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Biological production and recovery of 2,3-butanediol using arabinose from sugar beet pulp by *Enterobacter ludwigii*

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ABSTRACT

Sugar beet pulp (SBP) is a major byproduct from the sugar industries and consists of >20% w/w arabinose. The current work evaluated the potential of *Enterobacter ludwigii* assimilating pure arabinose and arabinose rich hydrolysate from SBP pellets for 2,3-butanediol (BDO) production. The hydrolysate was obtained through dilute acid pretreatment (DAP) with sulphuric acid. The process was optimized for acid and solid loading to obtain a hydrolysate free from furan derivatives. The effect of different levels of substrate (10–60 g/L) using pure arabinose was conducted in shake flask experiments, followed by co-fermentation with small amounts of glucose and SBP hydrolysate. After flask cultivations, BDO fermentations were carried-out in a bench-top bioreactor in batch and fed-batch modes using pure arabinose as well as SBP hydrolysate. The fed-batch culture led to BDO production of 42.9 and 35.5 g/L from pure arabinose and SBP hydrolysate with conversion yields of 0.31 and 0.29 g/g, respectively. Finally, BDO accumulated on pure arabinose and SBP hydrolysate were recovered using an aqueous two-phase extraction system. The recovery yield of BDO accumulated on arabinose and hydrolysate was ~97%. The work demonstrated the feasibility of using SBP as a suitable feedstock for manufacturing BDO.

1. Introduction

Industrial revolution, population growth, and urbanization has heavily increased reliance on crude oil-based chemical, and allied industries. The finite and non-sustainable resources like crude oil, have negative impacts on economy as well as environment, but the decades of research exploiting microorganisms to produce various value-added products using renewable feedstocks as the substrates, was observed to be a reliable approach for sustainable industrial sector. These microorganisms utilise the substrates and assimilate the carbon through various biochemical pathways and accumulate products via fermentative routes, which is an ideal alternative to fossil-based production of chemicals and combats the above associated problems. Various chemical building blocks can be produced by these microorganisms using both edible and non-edible feedstocks. Furthermore, the bioderived products are termed environmental friendly as they are biodegradable, reusable and generated through sustainable processes, contributing to carbon-neutral society [1–3]. In this era of circular bioeconomy, biogenic residues rich in fermentable carbon have become important resources to establish integrated biorefineries. This will not only result in the elimination of waste streams but would also lead to the development of low carbon biomanufacturing technologies [4,5].

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Lignocellulosic biomass (LCB) is the most abundant material on earth, inexpensive, and a rich source of fermentable sugars. Despite this, LCB based processes have not attained commercial significance. After glucose and xylose, arabinose is the third most abundant sugar in LCB [6]. The sugar beet pulp (SBP), a main byproduct from sugar industries that manufacture table sugar (sucrose) from sugar beet crop is analogous to sugarcane bagasse obtained from sugarcane-based sucrose manufacturing. Unlike sugarcane bagasse, the composition analysis of SBP reveals arabinose as of the the major sugars which is present in comparable amount to glucose [7]. In 2016, the total SBP production by EU-28 was 10.35 million tonnes. UK alone produces 8 million tons of sugar beet annually and fermentable sugar that can be obtained from SBP is 170,000 tons/year. The Wissington plant of British Sugar produces 400,000 tons of sugar and 350,000 tons of SBP every year [8,9]. SBP is largely composed of three biopolymers, cellulose, hemicellulose, and pectin. The composition of SBP is reported as follows: 22–24% cellulose, 30% hemicellulose, 15–25% pectin, 5.9% lignin, 10.3% total protein, 3.7% ash and 1.4% fat [10]. D-Glucose (from cellulose), L-arabinose and D-galacturonic acid (both from pectin) account for 85% of the total monosaccharides in SBP and constitutes 60–70% of dry matter. The biopolymers present in SBP can be solubilized, making it a rich source of fermentable sugars. The monosaccharide composition of SBP after hydrolysis with sulfuric acid is reported as follows: glucose (25.9%), L-arabinose (23%), galactouronic acid (14.4%), galactose (6.2%), rhamnose (2.4%), xylose (1.7%) and mannose (1.0%) [9]. Cellulose is a homopolymer of glucose that can be easily hydrolysed by cellulase to release glucose, which can then be fermented into a range of products because glucose is the preferred carbon source for most of the microorganisms. However, the main challenges arise in bioconversion of pentose sugars such as xylose and arabinose which are overlooked due to many factors such as the lack of transporters, pathways, less preference, and the presence of glucose [11].

