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Enhanced 2,3-Butanediol production by mutant *Enterobacter ludwigii* using Brewers' spent grain hydrolysate: Process optimization for a pragmatic biorefinery loom

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Abstract

2,3-Butanediol (BDO) is a fossil-based versatile bulk chemical with a multitude of applications. BDO can also be synthesized using microbial cell factories harnessing renewable feedstocks. However, the high cost of the substrate via microbial route impedes commercial manufacturing of BDO. Therefore, identification of cheaper substrates could make bio-based BDO production more cost-competitive. Brewers' spent grain (BSG), a major by-product of breweries, is an inexpensive source of fermentable sugars and proteins. In the present study, we have attempted the bioproduction of BDO by *Enterobacter ludwigii* using BSG as feedstock. A random *E. ludwigii* mutant obtained after treatment with ethyl methane sulfonate (EMS) resulted in a BDO titer (9.5 g/L), ~30% higher in comparison to the wild type strain with a yield of 0.48 $g_{BDO}/g_{Glucose}$ approaching the theoretical yield of 0.50 $g_{BDO}/g_{Glucose}$. The enzymatic hydrolysis of microwave-assisted alkali pretreated BSG was optimized using the statistical Taguchi design. The BSG hydrolysis under optimal conditions (pH: 6.0; temperature: 50 °C; BSG: 10% w/v; enzyme loading: 2% v/v) resulted in a glucose yield of 0.25 $g_{Glucose}/g_{Biomass}$. The uncontrolled pH was found to be more beneficial for BDO accumulation from BSG hydrolysate in batch bioreactor cultivation as compared with controlled one. The fed-batch cultivation with forced pH fluctuations at an aeration rate of 2.0 vvm resulted in BDO accumulation of 118.5 g/L from glucose-rich BSG hydrolysate with the yield and productivity of 0.43 g/g and 1.65 g/L.h, respectively. To the best of our knowledge, this is the first study on BDO production from BSG.

Key words: *Enterobacter ludwigii*; Random Mutagenesis; Brewers' Spent Grain; Enzymatic Hydrolysis; Glucose-rich BSG hydrolysate; 2,3-Butanediol

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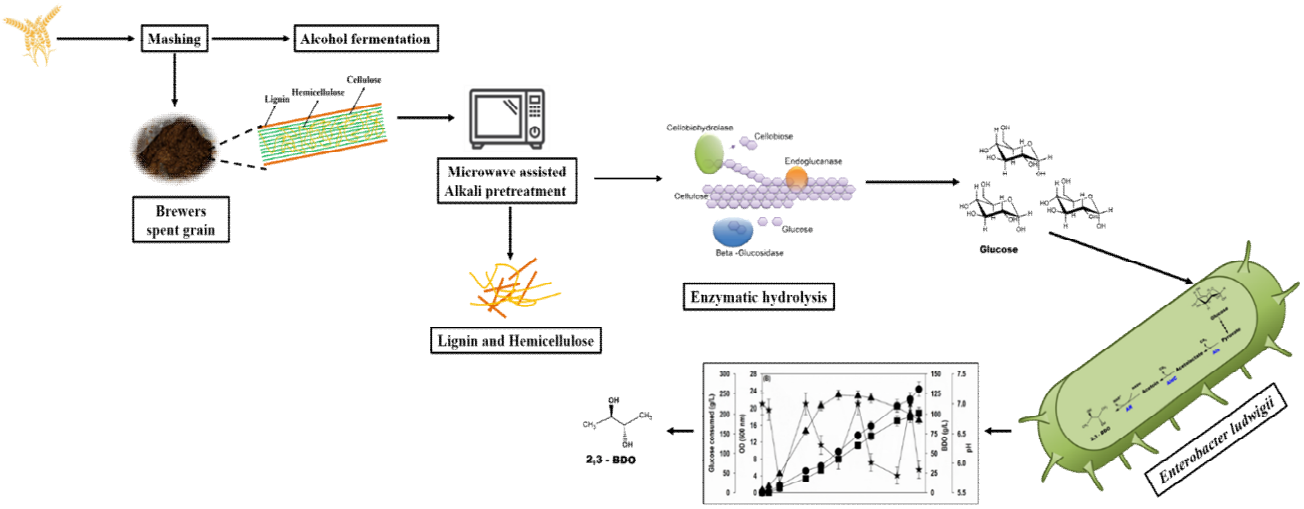
Highlights

- BSG was evaluated as promising feedstock for BDO production by *E. ludwigii*.
- Optimized enzymatic hydrolysis of BSG resulted in glucose yield of 0.25 g/g.
- Mutant strain of *E. ludwigii* accumulated BDO close to theoretical yield.
- BDO titer of 118.5 g/L with 0.43 g/g yield was obtained from BSG as feedstock.
- High BDO level was achieved without using any complex nitrogen source.

Graphical abstract



1. Introduction

Currently, the petrochemical route is predominantly preferred for producing a broad range of bulk, fine and specialty chemical products on an industrial scale. The non-sustainability and harmful impact of these petrochemical processes on the environment have ushered the search for alternative sustainable green routes making use of renewable sources [1]. As a result, the last few decades have witnessed an immense paradigm shift towards synthesis of high-value products through the fermentative route offering potential advantages such as environmental friendliness, biodegradability, and sustainability [2–5]. The demand for clean and sustainable production of chemicals along with the food versus feed debate has necessitated the use of agro-industrial by-products and waste streams rich in renewable carbon as feedstocks. Food loss across the supply chain including harvesting, transport, storage, processing, packing, distribution, marketing, and household consumption is considered as waste. About 1.3 billion tons of food is wasted globally every year accounting for ~ one-third of annual global food production. Due to high nutritional value of food waste, the disposal poses a potential threat and challenges to the ecosystems and causes environmental, economic, and social problems. Therefore, a global revolution is being initiated by promoting the activities indulging efficient recycling of residues generated from agro-industrial sectors. The conventional methods for treatment of food waste such as landfilling, incineration and anaerobic digestions do not harness the full potential. The conversion of food waste into value-added chemicals via microbial route is a more sustainable and profitable approach and contribute to efficient waste management [6–8].

Beer is one of the most consumed beverages in the world. During the brewing process, barley undergoes mashing that leaves behind an outer layer of the grain kernel, resulting in a material known as brewers' spent grain (BSG). BSG is the most abundant by-product and constitutes ~ 85% of the total by-products generated in the beer-brewing-process [6]. Global beer production was 1.94 billion hectoliters in 2018 and it has been estimated that every 100 L of brewed beer generates 15-20 kg of wet BSG [9]. This corresponds to ~39 million tonnes of wet BSG worldwide with 3.4 million tonnes produced in the EU, 0.5 million tonnes of which are generated by the UK [9–11]. Currently, the use of BSG is predominantly limited to animal feed with a market value of ~€35 per tonne [12]. BSG is a lignocellulosic material containing cellulose (12-33%), hemicellulose (19-42%), lignin (11-28%), proteins (14-31%) and lipids (6-13%). The fibre constitutes about 50% of the BSG composition (dry weight basis) while protein fraction can be up to 31% [11,13,14]. The high volume, low value, and viable composition of BSG has a strong potential to be recycled and serve as a promising feedstock for the fermentative production of chemicals.

2,3-Butanediol (BDO) is a C4 metabolite and the presence of two hydroxyl groups makes BDO a versatile molecule for the synthesis of numerous chemicals with huge market potential such as methyl

ethyl ketone, diacetyl, polyurethane, 1,3-butadiene etc. For example, dehydration of BDO gives 1,3-butadiene with a global production volume of 10 million tonnes [15,16]. BDO and its downstream chemicals have potential applications in manufacturing printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals [17]. Downstream products of BDO have an estimated global market potential of around 32 million tons per annum, valued at $43 billion [18]. In addition, BDO has several attractive properties to serve as fuel. The heating value of BDO (27.2 KJ/g) is comparable or better than other biofuels such as n-butanol (33.1 kJ/g), methanol (22.1 kJ/g) and ethanol (29.1 kJ/g). Due to high octane number, BDO acts as octane booster for gasoline [15,19]. BDO and n-butanol both are C4 metabolites, however, unlike BDO, biobutanol production suffer from low titer, yield and productivity due to co-production of acetone, ethanol and organic acids. Further, n-butanol production is severely impacted by end-product toxicity beyond 2 % (w/v) [20–22], which is far behind than BDO accumulation achieved (10-15% w/v) [23]. BDO derivatives also show immense potential as biofuels such as 2-ethyl-2,4,5-trimethyl,1,3-dioxolanes (TMED) which is dehydration product of BDO. The advantageous features of TMED as fuel are higher heat of combustion (28.3 MJ/L) than ethanol (21.1 MJ/L) and n-butanol (26.8 MJ/L) along with comparable anti-knock index (90.4) to n-butanol and high-octane gasoline. Further, TMED has much low miscibility in water (8 g/L) in comparison to n-butanol (77 g/L) and ethanol, which is another desirable property as presence of water leads to corrosion of engines [24].

