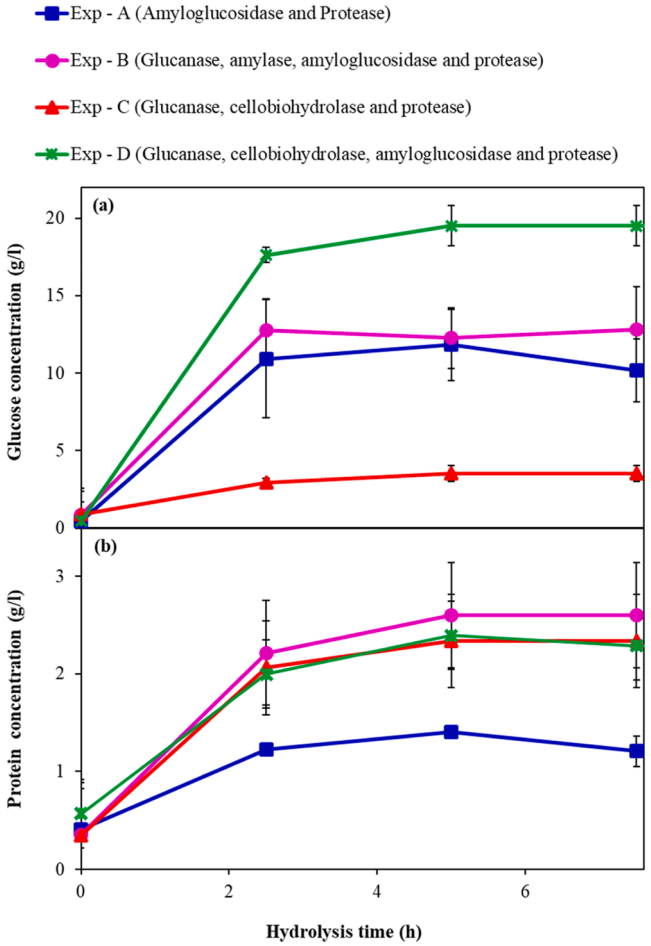
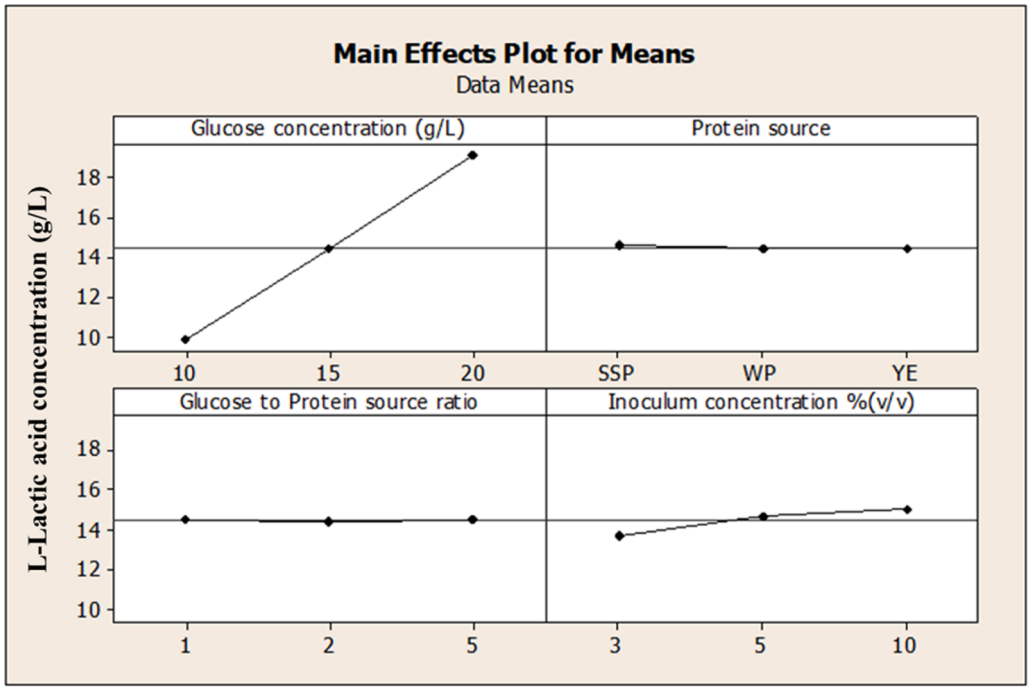