2,3-Butanediol (BDO) is a C4 diol with multitude of applications in various industrial sectors like pharma, food, and chemicals and has enormous commercial potential. BDO finds applications in the manufacturing of a range of products including softening agents, plasticizer, polyester, drugs, and cosmetics. BDO serves as a starting material for synthesis of several chemical products such as e.g.: 1,3-butadiene, methyl ethyl ketone etc. Owing to its high heating value and octane number, BDO and its derivatives find applications as fuel additive. The potential of BDO and its derivates have been valued at $43 billion with an annual global production of 32 million tonnes [12–15]. Various reports on use of fermentable sugars from SBP have focussed on bioethanol or biogas production [7,16]. The perusal of literature shows there are very few reports using arabinose as carbon source for fermentative production of chemicals unlike, glucose and xylose. The articles on arabinose-based bioproduction are even rarer than xylose, indicating not much effort has been made in this direction. To this end, the current work assessed the prospective of *Enterobacter ludwigii* as a biocatalyst for BDO production from arabinose. Initially the experiments were conducted in Erlenmeyer flasks (250 or 500 mL) with different levels of pure arabinose followed by co-fermentation with a small amount of glucose. SBP pellets were pretreated with sulphuric acid to obtain arabinose-rich hydrolysate which was later utilized for BDO production. The data from the shake flask was scaled up in a bioreactor and BDO fermentations were performed in batch and fed-batch modes using pure arabinose as well as SBP hydrolysate (rich in arabinose). Finally, the BDO rich fermented broth obtained from pure arabinose and the arabinose-rich hydrolysate was subjected to aqueous two-phase extraction system (ATPS) for recovery.

2. Materials and methods

2.1. Materials

The media components and other chemicals used in pretreatment, fermentation and downstream processing were procured from Sigma Aldrich (USA) and Fischer Scientific and were of analytical grade. Sugar beet pulp (SBP) pellets in dried form were purchased online from Supa beet, Trident Sugar beet pellets, UK. The pellets were mechanically ground, and 500 μm sieve was used to separate the fine powder and then stored at 4 °C until further use.

2.2. Dilute acid pretreatment (DAP) of SBP with optimization of acid and solid loading

The chemical composition of the SBP used in this study contained approximately, 23.3% cellulose, 37.6% hemicellulose, 20.6% pectin, <2% lignin, 10.9% protein, 3.3% ash, and 2.8% other components. In the DAP, the finely sieved SBP was added to known volume of deionized water containing dilute acid. The SBP slurry obtained was heated at 121 °C for 15 min and further cooled down at room temperature. The samples were further neutralised with 5M NaOH and stored at 4 °C for analytical and bioconversion purposes. In the initial set of experiment, the SBP slurry with solid loading of 10% w/v was supplemented with different concentrations of H_2SO_4 (1, 2, 3, 4, and 5% v/v) to understand the impact of acid loading on pretreatment efficiency. Next, pretreatment experiments were conducted at various solid loadings (5, 10, 15, 20, 25, 30% w/v) with optimal acid concentration (2% v/v). Furthermore, the SBP hydrolysis was carried out in a 1 L scale reactor using the optimal acid and solid loading. The rotary vacuum evaporation was employed to concentrate the hydrolysate to obtain the arabinose concentration of ~250 g/L. The hydrolysate was diluted to obtain the required concentration of arabinose for different experiments.