Enterobacter ludwigii is a subspecies of *Enterobacteriaceae* family and has been reported to accumulate large amount of BDO (50-120 g/L) from a variety of renewable carbon sources such as sugarcane molasses [25], cane sugar [26], fruit and vegetable waste [27], making it a potential biocatalyst for BDO production from crude renewable sources. In the present study, BDO production by *Enterobacter ludwigii* has been evaluated using BSG as the sole carbon source. To improve the BDO manufacturing, the strain was subjected to random mutagenesis by ethyl methane sulfonate (EMS), and the highest BDO yielding strain was isolated. The BSG was subjected to pretreatment and the enzymatic hydrolysis of pretreated BSG was optimized using the Taguchi method to maximize glucose release. The culture conditions were optimized in shake flask and bioreactor experiments for high-levels BDO production. The impact of substrate concentration (BSG hydrolysate) and pH on BDO production was investigated to find fermentation conditions favoring BDO production. Further improvement in BDO production was achieved by with fed-batch fermentation by interplaying between forced pH fluctuation and different aeration rates.

2. Material and Methods

2.1 Materials

All the chemicals used in this study were purchased from Sigma Aldrich (USA) and Fischer scientific and were of analytical grade. The enzyme cellulase from *Aspergillus sp* (1000 U/g) was procured from Sigma

Aldrich (USA). BSG was a generous gift from a local Brewery in Bedford, UK. Upon receipt of the BSG, it was subjected to oven drying at 60°C for 48 h. Following this, the dry BSG was grounded mechanically and sieved using a 500 µm sieve and stored at 4 °C [28].

2.2 Microorganism, culture maintenance, and inoculum preparation

The bacterial strain *Enterobacter ludwigii* has been isolated at the Laboratory of Food Microbiology and Biotechnology at the Agricultural University of Athens [29]. It was maintained in Tryptic Soy Broth: [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, pH 7.3] supplemented with 50% (v/v) glycerol at −80 °C. The seed culture was grown in a 250 mL Erlenmeyer flask containing 50 mL of complex medium with following composition (g/L): 10.0, glucose; 2.5, yeast extract; 5.0, peptone; 2.0, KH_2PO_4; 5.0, sodium acetate; 0.8, $MgSO_4$ $7.H_2O$; 0.05, $MnSO_4.H_2O$ [26]. The final pH of the medium before sterilization was adjusted to 6.6 using 5M NaOH. Cultivation was carried out for 12 h at 30 °C on a rotary shaker at an agitation speed of 180 rpm.

2.3. Microwave-assisted alkali pre-treatment of BSG

Microwave-assisted alkali (MAA) pretreatment of BSG was performed as per the procedure described by Ravindran et al., (2018). BSG (10% w/v) was suspended in 0.5 w/v% NaOH solution in a 1L pyrex bottle and subjected to microwave radiation at 400W for the 60s using a Russell Hobbs microwave (Digital Black, UK). After the pretreatment, the BSG was thoroughly washed with distilled water until the pH was reduced to 6.0 and dried in the oven at 80°C. The dried solid was used for enzymatic hydrolysis [28].

2.4. Taguchi method of an orthogonal array (OA) for optimization of enzymatic hydrolysis of BSG

In the present study, enzymatic hydrolysis of BSG was carried out using commercially available cellulase enzyme. We have adapted the Taguchi method of the orthogonal array (OA) for enzymatic hydrolysis, which employ the S/N (Signal: Noise) ratio to measure the interaction between controllable and uncontrollable factors and thereby reducing the effect of variance from the uncontrollable factors [30]. The S/N ratio is represented by equation 1.

$$\frac{S}{N} = 10log_{10}(\frac{\beta^2}{\sigma^2}) \qquad (1)$$

where the signal represents the mean value (β) while the variance (σ) present in the system is represented by noise.

For optimization of enzymatic hydrolysis, four parameters were considered: biomass loading, enzyme loading, pH, and temperature. The variables and the levels of the process parameters are shown in Table S1. The pretreated BSG suspension was prepared using 0.05M citrate buffer. The saccharification reaction was carried out in a 2 L glass Pyrex bottle with 1L working volume and incubated at a predetermined temperature with shaking at 150 rpm for 96 h. The cellulase concentration used for

this purpose was 100 U/mL. After the enzymatic hydrolysis, the hydrolysate was filtered using muslin cloth, and appropriate dilutions were made to quantify glucose using HPLC. The BSG hydrolysate obtained after pretreatment and enzymatic hydrolysis was concentrated by rotary vacuum evaporation to a final glucose concentration of 500 g/L and stored at 4^0C until further use. The hydrolysate was used as a sole carbon feedstock in a shake flask and the bioreactor experiments.

Independent variables	**Levels**		
Biomass loading (w/v %)	2	5	10
Enzyme loading (v/v %)	0.5	1	2
pH	5	6	7
Temperature (ºC)	50	55	60

Table S1: Parameters and their levels used for Taguchi (OA) optimization method for enzymatic saccharification of BSG

2.5 Submerged cultivations in shake flask

The synthetic medium used for fermentation had the following composition (g/L): 6, $(NH_4)_2HPO_4$; 7.2, $(NH_4)_2SO_4$; 0.45, KOH; 0.51, EDTA; 0.3, $MgSO_4 \cdot 7H_2O$; 0.09, $CaCl_2 \cdot 6H_2O$; 0.022, $FeSO_4 \cdot 7H_2O$; 0.0038, $MnSO_4 \cdot H_2O$; 0.0075, $ZnSO4 \cdot 7H_2O$ [26]. The medium was supplemented with 20 g/L pure glucose for initial media and mutant screening experiments. The starting pH was adjusted to 6.6 before inoculation by using 5M NaOH. The submerged cultivations were carried out in 500 mL shake flasks containing 100 mL working volume. The flasks were inoculated with 2% (v/v) of fresh inoculum prepared as mentioned in section 2.2 and kept at 30 °C under constant shaking at 180 rpm on a rotary shaker (Excella 24, New Brunswick).

2.6 EMS based mutagenesis of *E. ludwigii*

The EMS mutagenesis was performed according to Cupples and Miller, 1989 with slight modification [31]. The single colony from the freshly sub-cultured plate was inoculated into 10 mL sterile complex medium and grown till the OD (600 nm) reached 5-6 (mid log phase) by incubating at 30 °C with agitation of 180 rpm. The grown cells were harvested and subjected to centrifugation at 5000 ×g and then washed with 20 ml of buffer A [0.1M phosphate buffer (pH 7.0)]. The cells were resuspended in 1 ml of buffer A and exposed to different concentrations of EMS (10-100 mM) at 30 °C for 60 min in a static environment. The cells were centrifuged at 5000 ×g and then washed twice with 1 ml of buffer A and again suspended in 100 µL of the same buffer. The cells were spread on Tryptic Soy Agar plates and incubated at 30°C until colonies were formed. After incubation, the survival rate of *E. ludwigii* was calculated using total plate count method. The desired survival rate is expected to be less than 1%. The survival rate calculation was done according to Naveena et al., 2012 [32], using the following equation (2).

$$Survival\ rate\ (\%) = \frac{N_i - N_d}{N_i} \times 100 \quad (2)$$

Where N_i and N_d are the viable cell count, initially and after mutation in CFU/mL (colony forming unit/mL), respectively.