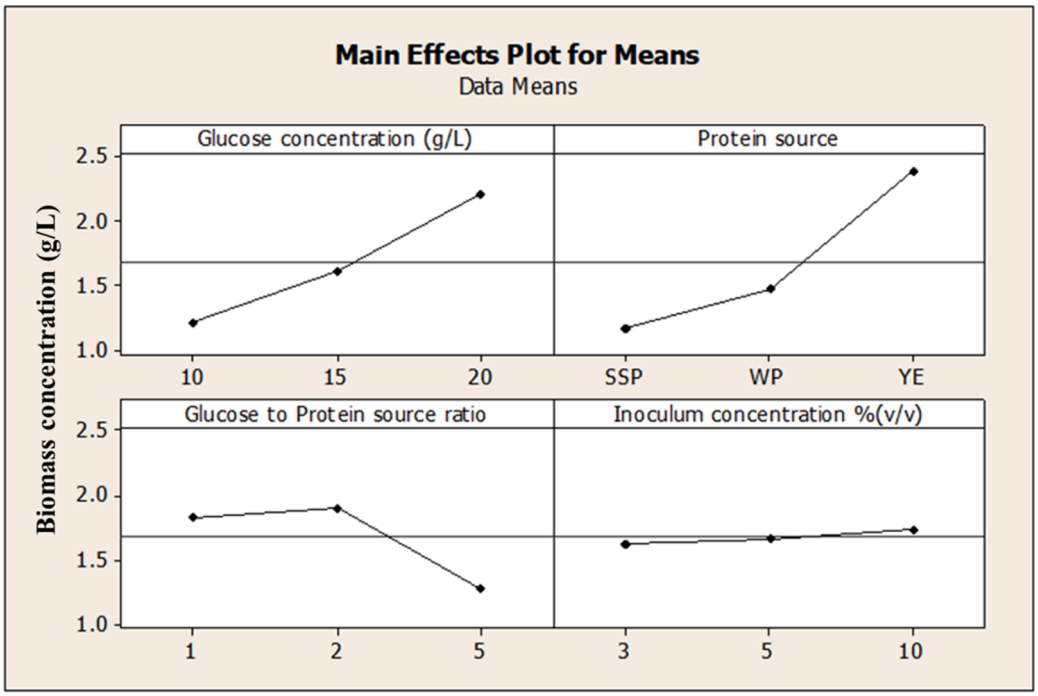
Fig. 1. Kinetic study on enzymatic hydrolysis of SBG with various enzymes and its combination as a function of time (enzymatic hydrolysis conditions: solid content of 15 wt% of blended BSG (dry basis), enzymes dosage of 0.1% v/v of respective enzymes, solution pH of 4 ± 0.5, solution temperature of 60 °C and an agitation rate of 120 rpm).

3. Results and discussion

BSG is a lignocellulosic biomass and therefore requires sophisticated pre-treatment strategies for hydrolysing the biomass to release simple sugars like glucose (Esquivel-Hernández et al., 2022; Zabed et al., 2016). Hydrolysis of biomass followed by the separate



(a)



(b)

Fig. 2. Mean effect plot of the different process parameters on (a) L-lactic acid and (b) biomass concentration using *L. rhamnosus*.

hydrolysis and fermentation for lactic acid using *Lactobacillus* sp. performed better than the solid-state fermentation (Rojas-Pérez et al., 2022). Thus, enzymatic hydrolysis was the first process in the present study to extract glucose and protein from BSG biomass.

3.1. Enzymatic hydrolysis of BSG

To promote BSG biomass as a self-sufficient feedstock and eliminate the process steps, simultaneous saccharification and proteolysis were carried out to extract fermentable sugars and proteins. Thus, Pappain - a protease enzyme, was added to all the enzymatic hydrolysis processes (Tacias-Pascacio et al., 2021). Fig. 1 suggests that the saccharification for glucose release and proteolysis for protein extraction of BSG attained equilibrium at the end of 5 h. At the end of the fifth hour, a maximum glucose concentration of 11.81 ± 2.31 g/L, 12.26 ± 1.95 g/L, 3.52 ± 0.53 g/L, and 19.5 ± 1.29 g/L was observed for Exp - A, B, C and D, respectively. Further increase in the hydrolysis time to 7 h did not show a significant variation in glucose and protein concentration. It can be inferred that the residual starch content in BSG biomass was effectively hydrolysed by amyloglucosidase in Exp – A, B and D and the addition of amylase to amyloglucosidase in Exp –B did not improve the starch hydrolysis. Furthermore, the cellulosic content of BSG was effectively hydrolysed by glucanase and cellobiohydrolase enzymes. Thus, highest glucose concentration of 19.5 ± 1.29 g/L was observed in Exp–D, in which glucanase, cellobiohydrolase, amyloglucosidase and protease were used for hydrolysis.