2.3. Microorganism, cultivation, and maintenance

A mutant *Enterobacter ludwigii* strain developed in our previous study [13], which has been evaluated as a potent BDO producer was used in the current study. The strain was maintained on Tryptic Soy (TS) agar plates with the following composition (g/L): 17 pancreatic digest of casein, 3 soybean meal, 5 NaCl, 2.5, KH_2PO_4, 2.5 glucose and the pH was adjusted to 7.3 prior to sterilisation. The culture grown on TS broth was centrifuged and the pellet was suspended in 50% (v/v) glycerol and stored at −80 °C as freeze dried samples. The seed cultures for the shake flask and bioreactor experiments were grown in a 500 mL Erlenmeyer flask containing 100 mL of TS broth. The TS broth was inoculated with freshly sub-cultured colony of *E. ludwigii* and incubated for 12 h at 30 °C with an agitation speed of 180 rpm on a rotary shaker (Excella 24, New Brunswick). The culture medium composition for fermentation is as follows: 6 g/L $(NH_4)_2HPO_4$; 7.2 g/L $(NH_4)_2SO_4$; 2 g/L yeast extract; 0.45 g/L KOH; 0.51 g/L EDTA; 0.3 g/L $MgSO_4 \cdot 7H_2O$; 90 mg/L $CaCl_2 \cdot 6H_2O$; 25 mg/L $FeSO_4 \cdot 7H_2O$; 3.8 mg/L $MnSO_4 \cdot H_2O$; 7.5 mg/L ZnSO4·7H2O [14]. The sugar concentration (arabinose and glucose) was adjusted as per the requirement and the final pH of the medium before sterilization was adjusted to 6.6 using 5M NaOH.

2.4. Shake flask cultivation

Prior to the bioreactor studies, the strain was cultured at different concentrations of arabinose in pure and crude form. In addition, co-fermentation experiments with different levels of arabinose and 5 g/L glucose were performed. The fermentations

were carried out in 500 mL shake flasks containing 100 mL of sterilised culture medium. The complex media (section 2.3) was supplemented with different concentrations of pure arabinose (10–60 g/L), glucose (5 g/L) and non-detoxified SBP (10–60 g/L) hydrolysate. The flasks were inoculated with 2% (v/v) freshly prepared seed inoculum as discussed in the previous section. The initial pH was adjusted to 6.6 before inoculation and adjusted manually in the range of 6.0–7.0 periodically during the cultivation by using 5M NaOH under sterile conditions to minimize the negative impact of continuous pH drop.

2.5. Bioreactor experiments

The batch experiments using the optimal arabinose concentration obtained in the shake flask experiments with pure arabinose and non-detoxified SBP hydrolysate were validated in a 2.5 L benchtop stirred tank bioreactor (Electrolab Bioreactors, UK) with a 1.0 L working volume. The agitation speed, aeration rate and temperature, were controlled at 180 rpm, 1.0 vvm and 30 °C, respectively. The medium pH was controlled at 7.0 using 5M NaOH throughout the fermentation. Further to the batch experiments, a fed-batch fermentation was carried out with intermittent feeding of arabinose using the concentrated (250 g/L) solution of pure arabinose and SBP hydrolysates to maintain the residual arabinose concentration above 10 g/L.

2.6. Separation of butanediol from the fermented broth

An aqueous two-phase system (ATPS) extraction system was employed to separate and purify BDO accumulated on arabinose and the non-detoxified arabinose rich SBP hydrolysate during fed-batch fermentation. Initially, residual microbial cells, and insoluble macromolecules are removed from the fermented broth through centrifugation at 8000 rpm for 10 min. Further, $(NH_4)_2SO_4$ as salting-out agent at 30% w/v was added to the clear supernatant followed by addition of 50% v/v isopropanol as the extractant [17]. The mixture was vortexed for 15 min and kept ideal at room temperature for 7 − 8h for separation of organic and aqueous phase. After the phase separation, the organic phase was separated from the aqueous phase and then the extractant was evaporated using vacuum distillation (Rotavapor, BUCHI UK Ltd) operated at 45 °C and 150 mbar pressure. Later BDO and other metabolites in the concentrated sample were quantified. The partition co-efficient (K) and recovery yield (Y), which indicates the efficiency of the process, were calculated using the following equations.

$$K = \frac{C_T}{C_B} \quad (1)$$

$$Y = \frac{C_T}{C_{FB}} X100\% \quad (2)$$

Where C_T, C_B, and C_{FB}, are the concentrations of BDO in top organic phase, bottom aqueous phase and the fermented broth, respectively.