2.7 Effect of initial BSG hydrolysate concentration and pH on BDO production

The effect of initial glucose concentrations in BSG hydrolysate (20, 40, 60, 80, 100g/L) and initial pH (5.0, 6.0, 7.0 & 8.0) on substrate consumption, cell growth and BDO production were investigated in shake flask using the synthetic medium mentioned in section 2.5.

2.8 Bioreactor studies

The batch experiments were performed in a 2.5 L benchtop bioreactor (Electrolab Bioreactors, UK) with 1.0 L working volume with an inoculum size of 10% (v/v). BSG hydrolysate and nutrient medium (Synthetic medium) were separately autoclaved at 121°C for 15 min. BSG hydrolysate adjusted to a glucose concentration of 40 g/L was used in batch cultivation. The temperature, agitation speed, and aeration rate were controlled at 30 ºC, 180 rpm, and 1.0 vvm, respectively. Two separate batch cultivations were performed and starting pH was 7.0 in both cases; in one reactor pH was controlled at 7.0 using 5 M NaOH throughout the fermentation while in the other case pH was not controlled. In fed-batch fermentation, the mutant strain *E. ludwigii* was cultivated at three different aeration rates (0.5, 1.0, and 2.0 vvm) by maintaining the residual glucose concentration at approximately 20 g/L with intermittent feeding using concentrated BSG hydrolysate containing 500 g/L glucose.

2.9 Analytical methods

The samples were withdrawn periodically and analyzed for OD_{600}, pH, glucose, BDO, acetoin, ethanol, succinic, lactic, and acetic acids concentration. The concentrations of BDO and glucose were measured by HPLC (Agilent Technologies 1200 series, USA) using Rezex ROA-Organic Acid H+ (Phenomenex, USA) column at 60 °C. The mobile phase and flow rate were 0.5 mM H_2SO_4 and 0.4 mL/min, respectively [33]. All measurements were conducted in triplicates and the values were averaged. The standard deviation was not more than 10 %. The statistical analysis was carried out using student t-test between individual experiment and found to be statistically significant with $p<0.05$.

3. Results

3.1 Shake flask cultivation of *E. ludwigii*

The wild type *E. ludwigii* strain was cultured in a synthetic and complex medium with pure glucose as a carbon source (Figure 1). The large fraction of glucose present in both media types was exhausted to zero level between 12-16 h of the fermentation period, which was concomitant with the cell growth. The glucose consumption was faster and cell growth (OD_{600}: 13.7) was significantly higher in the complex medium than the synthetic one where the maximum OD_{600} recorded was 8.8. The onset of BDO

production was observed during the early log phase and accumulation increased during the mid to late log phase. The highest BDO titer of 8.6 g/L and yield of 0.43 $g_{BDO}/g_{Glucose}$ was achieved on complex medium whereas in the case of the synthetic medium the titer and yield were 7.1 g/L and 0.36 g/g. The difference in the performance of *E. ludwigii* may be attributed to the rich composition of the complex medium, particularly in terms of complex nitrogen sources such as yeast extract and peptone which favors the cell growth and therefore, BDO production. The pH was reduced during fermentation and a rapid drop was observed during the late phase of the growth indicating the production of organic acid, subsequently, the BDO production was triggered to counteract the internal acidification [34,35]. The BDO production in both complex and synthetic medium was comparable. The low-cost of the synthetic medium, less complicated downstream processing due to the absence of complex nitrogen sources, and batch to batch variation make it an obvious choice. Further experiments were performed with the synthetic medium.

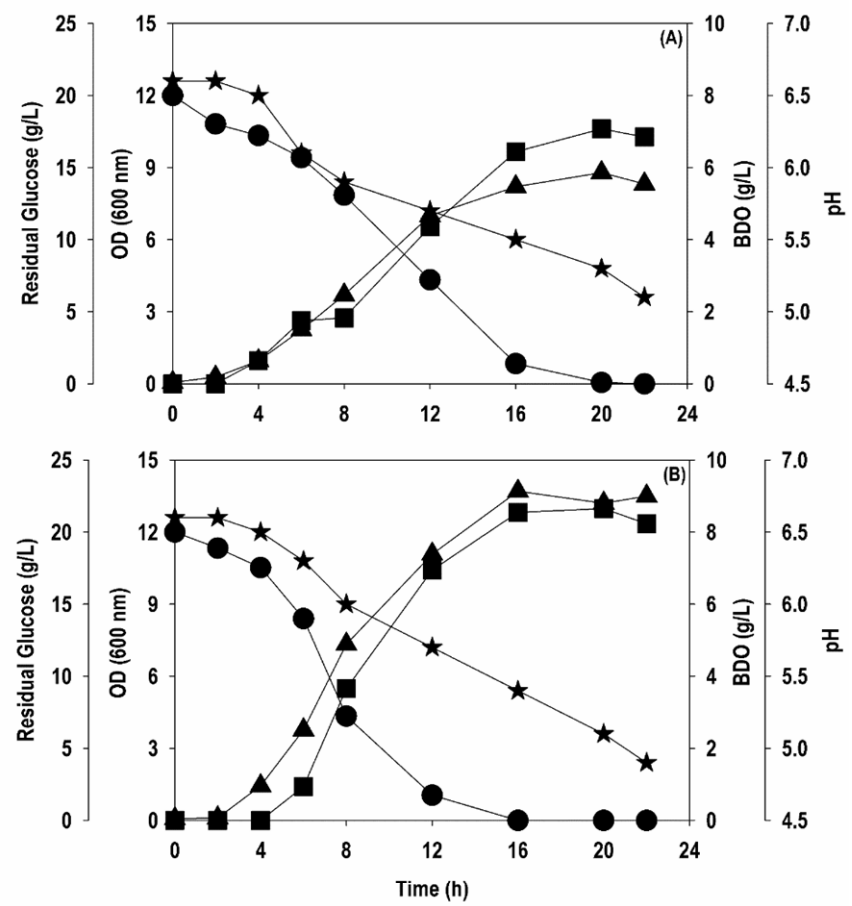


Figure 1: Time course profiles of glucose consumption, OD_{600}, pH and BDO production during shake flask cultivation of *E. ludwigii* on: (A) synthetic medium; (B) complex medium. Symbols: filled circle (glucose), filled triangle up (OD_{600}), filled square (BDO) and filled star (pH).

3.2 Strain improvement using EMS-based mutagenesis

Random mutagenesis using chemical/physical agents results in either insertion or deletion of nucleotides *in vivo*, generating strains with potentially superior characteristics. A key advantage of the technique is that it can be performed with a slight underlying knowledge of molecular biology [36]. In the current study, random mutagenesis was performed using EMS as chemical mutagen which relies on creating a lesion or a modification in the base sequence of the DNA molecule, and a mutation is caused if this lesion is not repaired [37]. The wild type strain of *E. ludwigii* was exposed to different concentrations of EMS. The EMS mutagenesis displayed a lower survival rate of bacteria at high EMS concentrations. Until 40 mM EMS, the survival rate was approximately about 3-4% and it reduced to <1% above 60 mM. The fewer survived bacteria were more likely the mutant displaying the required characteristics [38]. The procedure used to generate mutagens described in material and methods section resulted in the 2- to 3-fold decrease from the initial cells (wild type) used, giving an approximately 1000 colonies under different concentration of EMS. A total of 50 mutants were isolated among the colonies based on the phenotypic stability. The phenotypic stability was evaluated by subjecting the mutant strains to undergo two sequential culturing in above mentioned complex media in a 96 well cell culture plate and testing the quantitative BDO production against wild type strain. Among the 50 isolated strains, one strain with superior BDO production ability was selected to evaluate its growth and BDO production ability under shake flask studies.

The specific growth rate was affected with the mutations caused by a higher dose of EMS as it induces the predominant GC to AT transition causing the deleterious effect to cells [39]. The effect of EMS dosage on the phenotypic characteristics such as specific growth rate and BDO production was estimated by isolating best mutant from each concentration and results are depicted in Figure 2.