Proteolysis of BSG biomass resulted in a maximum protein concentration of 1.4 ± 0.01 g/L, 2.6 ± 0.07 g/L, 2.3 ± 0.1 g/L and 2.39 ± 0.29 g/L for Exp - A, B, C and D, respectively. It could be inferred that the presence of glucanase cleaved the cellulosic fibres and exposed the proteins present in the BSG biomass for proteolysis. It was previously reported in the literature that an aleurone layer encloses the protein in BSG biomass (Lynch et al., 2016; Rojas-Pérez et al., 2022). This also explains the least protein concentration of 1.4 ± 0.01 g/L observed in Exp – A, as the glucanase needed for cleaving the aleurone layer was absent only in this experimental condition. Paz et al., 2019 used an enzyme cocktail produced by *Aspergillus niger* for enzymatic hydrolysis of BSG, which resulted in a maximum glucose concentration of 18.45 ± 1.66 g/L (18.45 wt% of BSG biomass). Ravindran et al. (2013) reported 7.59 wt% extraction of reducing sugar from BSG biomass using cellulase and hemicellulase enzymes. However, the authors reported a 2.14-fold (16.2 wt% of BSG biomass) increase in reducing sugar yield by improving the enzymatic hydrolysis of BSG using non-thermal plasma. Yang et al. (2023) reported a maximum glucose concentration of 27 g/L (27 wt% of BSG biomass). However, the BSG biomass was treated using dilute sulfuric acid hydrolysis before enzymatic hydrolysis. The glucose (0.13 ± 0.01 g/g) and protein (15.6 ± 0.44 mg/g) yield with respect to dry BSG was observed at the end of enzymatic hydrolysis experiment -A. The yield reported here in the present study is slightly lesser than the one reported in the literature. This could be attributed to the absence of alkali or acid pre-treatment before the enzymatic hydrolysis. Thus, an alkali pre-treatment before enzymatic hydrolysis should be considered. However, the simultaneous saccharification and proteolysis proposed in the study are expected to fulfil the carbon and protein requirement for *L. rhamnosus* growth uniquely, and the same is substantiated in section, 3.2.

3.2. Screening of process parameters using Taguchi experimental design

Increasing the carbon source concentration and maintaining a proper balance between the carbon and protein source ratio maximised the lactic acid concentration. Thus, the glucose concentration varied between 10 and 20 g/L, and the glucose-to-protein source ratio was controlled between 1:1 and 5:1. In addition, the performance of protein stemming from the BSG was compared to that of the other conventional protein source like YE and WP. Finally, the effect of inoculum concentration on lactic acid and biomass growth was also studied. The effect of all the different process parameters and their various levels on *L. rhamnosus* biomass growth and lactic acid production is displayed in Table 1. Exp run no. 8 exhibited the highest lactic acid concentration of 19.8 ± 0.2 g/L with glucose concentration of 20, glucose to protein ratio of 10:1 and protein source stemming from WP. Statistically similar lactic acid concentration of 19.4 ± 0.2 g/L was observed with the glucose concentration of 20 g/L, glucose to protein source ratio of 5:1 and protein source stemming from BSG (SSP). However, the WP concentration in this run, due to which an elevated bacterial biomass

Table 2
Signal-to-noise (S/N) ratio values of various process parameters and their ranking based on delta S/N ratio for (a) L-lactic acid and (b) biomass concentration.

(a) L-lactic acid concentration				
Level	**Glucose concentration (g/L)**	**Protein source**	**Glucose to protein source ratio**	**Inoculum concentration (% v/v)**
1	19.91	22.91	22.86	22.45
2	23.17	22.85	22.92	23.00
3	25.64	22.97	22.95	23.28
Delta	5.73	0.11	0.08	0.83
Rank	**1**	**3**	**4**	**2**
(b) Biomass concentration				
Level	**Glucose concentration (g/L)**	**Protein source**	**Glucose to protein source ratio**	**Inoculum concentration (% v/v)**
1	1.35	1.10	4.34	2.58
2	3.52	2.83	4.85	3.97
3	6.34	7.27	2.01	4.66
Delta	4.99	6.17	2.84	2.08
Rank	**2**	**1**	**3**	**4**

concentration of 2.2 ± 0.1 g/L was observed in exp run no. 8.

Fig. 2a and b show the effect of different process parameters on lactic acid and biomass concentration, respectively. An increase in the glucose and inoculum concentration enhanced both the lactic acid and biomass concentrations. However, the glucose-to-protein source ratio and the protein source used in different experiments strongly affected the biomass concentration but not the glucose concentration (Fig. 2). Use of WP and YE directly increased the biomass concentration, which however was not the product of interest in the present study. Moreover, the ranking of various factors was determined through delta value analysis. Delta is the difference between the highest and lowest average response values for each factor. Thus, glucose concentration was ranked 1 for lactic acid concentration and protein source with high delta value emerged to 1st rank for biomass concentration (Table 2). This elevated biomass concentration with the usage of YE was previously reported in the literature (Oleksy-Sobczak and Klewicka, 2020), and the product of focus in those studies was *Lactobacillus* biomass rather than lactic acid. Thus, using inherent protein concentration originating from BSG biomass can be considered sufficient for the fermentation without any requirement for supplementing additional nitrogen source.

Fig. 3 shows the specific growth rate and L-lactic acid productivity observed in various experimental runs. L-lactic acid productivity ranged between 0.62 and 1.53 g/Lh^{-1}, and the specific growth rate was observed to vary between 0.04 and 0.19 h^{-1}. Lower L-lactic acid productivity and specific growth rate can be attributed to the lower glucose concentration considered in the screening study. Further, it can be witnessed from Fig. 4a that the three different plateaus observed for L-lactic acid concentration around 10, 15 and 20 g/L directly correlates with glucose concentration profile (Fig. 4c) originating at 10, 15 and 20 g/L, respectively. Such results of increased lactic acid productivity by *Lactobacillus delbreuckii* and increased glucose concentration were previously reported in the literature by Balakrishnan et al. (2018) when kodo millet was used as the substrate. The Taguchi experimental analysis identified glucose as the influential process parameter. Consequently, the glucose concentration was increased in batch bioreactor studies (Section 3.3) to achieve higher L-lactic acid concentration and productivity.