2.7. Analytical methods

During the submerged fermentation experiments the samples were withdrawn periodically and were analysed for cell growth, residual arabinose, BDO, acetoin, ethanol, lactic acid (LA), succinic acid (SA), and acetic acid (AA). Initially the samples were centrifuged at 8000 rpm for 10 min. The cell free supernatant was used for quantification of metabolites and the acquired cell pellet was washed with Millipore water followed by dilution of sample with the same volume of Millipore water. The bacterial growth was measured using spectrophotometry by quantifying the optical density at 600 nm. One unit of OD at 600 nm corresponded to a cell dry weight 0.31 g/L. An average molecular weight of 24.6, which corresponds to an average cell with a molecular formula of $CH_{1.8}O_{0.5}N_{0.2}$ was used for carbon analysis [18]. The sample processing for the High-Pressure Liquid Chromatography (HPLC) analysis were discussed elsewhere [13,14]. The samples were loaded into a HPLC system equipped with a Rezex ROA-Organic Acid H + (Phenomenex, USA) column for elution connected with Refractive Index Detector (RID) for sugars, diols, alcohols, and a Diode Array Detector (DAD) for organic acids. The mobile phase was 10.0 mM and 5.0 mM H_2SO_4 with flow rates of 0.4 and 0.6 mL/min for RID and DAD methods, respectively. For the inhibitor analysis, the hydrolysate samples were diluted (10x), filtered and eluted through same column connected with a Diode Array Detector (DAD) using Millipore water as the mobile phase with a flowrate of 0.6 mL/min. Except the bioreactor runs which were performed in duplicates, all other experiments were carried out in triplicates with the calculated standard deviation less than 10%.

3. Results

3.1. Shake flask cultivation of E. ludwigii on pure arabinose

E. ludwigii is Gram-negative bacterium with a strong capability to manufacture BDO from a variety of carbon sources including glucose, fructose, xylose, arabinose, mannose, galactose, sucrose, glycerol etc. In our previous work, we have reported high fermentation efficiency of the bacterium to generate BDO using pure as well as crude glucose and xylose from brewer's spent grains and sugarcane bagasse, respectively [13,14]. In the current work, the bacterium was cultured on different concentrations of pure arabinose (10, 20, 30, 40, 50 and 60 g/L). Fig. 1 demonstrates the time-course profiles for arabinose consumption, cell growth, BDO formation and change in pH. BDO fermentations by *E. ludwigii* are accompanied by a drop in pH. The degree of pH reduction amplify with increase in substrate levels and the metabolism almost ceases when the pH falls below 5.0 [13,14]. The pH was manually adjusted by addition of alkali (5M NaOH) at regular intervals during the course of fermentation to prevent the pH falling below 6.0. With this approach, the substrate assimilation was quite fast and 90–100% of substrate at an initial arabinose concentration of 10–50 g/L was exhausted within 24 h. In the case of 60 g/L arabinose, a gradual decrease in the substrate uptake rate was observed resulting in the prolonged fermentation period and a residual arabinose concentration of 2.9 g/L was observed even after 32 h. The cell growth was active from the beginning and the maximum OD_{600} (8–14) was recorded within 10–24 h at 20-60 g/L arabinose. BDO accumulation was concomitant with arabinose consumption and enhanced with an increment in arabinose levels from 10 to 50 g/L, and no further improvement was noticed at 60 g/L. The highest BDO achieved with arabinose concentrations of 10, 20, 30, 40, 50 and 60 g/L arabinose was 3.8, 7.5, 11.2, 17.5, 20.0 and 20.1 g/L with conversion yield of 0.38, 0.33, 0.39, 0.43, 0.39 and 0.34 g/g, respectively. Acetoin, SA, AA, LA, and ethanol were obtained as byproducts and their concentrations were less than 5.0 g/L. Fig. 2 shows the profiles of these byproducts during the course of fermentation. Acetoin is a precursor of BDO and generated from the same pathway while the presence of other metabolites indicates a substantial carbon loss at the phosphoenol pyruvate/pyruvate node. These byproducts (organic acids and alcohols) not only reduce the yield of the main product but are also toxic, thereby negatively impacting the cell growth and product formation through multiple product mediated inhibitions arising from their coexistence.