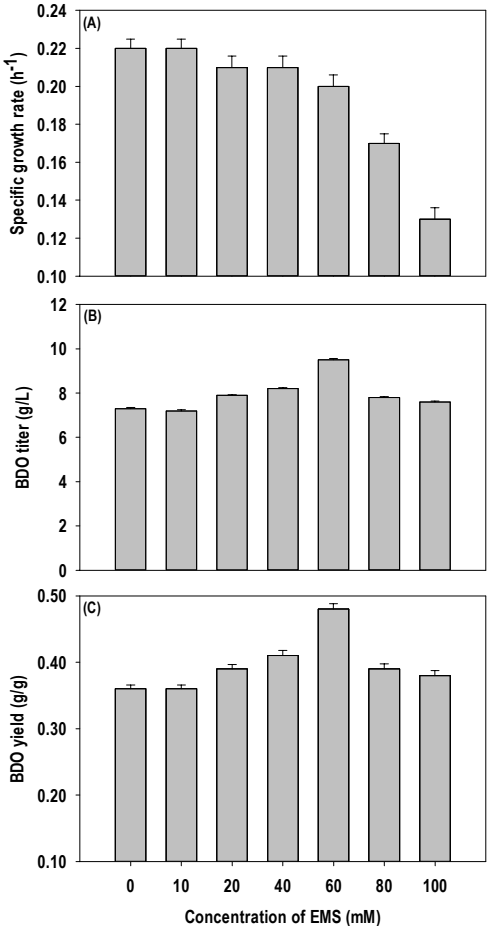


Figure 2: Phenotypic changes of an isolated mutant of *E. ludwigii* subjected to varied concentration of EMS (A) specific growth rate; (B) BDO titer and (C) BDO yield.

The decrease in specific growth rate was marginal at EMS concentrations below 60 mM with almost no effective impact on cell growth and thereafter, a significant drop was noticed. The specific growth rate of the wild type strain of *E. ludiwgii* was 0.22 h^{-1} and the lowest value of 0.13 h^{-1} was observed with the strain treated with 100 mM EMS (Figure 2A). The superior mutant was selected based on the amount of BDO accumulated. The highest BDO production (9.5 g/L) and yield (0.48 g/g) were observed with the mutant strain treated with 60 mM EMS (Figure 2B and 2C). The titer was~30% higher in comparison to control which produced 7.3 g/L of BDO and the yield of the mutant strain was near to theoretical maximum yield of 0.50 g/g (equation 3 and 4).

$C_6H_{12}O_6$ (Glucose) + NAD^+ = $C_4H_{10}O_2$ (BDO) + $2CO_2$ + NADH + H^+ (3)

$C_6H_{12}O_6$ (Glucose) + $0.5O_2$ = $C_4H_{10}O_2$ (BDO) + $2CO_2$ + H_2O (4)

However, specific growth rate of mutant was reduced by ~9% in comparison to control. The increase in mutagen concentration beyond 60 mM resulted in reduced BDO titer and yield, which can be due to the deleterious effects on impaired growth which in turn negatively affected the BDO production, however

the mutant strain still displayed a marginally higher BDO yield than wild type strain even beyond 60mM concentration. The mutant strain yielding the highest titer and yield was selected for further experiments.

3.3 Optimization of enzymatic hydrolysis of BSG by Taguchi orthogonal array method

The pretreatment and method for enzymatic hydrolysis play a very important role in determining the overall efficiency of the saccharification process from the lignocellulosic feedstocks [40]. The efficiency of the pretreatment will be reflected in terms of sugar recovery after enzymatic hydrolysis of the pretreated lignocellulosic biomass. BSG samples were pretreated according to the MAA method as described in section 2.3 and after the pretreatment, the BSG was dried and used for enzymatic hydrolysis. The enzymatic hydrolysis process was optimized using Taguchi (OA) methodology to ensure maximum sugar release from the pretreated BSG. We have adopted a standard orthogonal array $L_9(3^4)$, total of 9 trials with three levels were conducted according to the experimental design (Table 1) generated by the statistical software Minitab (version 16, PA, USA). The factors such as biomass loading, enzyme loading, pH, and temperature were chosen as essential parameters influencing the hydrolysis process. To maximize the glucose release, the quality characteristics "Bigger is Better" S/N ratio was set. The predicted glucose yield shown in Table 1 was calculated by substituting the individual variables in the polynomial model given in equation (5).

$$Glucose\ \left(\frac{g}{g}\right) = 0.256 + 0.0036\ Biomass\ (\%) + 0.039\ Enzyme\ loading\ (\%) - 0.003\ pH - 0.0022\ Temperature\ (°C) \quad (5)$$

The increased number of S/N ratio indicates higher glucose release. The optimal values of pH, temperature, biomass, and enzyme loading were found to be 6.0, 50 °C, 10%, and 2%, respectively, which resulted in a glucose yield of 0.25 g/g BSG. The S/N ratio of the factor indicates its relative significance over the others for a given response. A higher delta value of the factor signifies a higher effect as compared to the other factors. The effect of each of these parameters on glucose release was evaluated based on the ranking system calculated based on the delta S/N ratio [41]. The delta S/N ratio and the corresponding ranking of the factors affecting sugar release are shown in Table S2. The enzyme loading showed a maximum influence on glucose release followed by biomass loading. The model adequacy and statistically significant factors were determined by ANOVA (analysis of variance). Table S3 represents the ANOVA and regression coefficients of individual factors affecting glucose release. All the factors except pH showed high precision with $p<0.05$ and the model F-value was found to be 28.71 ($p<0.05$) indicating the model is statistically significant. The relation between factors was fitted using a polynomial model and is given in equation 5. Further, the coefficient of determination (R^2) was found to be 96.6% illustrating that the model is adequately fitting the data [42]. The main effect S/N ratio plot of the individual factor is represented in Figure S1. The increase in the biomass and enzyme loading

supported the glucose release, whereas the increase in pH and temperature showed an adverse effect on the liberation of the sugar from BSG.

Table 1: $L_9(3^4)$ orthogonal array of Taguchi experimental design for optimization of enzymatic hydrolysis of BSG

Experiment no.	Biomass loading (w/v %)	Enzyme loading (v/v %)	pH	Temperature (°C)	Glucose yield (g/g) (expt)[a]	Glucose yield (g/g) (pred)	S/N ratio
1	2	0.5	5	50	0.17±0.014	0.16	-15.39
2	2	1	6	55	0.16±0.008	0.18	-15.92
3	2	2	7	60	0.2±0.020	0.19	-13.98
4	5	0.5	6	60	0.16±0.064	0.15	-15.92
5	5	1	7	50	0.19±0.011	0.19	-14.42
6	5	2	5	55	0.23±0.016	0.22	-12.77
7	10	0.5	7	55	0.18±0.005	0.17	-14.89
8	10	1	5	60	0.19±0.019	0.19	-14.42
9	10	2	6	50	0.25±0.013	0.24	-12.04

[a]The observed values of glucose concentration, were the mean values of triplicates with standard deviation (mean±SD)

Table S2: Composition of native BSG and treated BSG

Components	Native BSG	MAA treated BSG
Cellulose	19.21	43.67
Hemicellulose	26.94	-
Xylan		11.53
Galactan		0.7
Arabinan		5.35
Mannan		0.46
Lignin	30.48	-

*****BSG: Brewers' spent grain; MAA: Microwave assisted alkali pretreatment.

Table S3: Response table for S/N ratio (in decibels) and relative ranking of variables for enzymatic hydrolysis of BSG

Level	Biomass loading(w/v %)	Enzyme loading(v/v %)	pH	Temperature (°C)
1	-15.1	-15.4	-14.19	-13.95
2	-14.37	-14.92	-14.63	-14.53
3	-13.79	-12.93	-14.43	-14.77
Delta	1.31	2.47	0.43	0.82
Rank	2	1	4	3