3.3. Batch fermentation kinetics with varying glucose concentration

The enzymatic hydrolysate obtained in Section 3.1 comprising of 19.47 ± 1.19 g/L of glucose and 2.34 ± 0.07 g/L of protein concentration was used in the bioreactor studies without any additional nitrogen source. However, the glucose and the inherent protein concentration were enhanced by distillation and the concentrations varied between 60 and 120 g/L. Unlike the screening experiments, batch fermentation was not supplemented with any mineral source. BSG was reported to possess several minerals including magnesium, calcium and silicon (Rojas-Pérez, L.C et al., 2022). Fig. 5 shows the lactic acid production, bacterial biomass growth and glucose consumption by *L. rhamnosus*. A lag phase of 4 h was noticed on all the glucose consumption profiles, with a glucose concentration between 60 and 120 g/L (Fig. 5c). A steady lactic acid profile was observed around 24 h. For instance, a maximum lactic concentration of 59.4 ± 2.1, 69.3 ± 0.6, 99.5 ± 6.6 and 114.4 ± 3.5 g/L was observed at 23, 24, 23 and 23 h for an initial glucose concentration of 60, 70, 100 and 120 g/L, respectively. Fig. 5b showed that a similar biomass growth profile was observed irrespective of the initial glucose concentration in batch fermentation. The similar biomass growth profile corroborates with the complete conversion of glucose to lactic acid found in Fig. 4a. This could be attributed to the constant glucose-to-protein source ratio (5:1) maintained throughout all the experiments. Similar results of constraining the C/N ratio to divert the carbon flux from producing bacterial biomass to products like polyhydroxybutyrate and bacterial lipids by *Ralstonia eutropha* and *Rhodococcus opacus* are observed in the literature (Nair and Sivakumar, 2022; Subagyo et al., 2021).

The specific growth rate of the batch bioreactor experiments were 0.14 ± 0.06, 0.12 ± 0.01, 0.15 ± 0.01 and 0.14 ± 0.01 for an initial glucose concentration of 60, 70, 100 and 120 g/L, respectively (Table 3). Kachrimanidou et al. (2022) studied the cheese whey valorisation using *L. rhamnosus* and *L. fermentum* and reported a maximum specific growth rate of 0.14 and 0.145 h^{-1}, respectively.

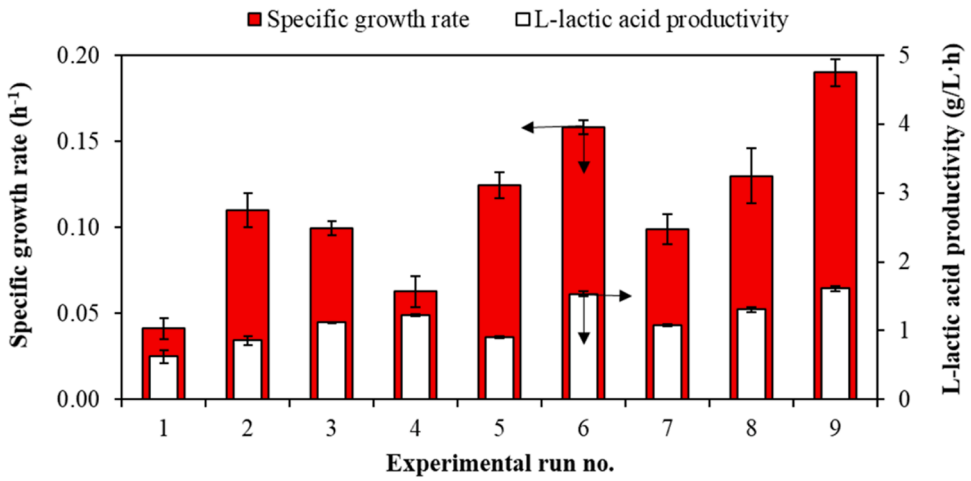


Fig. 3. L-lactic acid productivity and the specific growth rate of *L. rhamnosus* estimated as a response to varying process parameters displayed in the orthogonal Taguchi experimental design.