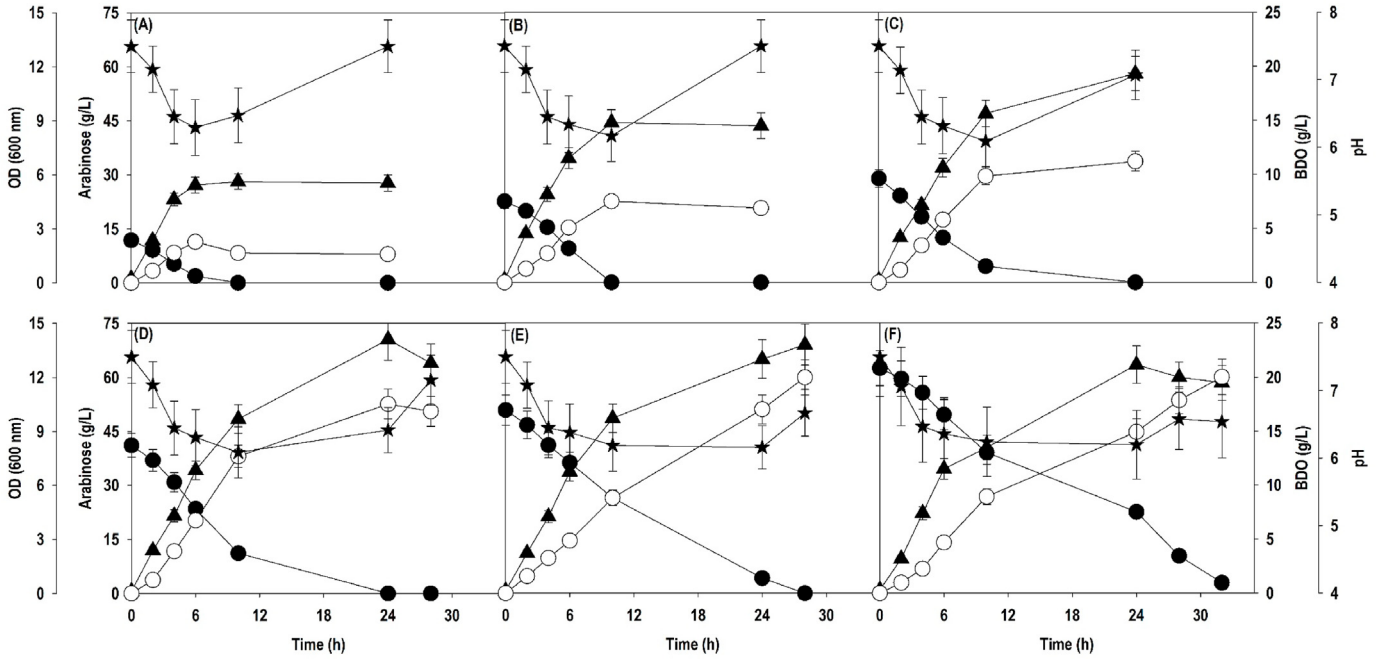

Fig. 1. Time course profiles for arabinose uptake, OD_{600}, BDO production and pH during shake flask cultivation at different levels of arabinose: (A) 10 g/L; (B) 20 g/L; (C) 30 g/L; (D) 40 g/L; (E) 50 g/L; (F) 60 g/L. Symbols: filled circle (arabinose), filled triangle (OD_{600}), empty circle (BDO) and filled star (pH).