3.4 Effect of glucose concentration in BSG hydrolysate on cell growth and BDO production

The initial substrate concentration has a substantial impact on cell growth and product formation. Therefore, it is important to determine an optimal substrate concentration which not only favors biomass growth but also leads to high rate of product accumulation [43]. The effect of BSG (glucose-rich) hydrolysate on BDO production was examined by growing the mutant strain in a medium supplemented with different concentrations of glucose (20-100 g/L) from BSG hydrolysate in a shake flask experiment. The time course profiles of cell growth (OD_{600} nm), substrate uptake, product formation, and pH profile are shown in Figure 3. Variation in the initial glucose concentrations had a marginal impact on the highest value of OD_{600} obtained, which was in the range of 6.7-8.2. However, the rate of biomass formation was reduced at high glucose concentrations (80 and 100 g/L). The glucose uptake rate was high, and all the supplied glucose was completely depleted within 20 h at an initial concentration of 20-60 g/L, while in the case of 80 and 100 g/L, there was a significant decline in the substrate consumption rate. As a result of this, a large amount of glucose was left unconsumed and the amount of residual glucose at 80 and 100 g/L was 14.0 and 44.1 g/L, respectively. The maximum BDO titer obtained at an initial glucose levels of 20, 40, 60, 80 and 100 g/L were 9.5, 13.9, 15.9, 14.0, and 14.4 g/L, respectively. However, the time of onset of BDO formation was delayed and productivity was reduced at 80 and 100 g/L glucose level. Increasing the glucose concentration caused impedance in BDO yield which dropped from 0.47 to 0.28 g/g when glucose level was increased from 20 to 100 g/L which is clear evidence of substrate inhibition. The initial pH in all the experiments was set to 6.6 which dropped below 4.5 after 14-16 h of cultivation and could be one of the reasons for low performance at higher concentrations as bacteria are quite sensitive to pH fluctuations. Since the yield of BDO achieved at 20 and 40 g/L glucose concentration were similar, 40 g/L was chosen as initial concentration for further experiments.

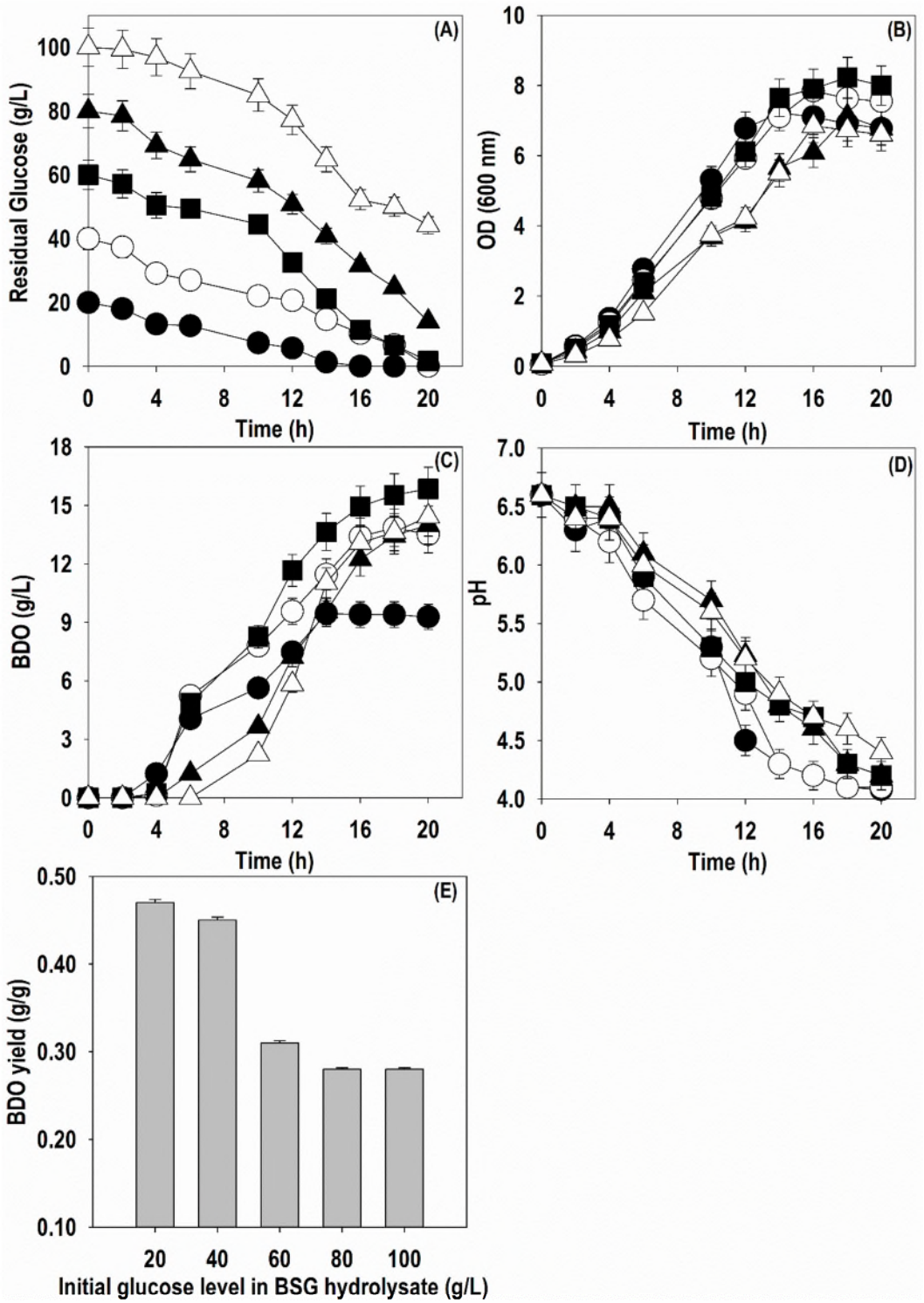


Figure 3: Kinetics of residual glucose, OD_{600}, pH and BDO production by mutant strain of *E. ludwigii* in shake flask at different initial glucose levels in BSG hydrolysate: (A) residual glucose; (B) OD_{600}; (C) BDO titer; (D) pH; (E) BDO yield. Symbols: filled circle (20 g/L), empty circle (40 g/L), filled square (60 g/L); filled triangle up (80 g/L) and empty triangle up (100 g/L).

3.5 Impact of initial pH on BDO synthesis from BSG hydrolysate

In microbial fermentation, pH plays a very important role in maintaining the physiological state of the microbial strain, and thereby influencing the growth and metabolite production. In case of microbial BDO

synthesis, pH is the crucial factor ruling the production as the most prominent role of the BDO pathway is to prevent intracellular acidification by diverting the metabolism from acid production towards the formation of neutral metabolites [15,23]. To investigate the impact of pH on BDO accumulation, the previously selected mutant strain of *E. ludwigii* was cultivated in a shake flask with different initial pH values and the time course profiles are shown in Figure 4. The strain grew poorly at pH 5.0 with no BDO production and more than 90% of glucose carbon was left unconsumed. The situation at pH 6.0 was better but the situation deteriorated after 6 h when the pH was below 5.5 and continued to drop during fermentation. Thereafter, cell proliferation and BDO production did not pick up as expected at an optimal pH. The results obtained in terms of cell growth and BDO synthesis was similar at pH 7.0 and 8.0. At pH 7.0, the maximum OD_{600}, BDO titer, and yield obtained were 8.1, 15.7 g/L, and 0.42 g/g, respectively, whilst at pH 8.0 the highest OD_{600} was 9.0 with a BDO titer of 14.1 g/L with conversion yield of 0.38 g/g. These results show that initial pH in the range of neutral to slightly alkaline favor BDO production along with good cell growth. However, if the fermentation begins with weakly/moderately acidic conditions, the pH slips quickly below 5.0, and cells stop assimilating glucose and the metabolism is halted. In subsequent studies, an initial pH of 7.0 was used.