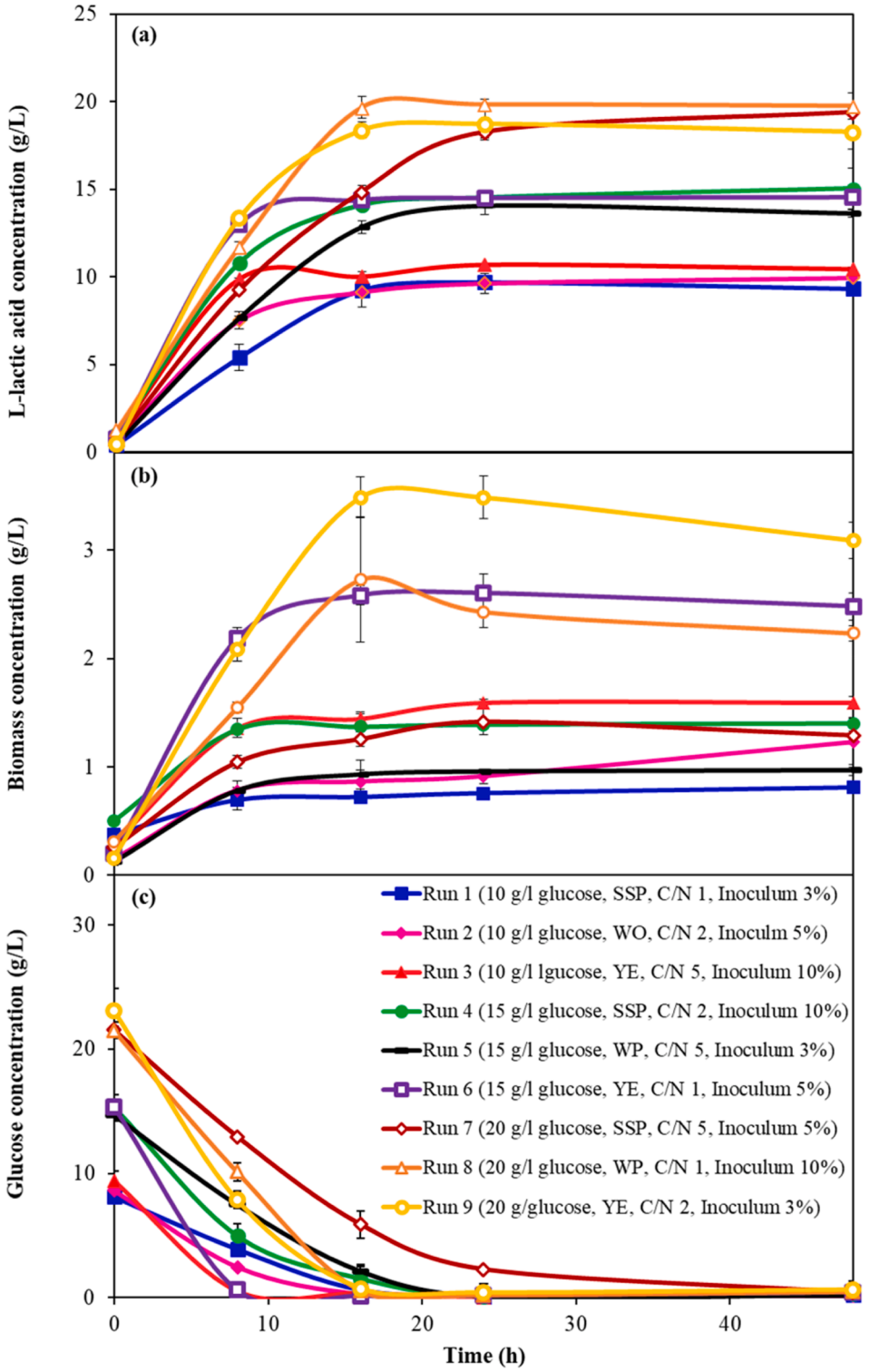


Fig. 4. Fermentation kinetics of (a) L-Lactic acid production (b) *L. rhamnosus* biomass growth, and (c) glucose consumption observed in different experimental runs of varying process parameters as per the orthogonal Taguchi experimental design. (Fermentation temperature: 37 °C, static flask condition).

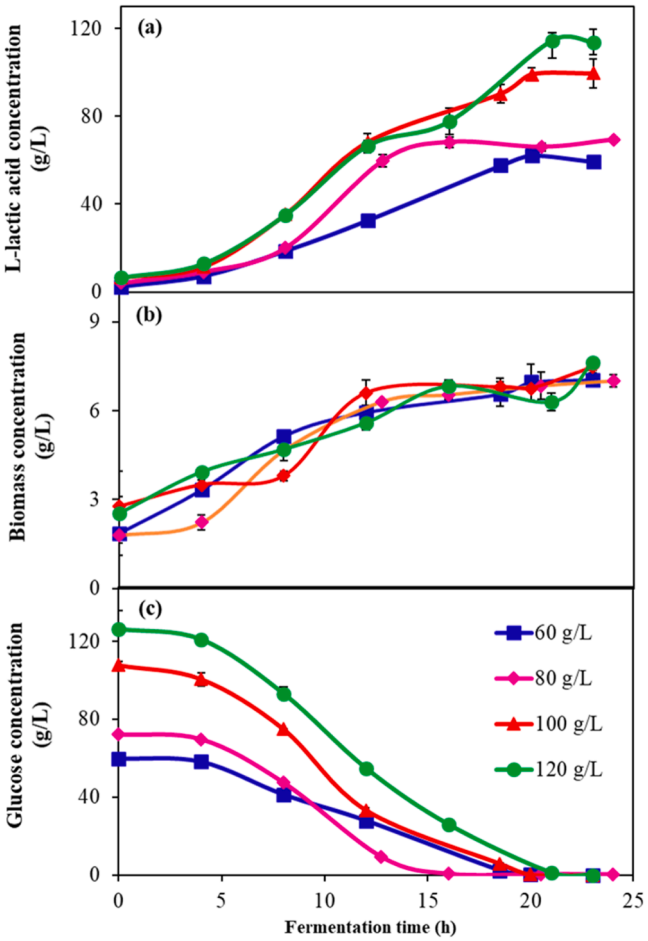


Fig. 5. Batch bioreactor kinetics showing (a) *L. rhamnosus* biomass growth, (b) glucose consumption and (c) L-lactic acid production with varying glucose concentrations from 60 to 120 g/L (fermentation temperature: 37 °C, stirring speed: 250 rpm, protein source: SSP, glucose to protein source ratio: 1, inoculum concentration: 10% v/v).