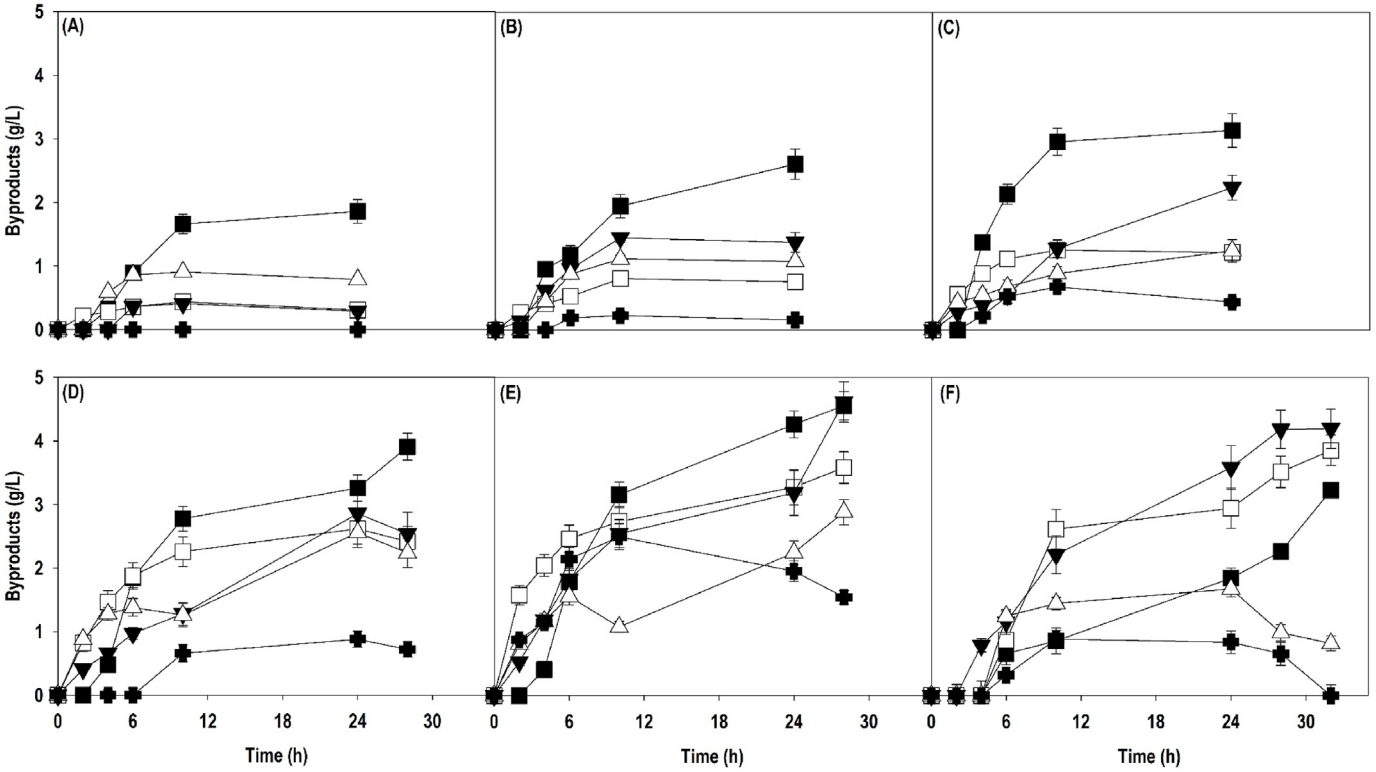

Fig. 2. Byproduct profiles during *E. ludwigii* cultivation at different levels of arabinose: (A) 10 g/L; (B) 20 g/L; (C) 30 g/L; (D) 40 g/L; (E) 50 g/L; (F) 60 g/L. Symbols: filled square (acetoin), empty square (LA), filled triangle down (AA), empty triangle up (SA) and filled cross (ethanol).

3.2. Co-fermentation of glucose and arabinose by E. ludwigii in shake flask

Pentose-rich biomass hydrolysate often contains small amount of glucose, therefore, to mimic the hydrolysate concentrations, co-fermentation experiments with different levels of arabinose and 5.0 g/L glucose were performed. After BDO production from pure arabinose, the ultimate goal was to use arabinose rich SBP hydrolysate to manufacture BDO which is always accompanied with small amount of glucose. Fig. 3 shows the co-fermentation profiles

for arabinose, OD_{600}, BDO and pH, while the byproducts profile is presented in Supplementary Fig. 1. The amount of glucose supplemented was completely utilised within 2–4 h. Since the concentration of glucose was lower, its presence did not suppress the utilization of arabinose which was fast from the beginning. The arabinose concentration of 10 g/L arabinose was assimilated within 10 h while the initial level of 20–50 g/L was exhausted within 24 h. At 60 g/L, ~6% of arabinose was left unconsumed even after 32 h indicating an early sign of substrate inhibition. The uptake of arabinose was in line with cell growth and BDO accumulation. The cell growth was enhanced with an increase in arabinose concentration up to 30 g/L (OD_{600}: 8–18), further increases in the substrate concentration had no significant impact on cell growth. Unlike the biomass, BDO production continuously improved with an increase in arabinose levels and highest BDO titer of 23.8 g/L with a conversion yield of 0.47 g/g was achieved at 50 g/L arabinose, whereas a decline in cell growth (12.7) and BDO (22.3 g/L) production was noticed at 60 g/L arabinose. The presence of glucose caused significant increment in cell growth and substantial improvement in BDO levels. In comparison to fermentation with only arabinose, higher cell growth and BDO titer were obtained during co-fermentations indicating beneficial effect from the presence of a small amount of glucose. The pH was manually controlled and maintained between 6 and 7. The byproducts profile was similar to pure arabinose with acetoin as the major component and the concentration of all the other metabolites were quantified to be less than 5.0 g/L (Supplementary Fig. 1).