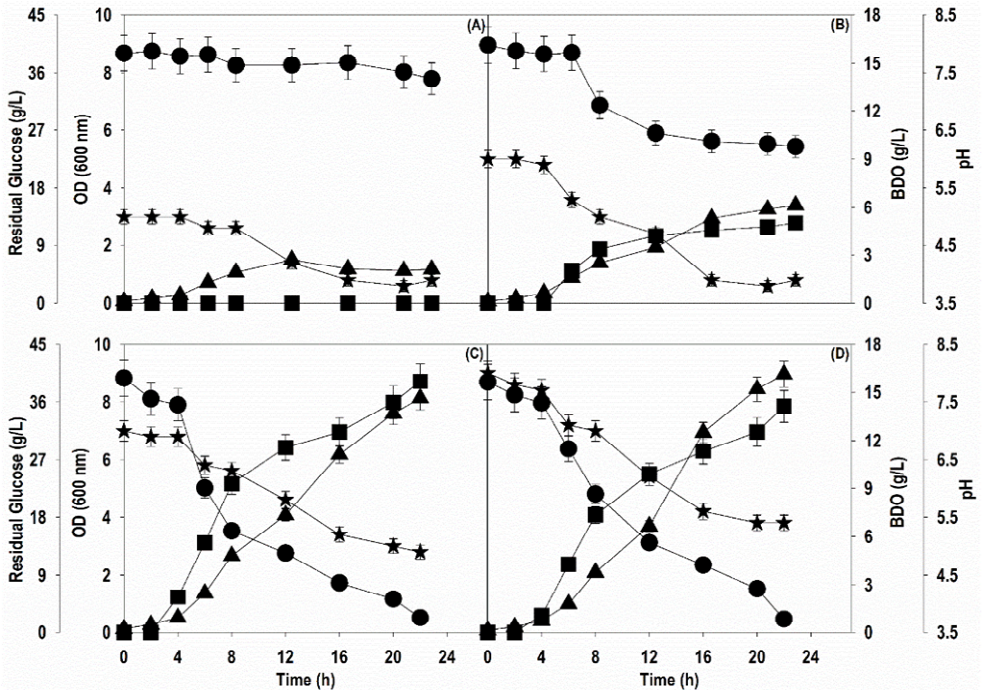


Figure 4: Batch cultivation of mutant strain of *E. ludwigii* in shake flask with different initial pH values: (A) 5.0; (B) 6.0; (C) 7.0; (D) 8.0. Symbols: filled circle (glucose), filled triangle up (OD_{600}), filled square (BDO) and filled star (pH).

3.6 Batch cultivation of mutant *E. ludwigii* strain in a bioreactor

After the shake flask cultivation, the mutant *E. ludwigii* was assessed in batch bioreactor for BDO production using BSG hydrolysate with a glucose concentration of 40 g/L. Two separated batch

fermentation were run, one with controlled pH and in another case, pH was not controlled and the initial pH in both the reactors was adjusted to 7.0. The time-course profiles for the fermentation runs are shown in Figure 5. The glucose was completely exhausted within 14-16 h in both cases, concomitant with maximum cell mass formation. The maximum cell OD_{600} obtained was 12.9 and 14.9 in uncontrolled and controlled pH batch fermentation, respectively, which was higher than achieved during shake flask cultivation. The BDO accumulation in case of uncontrolled pH was significantly higher than controlled fermentation where pH dropped from 7.0 to 5.8. In controlled cultivation, the maximum BDO titer of 12.7 g/L and yield of 0.32 g/g was achieved, while the uncontrolled fermentation resulted in 16.4 g/L BDO with a yield of 0.41 g/g which was ~29% higher than controlled experiment. The observation of higher BDO accumulation with a decrease in pH indicates that regular drop in pH (slightly acidic) in a systematic manner would divert the carbon flux towards BDO synthesis so that the intracellular pH could be maintained. Therefore, a fed-batch mode of cultivation with cyclic pH alterations was performed to improve the BDO titers and yield.

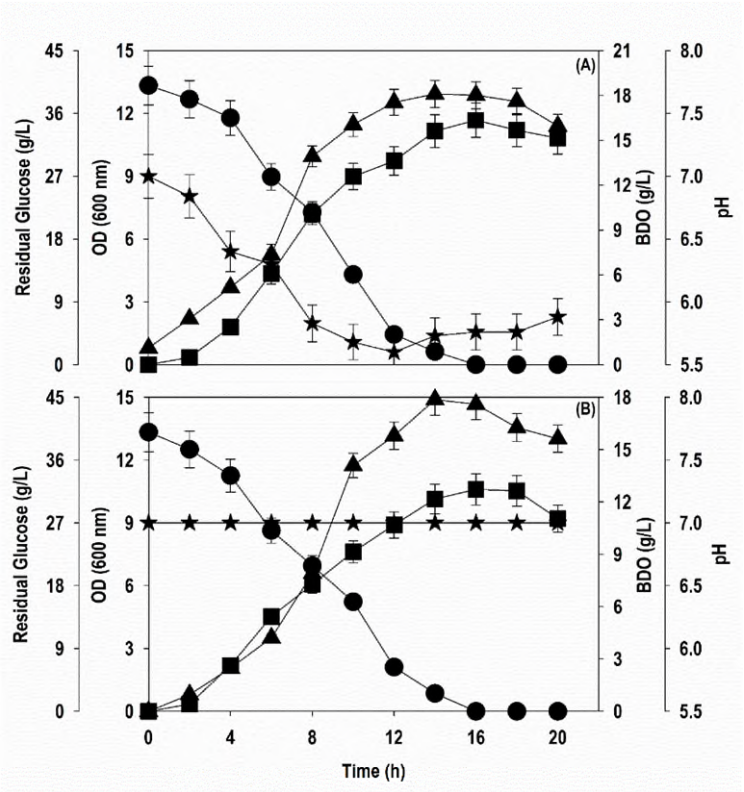


Figure 5: Effect of initial pH on glucose consumption, cell growth and BDO formation from batch cultivations of mutant strain of *E. ludwigii* in bioreactor: (A) uncontrolled; (B) controlled. Symbols: filled circle (glucose), filled triangle up (OD_{600}), filled square (BDO) and filled star (pH).

3.7 Fed-batch cultivation of mutant *E. ludwigii* strain in a bioreactor

Like pH, oxygen supply is another critical variable in the BDO fermentation, and therefore, it is very important to fine-tune oxygen level for optimal production of BDO [15,23]. The effect of the aeration

rate on the biomass and BDO production ability of mutant *E. ludwigii* under fed batch cultivation was examined. BDO is a neutral metabolite and countermeasure against acidification. It has been found that continuous drop in pH enhance BDO accumulation. Nevertheless, pH should not reduce to a value where metabolism is completely shut down [34,35,44]. Therefore, pH was controlled in a cyclic mode where forced pH fluctuations were implemented to enhance the BDO accumulation. The initial pH was 7.0 and when the pH dropped below 5.0 during the course of fermentation, it was adjusted back to 7.0 using 5M NaOH. This process was repeated many times during the course of fed-batch cultivation. After the depletion of initial glucose concentration (40 g/L), glucose was intermittently replenished using concentrated BSG hydrolysate (500 g/L glucose) when glucose level decreased to 20 g/L or less during the fermentation. The comparative kinetics of glucose assimilation, cell growth, and BDO formation during fed-batch fermentation are shown in Figure 6.

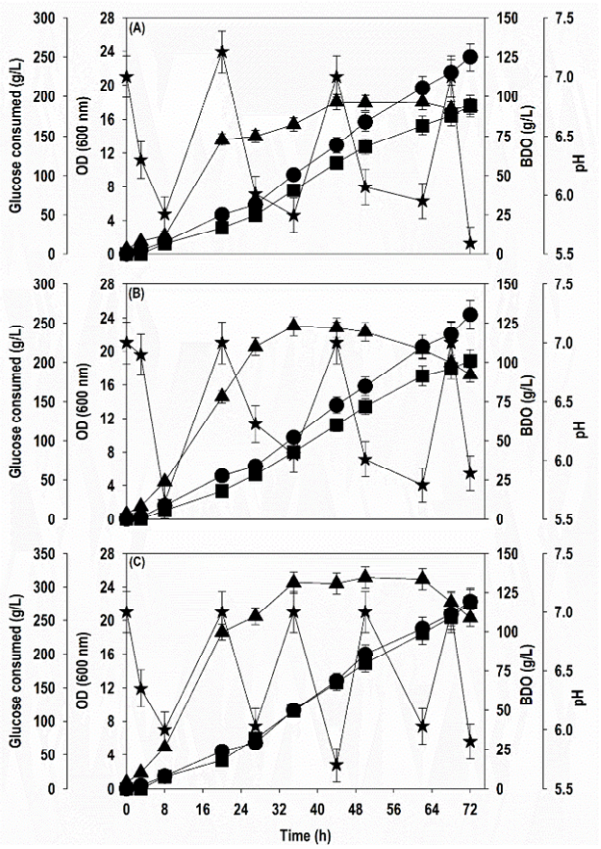


Figure 6: Fed-batch cultivation of mutant strain of *E. ludwigii* in bioreactor at different aeration conditions: (A) 0.5 vvm; (B) 1.0 vvm; (C) 2.0 vvm. Symbols: filled circle (glucose), filled triangle up (OD_{600}), filled square (BDO) and filled star (pH).