Table 3
Summary of batch bioreactor experiments observed at the end of the fermentation using *L. rhamnosus*.

Glucose concentration (g/L)	Batch fermentation				
	Specific growth rate (h^{-1})	L-lactic acid productivity (g/L·h)	Final biomass concentration (g/L)	Final L-lactic acid concentration (g/L)	Optical purity (%)
60	0.14 ± 0.06	3.48 ± 0.14	7.05 ± 0.21	59.4 ± 2.1	98 ± 1
70	0.12 ± 0.01	4.36 ± 0.21	7.0 ± 0.2	69.3 ± 0.6	96 ± 1
100	0.15 ± 0.01	5.44 ± 0.34	7.47 ± 0.12	99.5 ± 6.6	98 ± 1
120	0.14 ± 0.01	5.14 ± 0.2	7.63 ± 0.06	114.4 ± 3.5	99 ± 1

Anh Ngoc Le et al. (2023) stimulated the lactic acid production in *L. planatarum* using starch extracted from jack fruit resulting in a maximum specific growth rate of 0.16 h-1. Thus, the specific growth rate observed here is comparable with that of the literature reported for the valorisation of agro-industrial wastes. L-lactic acid productivity of 3.48 ± 0.14, 4.36 ± 0.21, 5.44 ± 0.34 and 5.14 ± 0.2 g/L·h was observed in the batch fermentation with varying glucose concentrations of 60, 70, 100 and 120 g/L, respectively. The maximum L-lactic acid productivity observed in the batch fermentation study is strikingly high compared to that of the productivity observed in the screening studies. The optical purity of lactic acid was found to be beyond 96 ± 1% (Table 3) for all samples, attributed to the fact that *L. rhamnosus* is classified under homofermentative bacterium (Klongklaew et al., 2021).

3.4. Characterisation of L-lactic acid and its comparison with pure L-lactic acid

L-lactic acid produced by the fermentation of BSG hydrolysate was refined using conventional downstream processing techniques.

Characterization of the L-lactic acid produced from BSG and commercially available L-lactic acid was carried out using the FTIR and Proton-NMR spectra. Downstream processing of L-lactic acid and its characterization was carried out to test the potential challenges that could result due to the intrusion of impurities from BSG. Fourier transform infrared (FTIR) analysis of L-lactic acid produced using BSG hydrolysate showed close similarity to pure L-lactic acid (Fig. 6). The broad peak observed around 3400 cm^{-1} can be attributed to the hydroxyl (-OH) groups stemming from the lactic acid and the residual moisture present inherently in the L-lactic acid. Since lactic acid is a carboxylic acid, the carboxyl (C═O) group was identified with a sharp peak at 1720 cm^{-1} (Fig. 6). The peak at 1127 cm^{-1} can be attributed to the stretching vibration originating from C-O. The asymmetric bending due to the methyl (-CH_3) group was visualised at 1402 cm^{-1}. Further, the peak at 1232 cm^{-1} was due to the asymmetric rocking by the methyl (-CH_3) group linked with the asymmetric vibration by C-O. All the aforementioned peaks in the lactic acid derived from BSG biomass matched well with the pure L-lactic acid and the spectrum reported in the literature (Kumar et al., 2020).

Proton-NMR analysis of L-lactic acid produced using BSG biomass was compared with pure L-lactic acid in Fig. 7. The strong signal visualised at 4.79 ppm is due to the solvent (D_2O) used in both samples. The proton signals due to methyl (CH_3) and methimine (CH) was observed as a doublet and a quartet centred around 1.3 and 4.2 ppm, respectively. The additional signals observed at 1.99 ppm only in lactic acid produced by BSG biomass could be due to the residual ethyl acetate used during the downstream processing steps. This residual ethyl acetate can be removed during the subsequent process of lactide preparation from lactic acid by vacuum distillation using Schlenk line. All the values above agree well with the data reported in the literature. For instance, the signals due to methyl and methine observed in the present study matched with the lactic acid extracted from fermentation broth using butanol (Kumar et al., 2020). The downstream processing and characterisation study revealed the suitability of the L-lactic acid produced from BSG biomass for subsequent distillation in lactide preparation. However, improvement in the downstream processing could be considered by integrating membrane separation or chromatography to produce food-grade lactic acid. Additionally, an average lactic acid loss of 20% was observed during activated carbon adsorption and liquid-liquid extraction and a 10% (w/w) lactic acid yield was obtained with reference to BSG biomass (dry basis). This emphasizes the need for loss reduction and enhancement in overall process efficiency.