3.3. Pretreatment and saccharification of SBP pellets

SBP, a byproduct of the sugar industries in Europe and USA, contains significant amount of arabinose unlike majority of lignocellulosic feedstocks. Conventional pretreatment techniques like dilute acid, liquid hot water, hydrothermal and steam explosion cause solubilization of the hemicellulose fraction and partial lignin removal. However, these processes, along with fermentable sugars, release products considered as fermentation inhibitors such as AA, phenols, furfurals, hydroxymethyl furfural (HMF) etc [4]. For the extraction of arabinose, SBP pellets were subjected to DAP. Initial experiments were carried out at 10% (w/w) solid loading with different acid loadings (1, 2, 3, 4 and 5% v/v H_2SO_4). The arabinose released at 1 and 2% acid loading were 16.9 and 18.8 g/L, respectively, with a little amount of glucose (3.0–4.5 g/L) and AA (5.0–6.0 g/L) and no furfural (Table 1).

At higher acid loadings, the amount of arabinose (17–19 g/L) and glucose (4.0–5.0 g/L) extracted was almost similar but with a higher amount of AA (6.0–7.0 g/L) and a substantial level of HMF (0.5–2.0 g/L). After optimizing the acid loading, the next set of experiments were conducted at various solid loadings (5, 10, 15, 20, 25 and 30% w/v) using 2% acid (Table 2). There was a continuous improvement in amount of arabinose released, however, the sugar yield decreased with an increase in solid loading. The arabinose extracted at solid loading of 5, 10, 15, 20, 25 and 30% were 8.2, 17.9, 25.0, 31.6, 36.7 and 42.9 g/L with yields of 0.16, 0.18, 0.17, 0.16, 0.15 and 0.14 g arabinose/g SBP, respectively. The glucose and AA also increased with solid loadings while small amount HMF (0.30–0.60 g/L) was noticed at higher levels of SBP. For example, glucose, AA, and HMF obtained at 30% solid loading were 12.6, 14.4 and 0.53 g/L, respectively. The presence of glucose is desirable for BDO production but AA being an organic acid exerts a toxic effect on the fermentation even at a concentration as low as 10 mM. Thus, an acid concentration of 2.0% v/v and solid loading of 10% w/w were identified to be the optimal concentrations.

3.4. BDO production from SBP hydrolysate

After culturing *E. ludwigii* on pure carbon sources, arabinose rich hydrolysate obtained from SBP via DAP was employed for BDO production. With the optimal acid loading of 2% v/v H_2SO_4 and 10% w/v solid loading and pretreatment conditions of 121 °C, 15 min, the DAP of SBP resulted in 69.9% arabinan, 13.7% glucan, and 0.24% of acid soluble lignin removal. The bacterium was cultivated at