At 0.5 vvm, the period of active biomass formation was 8-20 h where OD_{600} reached ~14. Post 20 h, cell growth slowed down, steadily increased and the maximum OD_{600} obtained was 18, thereafter, no cell growth was observed. After the initial period of biomass accumulation, the rapid BDO production was noticed and cells continued to manufacture BDO till the end of fermentation. The final titer of BDO

recorded was 94.0 g/L at 72 h. On increasing the aeration rate from 0.5 to 1.0 vvm, a similar pattern was obtained. The highest cell growth and BDO titer obtained at 1.0 vvm were moderately higher than that at 0.5 vvm, reaching 23.0 and 101.1 g/L, respectively. The conversion yield and productivity of BDO accumulation were 0.39 g/g and 1.40 g/L.h, respectively. Further increment in aeration rate (2.0 vvm) resulted in slightly higher biomass formation and significant acceleration in BDO synthesis which was also evidenced by an increase in the number of rounds of forced pH fluctuations. The final amounts of BDO with 2.0 vvm observed was 118.5 g/L with yield and productivity of 0.43 g/g and 1.65 g/L. h. Besides BDO titer, yield and productivity improved with an increase in aeration rate, and a significant hike was observed when aeration was enhanced from 1.0 to 2.0 vvm (Figure 7). The results indicate that a high aeration rate is favourable for cell growth, active metabolism, and BDO production.

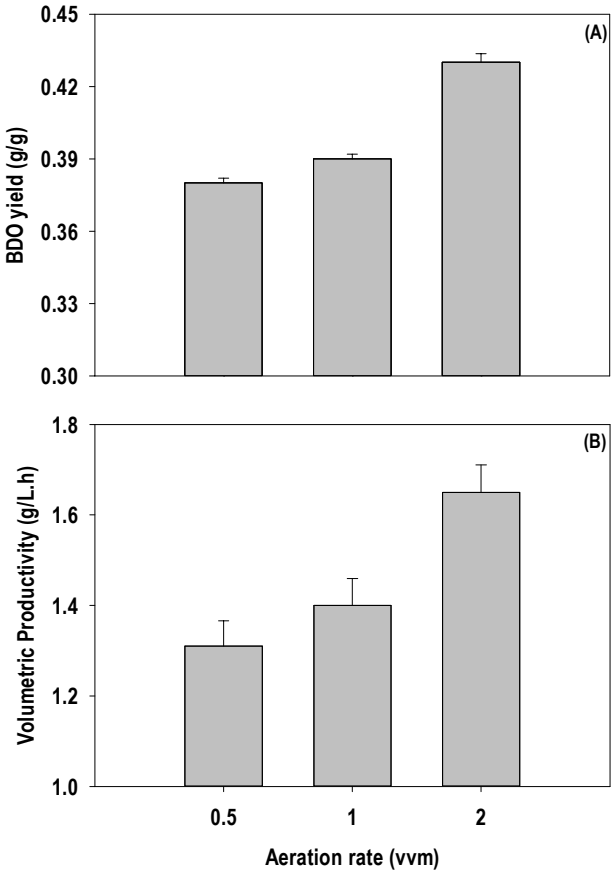


Figure 7: Impact of aeration on yield and volumetric productivity of BDO accumulation from fed-batch cultivation of mutant strain of *E. ludwigii* in bioreactor.

4. Discussion

Agro-residues and industrial by-products can be an attractive renewable source if utilized perceptively. BSG is a by-product of brewing process, generated in huge quantities and is rich in cellulosic and non-cellulosic components making it an interesting feedstock for biorefineries. However, it has not received

much attention and its disposal is often an environmental problem [10,11]. The European Union is the second-largest beer producer in the world, producing approximately 383 million hectolitres of beer in 2014, behind China (approximately 465 hectolitres) [45]. In 2008/09 the EU (European Union) introduced the "New waste framework directive" to develop processes such as reuse and recycle to curb the disposal of residues like BSG [40]. In this regard only, limited work has been carried out on utilizing BSG for the bioproduction of chemicals such as ethanol, xylitol, lactic acid, butanol, hydrogen, carboxylic acid, enzymes, biogas, prebiotics, PHA, PHB, etc [9,10].

BDO is a valuable biochemical which is generally produced from hydrocarbons by the cracking of butane and 2-butene where intermediate is further hydrolyzed to BDO. In the microbial route, BDO is produced from pyruvate in a mixed-acid fermentation process with acetolactate, acetoin, and diacetyl as intermediates (Supplementary Figure 2). The bioproduction of BDO is still in infancy but with strong prospects of growth. One of the factors strongly influencing the process economics of bio-based BDO production is the substrate cost which accounts for more than 50% of total production cost [15,23,46]. Although several studies have been performed on fermentative production of chemicals using BSG as a substrate, the authors did not find any report based on the BDO production from BSG. To this end, BDO production from BSG as a source of fermentable carbon by *E. ludwigii* was investigated in the present study.

We started with culturing *E. ludwigii* on complex and synthetic medium and a comparable BDO production was achieved on both (Figure 1) and our results are in agreement with Maina et al., (2019) [26]. The complex medium contains a high concentration of yeast extract and peptone contributes to a significant enhancement in the overall production cost of the BDO and would make the process expensive and unviable for commercial scale [16]. Therefore, it is envisaged that the use of the inexpensive synthetic medium with BSG as a carbon source would significantly reduce the cost for industrial BDO production. EMS is a common alkylating agent and is well known to induce the overproduction of metabolites in microbial cultures [47–49]. The random mutagenesis of wild type *E. ludwigii* strain by EMS elevated the BDO titer as well as yield by 30-32%. The yield reached close to the theoretical maximum without compromising the growth (Figure 2). Similar approaches have been used for other *Enterobacter* species to ameliorate the efficiency of BDO fermentation. For example, Xin et al., (2016) subjected hyper BDO producing wild-type *Klebsiella* sp. strain XRM21 to random mutagenesis using EMS which resulted in a highly productive mutant strain accumulating 119.4 g/L in 65 h against 91.2 g/L BDO generated by wild type strain [50]. In another report by Bustamante et al., (2019), two mutant strains were identified after treatment with EMS accumulating 30% more BDO (~31 g/L) than the wild type, with yield approximating the theoretical maximum [51].

For effective utilization of a lignocellulosic feedstock through the fermentative route, it is necessary to maximize exposure of the cellulosic fraction along with the removal of lignin fractions. Several reports have been published on pretreatment of BSG by microwave radiation, mild acid treatment, and acid-alkali treatment. The MAA pretreatment method employed is the best to improve the cellulosic content (~44% w/w) and effectively remove lignin fractions [28,52,53]. The enhancement in cellulosic content coupled with optimized enzymatic hydrolysis by Taguchi method resulted in a glucose yield of 0.25 g/g which is comparable to the yield (0.23 g reducing sugars/g) obtained after RSM optimized enzymatic hydrolysis of BSG pretreated with MAA by Ravindran et al., (2018). After saccharification, the glucose tolerance from BSG hydrolysate was tested by growing mutant *E. ludwigii* strain at different concentrations of glucose (20-100 g/L). Beyond 60 g/L, there was a significant drop in BDO yield and a large amount of residual glucose was observed (Figure 3). Contrary to our results, ascending substrate (glucose/sucrose/molasses) concentration up to 100-200 g/L did not have any negative impact on BDO production (titer, yield, and productivity) by *Enterobacter* species in previous works [50,54,55]. For example, BDO production by *E. ludwigii* increased continuously on enhancing initial molasses concentration from 40 to 200 g/L while yield and productivity remained unaffected up to 160 g/L indicating a strain with high osmotolerance [25]. Unlike our work, other studies were conducted in a bioreactor in a pH-controlled environment [56], while we experimented with a shake flask without pH control. The pH decreased during course of fermentation, reaching below 5.0 and causing the cells to stop assimilating glucose. This large drop in pH could be one of the reasons for the low performance of strain rather than high glucose concentration. Another factor could be an enhancement in the level of fermentation inhibitors with an increase in glucose concentration from BSG hydrolysate.