4. Future perspectives

A novel approach for lactic acid production was proposed, using Brewer's spent grain as a self-sufficient feedstock for optically pure L-lactic acid production. This study proposes that an elevated glucose concentration is directly proportional to L-lactic acid concentration. In addition, the glucose to protein source ratio was found to be crucial for biomass production rather than L-lactic acid concentration. However, oxygen being a limiting substrate, higher biomass production is achieved not only through higher protein concentration but also with the presence of higher amount of oxygen. This inference is supported through the results presented in Fig. 8, which shows biomass concentrations in aerobic, microaerophilic, and anaerobic cultivation were observed to be 4.05 $\pm$ 0.01, 3.19 $\pm$ 0.07 and 3.16 $\pm$ 0.08 g/L, respectively. Meanwhile, L-lactic acid concentrations were found to be 11.95 $\pm$ 0.25, 15.39 $\pm$ 0.18 and 15.32 $\pm$ 0.45 g/L, respectively. Furthermore, the variation in biomass concentration and L-lactic acid production observed in aerobic cultivation was found to be statistically significant ($P < 0.05$) compared to microaerophilic, and anaerobic cultivations.

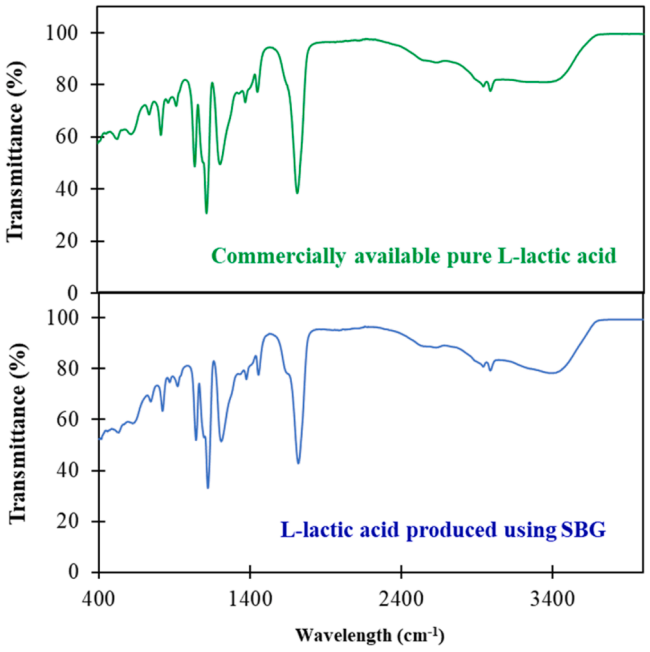


Fig. 6. Fourier transform infrared (FTIR) analysis of L-lactic acid produced during upstream processing of SBG alongside a comparison with the commercially available pure compound.