Oxygen and pH are the two important parameters that have tight control over BDO synthesis with oxygen supply being the most important variable in BDO fermentation [15,23]. In the current study, we found that BDO production by *E. ludwigii* strongly depends on the pH value and is inversely linked to pH (Figure 5). The results obtained in the present study are in complete agreement with previous reports. Biebl et al., (1998) reported that the lower the pH, more the BDO generated by the cell [34], and the highest titer of BDO was obtained with uncontrolled pH. Similar observations were made by Durgapal et al., (2014) when mutant *K. pneumoniae* strain was cultured at different pH's with the highest BDO titer (18.0 g/L) obtained when pH was not controlled, and metabolic activities of cells came to halt when pH dropped below 5.0 [57]. It has been hypothesized that in the absence of external maintenance, some microorganisms attempt to control pH through switching metabolism to the production of less toxic compounds such as alcohols in response to spontaneous pH drops [15,23]. However, our finding is in disparity with Chan et al., (2016) who observed that when pH was gradually lowered in an uncontrolled pH experiment, maltodextrin was not efficiently utilized by *Klebsiella oxytoca* strain [55]. The fed-batch

cultivation at different aeration rate revealed that as the aeration rate was increased, a continuous improvement in biomass and BDO concentration was obtained. The differences in results at 0.5 and 1.0 vvm were marginal, however, a momentous leap was noticed on further augmentation in aeration rate from 1.0 to 2.0 vvm (Figure 6). The forced pH fluctuation with high aeration (2.0 vvm) resulted in a very high level of BDO accumulation of 118.5 g/L with 0.43 $g_{BDO}/g_{Glucose}$ yield and productivity of 1.65 g/L.h. Our results are similar to previous reports where high oxygen supply was found to be beneficial for biomass and BDO synthesis [55,58]. It is important here to discuss the study by Petrov and Petrova (2010) as our findings are in complete accord with their findings [44]. They investigated the impact of oxygen and pH on BDO production by *K. pneumoniae*. The regime of intensive aeration led to significant improvement in the yield and productivity of BDO. An aeration rate of 2.2 vvm was recorded as most beneficial for BDO accumulation and diminished the production of by-products. The further increment in titer was brought by forced pH fluctuations by consecutive raisings with definite ΔpH value (1.0, 2.0, and 3.0). The maximum amount of BDO (70.0 g/L) was produced when pH was increased by one unit after every 12 h. In current study, acetoin, ethanol, succinic, lactic, and acetic acids were obtained as byproducts. Succinic acid was the major product while the other metabolites were recorded < 10 g/L (Table 2). Similar to Maina et al., and Psaki et al., (2019), the concentration of these byproducts was fluctuating during fermentation, possibly assimilated as carbon source [25,26]. The elimination of biochemical pathways leading to these byproducts could result in further improvement in yield.

Table 2: Byproduct accumulation at the end of fed-batch cultivation of *E. ludwigii* on BGS at different aeration rates

Aeration rate	Acetoin (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)
0.5 vvm	4.0±0.22	10.6±0.62	9.6±0.86	4.4±0.28	12.8±0.65
1.0 vvm	5.9±0.34	13.4±0.94	5.8±0.33	4.1±0.22	8.8±0.52
2.0 vvm	4.9±0.15	16.7±1.22	6.9±0.59	7.0±0.42	5.5±0.48

A large part of the cost for microbial BDO production stems from substrate price and in the last two decades, significant work has been done on BDO production from cheap renewable feedstocks to improve the process economics. Table 3 compares our results with some of the potentials studies based on BDO accumulation from crude renewable sources. The results obtained in the current study are comparable or even better than some of the reported results. Most of the studies shown in Table 3 is based on media containing complex and rich nitrogen sources like yeast extract, peptone, meat extract, casamino acid hydrolysate and corn steep liquor which not only contribute to high production cost but also complicate product recovery [55]. For example, Häßler et al. (2012) used massive amount of YE (60

g/L) to accumulate 111 g/L BDO by *Paenibacillus polymyxa* [56]. In the current study, we made use of an inexpensive synthetic medium devoid of any complex nitrogen source and supplemented with BSG hydrolysate as a source of fermentable carbon. Further, the majority of reports controlled the pH while in current study cyclic control of pH was performed with forced pH fluctuations which is an added advantage due to significant reduction in amount of expensive alkali addition for pH maintenance during the course of fermentation.

Table 3: Summary of microbial BDO production from different carbon sources by various microorganisms*

Microorganism	Feedstock	Complex nitrogen source(s) (g/L)	pH	BDO			Reference
				Titer (g/L)	Yield (g/g)	Productivity (g/L. h	
Paenibacillus polymyxa	Sucrose	YE: 60	Controlled	111.0	-	2.06	[58]
Klebsiella oxytoca	Glucose	YE: 5; CAH: 10	Controlled	142.5	0.42	1.47	[57]
Klebsiella pneumoniae	Jerusalem artichoke tubers	None	Controlled	91.6	0.32**	2.29	[59]
Klebsiella pneumoniae	Corncob molasses	CSLP: 8.3	Controlled	78.9	0.41	1.30	[46]
Enterobacter cloacae	Cassava powder	CSLP: 10	Controlled	93.9	-	2.00	[60]
Bacillus licheniformis	Inulin hydrolysate	YE: 5.8; CSLP: 14.7	Controlled	103.0	-	3.4	[61]
Enterobacter cloacae	Corn stover hydrolysate	CSLP: 10	Controlled	119.4	0.48	2.30	[62]
Bacillus licheniformis	Apple pomace + Glucose	YE: 3.2	Uncontrolled	113.0	0.49	0.69	[63]
Klebsiella oxytoca	Maltodextrin	None	Controlled	88.1	0.41	1.13	[55]
Enterobacter ludwigii	Fruit extract	YE: 2.5; P: 5; ME:5	Controlled	50.0	0.40	0.41	[27]
Enterobacter ludwigii	VHP Cane sugar	None	Controlled	86.8	0.37	3.95	[26]
Enterobacter ludwigii	VHP Cane sugar	None	Controlled	108.8	0.38	1.15	[26]
Enterobacter ludwigii	Sugarcane molasses	None	Controlled	50.6	0.31	2.66	[25]

Enterobacter ludwigii	BSG hydrolysate	None	Cyclic control	118.5	0.43	1.65		This study

CAH – Casamino acid hydrolysate; CSLP – Corn steep liquor powder; ME – Meat extract; P – Peptone; YE – Yeast extract

*All the data shown in Table is from fed-batch cultivation.

**Yield of BDO + acetoin

5. Conclusions

Despite a large available body of knowledge about the biosynthesis of BDO via a fermentative route, industrial microbial production is still in nascent stage. Robust microorganisms and the use of inexpensive substrates and media are the main drivers to ensure the economic feasibility of BDO production. In the current study, *E. ludwigii* was investigated as a potential effective BDO producer from BSG, an abundant, renewable agro-industrial residue. Enzymatic hydrolysis of BSG was successfully used to achieve a high-level of fermentative BDO production by *E. ludwigii*. The BDO accumulation achieved under optimal conditions is in significant titers and appears to be the highest BDO production from BSG by mutant *E. ludwigii* strain. The fast growth, active sugar metabolism, resistance to fermentation inhibitors, and high bioconversion rate makes *E. ludwigii* an excellent biocatalyst for large scale BDO production. The use of inexpensive medium with high product accumulation and a reduced requirement of neutralizing agent represents a significant reduction in operating costs. The results obtained in the present study demonstrate the feasibility of high-level production of BDO from BSG for industrial applications.

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Contributions

YA, AAP and VN carried out all the experimental work and collected the data. VK analyzed the data and wrote the manuscript. FC, AKC, NW, SJ and AK were involved in proofreading the manuscript and revised the manuscript critically. All authors read and approved the final manuscript.

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Correspondence to Vinod Kumar.

Consent for publication and Competing interests

The authors declare that they have no competing interests and give consent for publication.

Availability of Data and Materials

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

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