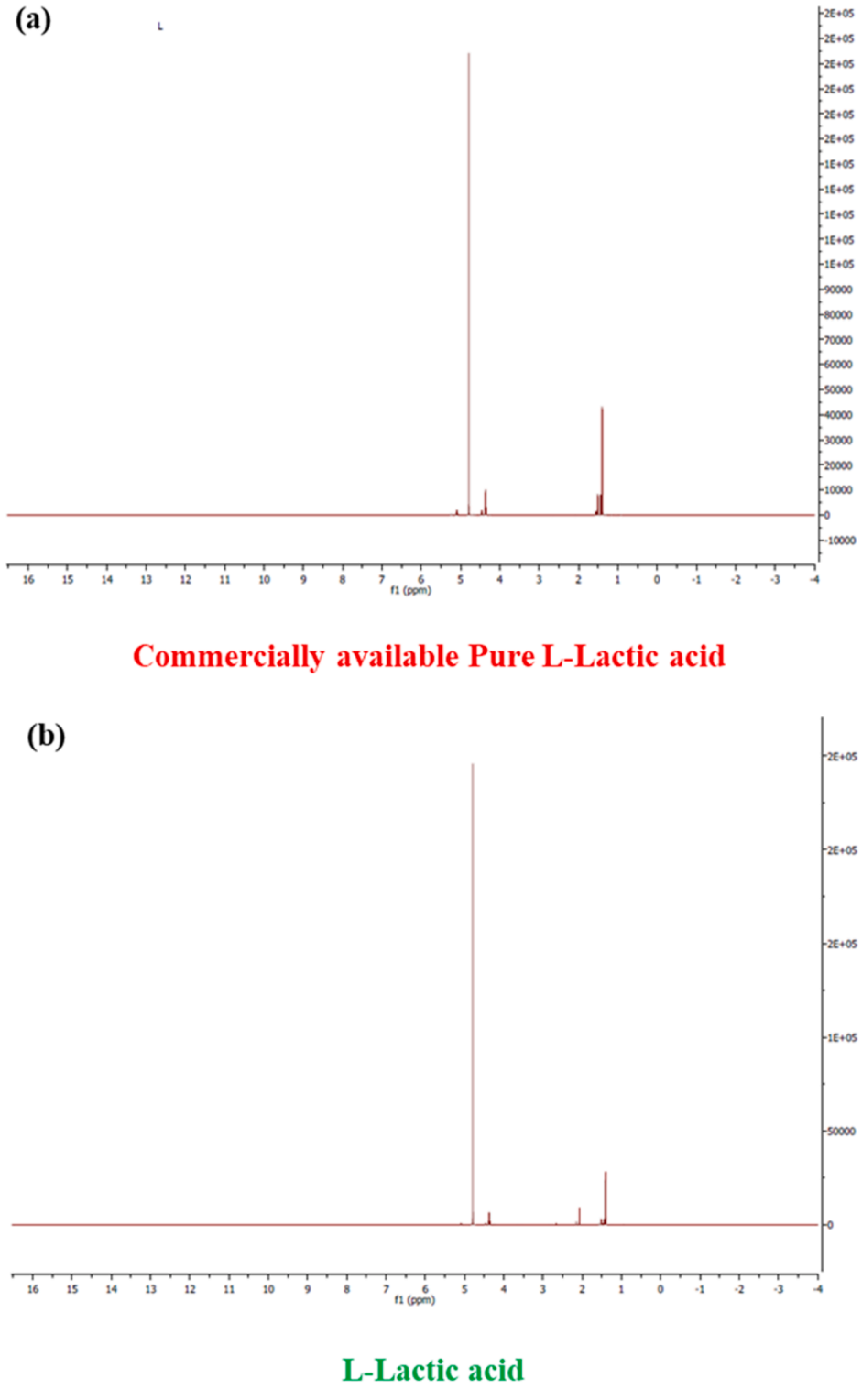


Fig. 7. Proton-NMR analysis of (a) L-lactic acid produced using SBG and (b) commercially available pure L-lactic acid.

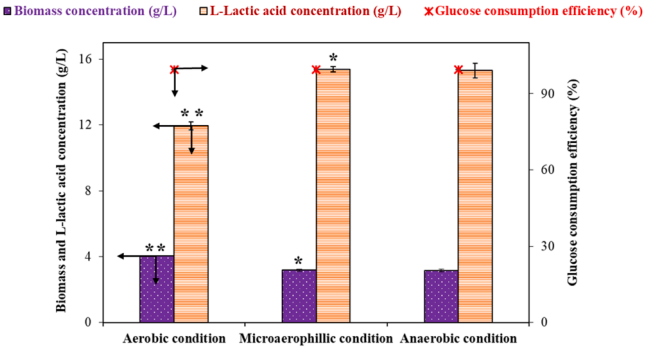


Fig. 8. Effect of oxygen on biomass and L-lactic acid production by *Lactobacillus rhamnosus* cultivated under aerobic, microaerophilic and anaerobic conditions. Experimental conditions: Glucose concentration – 15 g/L, glucose to protein source ratio - 0.075 and fermentation temperature – 37 °C and fermentation pH – 6. (** in the figure shows that the variations are statistically significant at $P < 0.05$ and * represents that the variations are statistically insignificant at $P > 0.05$ using Tukey's test).

Although an elevated biomass concentration at the cost of reduced L-lactic acid production is not of present interest. Such results would be of interest for functional food production through *Lactobacillus sp*. based bacterial fermentation (Sørensen et al., 2022). Thus, the present study has opened the door for L-lactic acid production by *Lactobacillus rhamnosus* under microaerophilic condition and biomass production under aerophilic condition using BSG as the self-sufficient feedstock.

5. Conclusions

The study demonstrated BSG comprising carbon, nitrogen, and mineral sources as a self-sufficient feedstock for L-lactic acid production. Simultaneous saccharification and proteolysis showed the need for amyloglucosidase, glucanase and cellobiohydrolase to extract glucose and papain to extract protein. Screening of process parameters using Taguchi experimental design outlined a linear relationship between glucose concentration and lactic acid production. Using SSP from BSG was adequate rather than using costly YE in the fermentation broth. The batch bioreactor study showed the linearity between glucose concentration and lactic acid production was continued even at a maximum glucose concentration of 120 g/L. Characterisation studies using FTIR and proton-NMR revealed the similarity of the L-lactic acid produced using BSG to that of the commercially available pure L-lactic acid. Thus, the present study strategized a novel bioprocessing route for using BSG biomass, which can be extended to produce other bioproducts such as ethanol, succinic acid, polyhydroxyalkanoic acid and others.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

CRediT authorship contribution statement

Keith D. Rochfort: Writing – review & editing, Funding acquisition. **Jennifer Gaughran:** Writing – review & editing, Funding acquisition. **Susan Kelleher:** Writing – review & editing, Funding acquisition. **Michal Dabros:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Samantha Fahy:** Funding acquisition. **N. Arul Manikandan:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **K. Amulya:** Writing – review & editing. **Coralie Granget:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Brian Freeland:** Writing – review & editing, Supervision, Methodology, Conceptualization, Funding acquisition.

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

Data availability

Data will be made available on request.

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