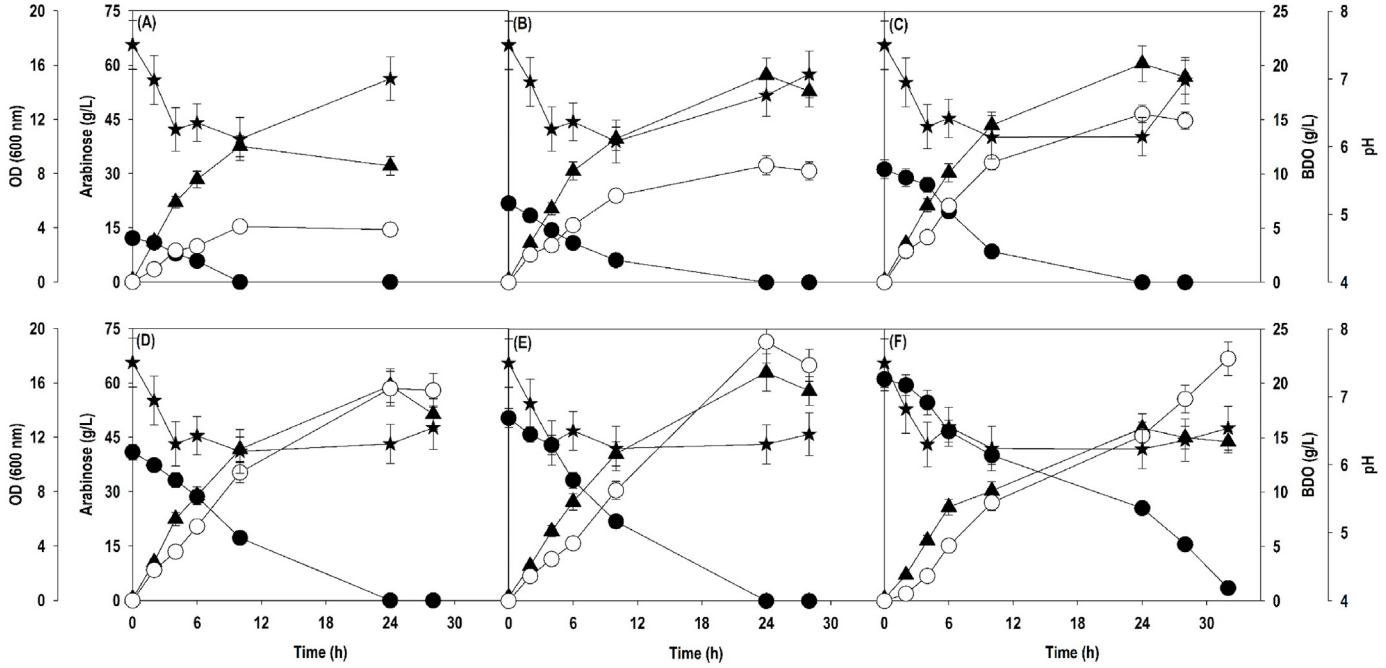

Fig. 3. Arabinose and glucose (5 g/L) co-fermentation in shake flask culture at various arabinose concentrations: (A) 10 g/L; (B) 20 g/L; (C) 30 g/L; (D) 40 g/L; (E) 50 g/L; (F) 60 g/L. Symbols: filled circle (arabinose), filled triangle (OD_{600}), empty circle (BDO) and filled star (pH).

Table 1
Optimization of acid loading for release of arabinose from sugar beet pulp pellets with 10% (w/v) solid loading.

Acid loading (%w/v)	Arabinose (g/L)	Glucose (g/L)	AA (g/L)	Furfural (g/L)	HMF (g/L)
1.0	16.86	3.25	5.35	ND	0
2.0	18.81	4.2	5.72	ND	0
3.0	17.31	4.01	6.41	ND	0.53
4.0	18.17	4.7	6.76	ND	1.44
5.0	17.8	4.6	6.96	ND	1.94

*ND: Not detected.

Table 2
Effect of solid loading on release of arabinose from sugar beet pulp pellets through acid hydrolysis (2.0% v/v H_2SO_4).

Solid loading (%w/v)	Arabinose (g/L)	Glucose (g/L)	AA (g/L)	Furfural (g/L)	HMF (g/L)
5	8.24	1.48	3.26	ND	ND
10	17.86	3.19	6.15	ND	ND
15	24.99	6.12	8.87	ND	ND
20	31.56	9.19	10.03	ND	0.32
25	36.66	9.32	11.98	ND	0.46
30	42.85	12.57	14.41	ND	0.53

*ND: Not detected.

different concentrations of crude arabinose (10–60 g/L) from SBP. The outcome was comparable to the results obtained with pure arabinose in terms of substrate uptake, BDO and byproducts synthesis. The BDO titer obtained with a substrate range of 10–45 g/L were 3.4, 9.7, 12.5 and 15.1 g/L, respectively. The best results were obtained at 50 g/L where large amounts of arabinose (>85%) was metabolized within 24 h. The maximum titer of BDO achieved was 21.2 g/L with a yield of 0.41 g/g at 28 h (Fig. 4). The byproducts obtained were as follows: acetoin (5.2 g/L), LA (3.7 g/L), AA (7.0 g/L), SA (4.0 g/L) and ethanol (1.2 g/L) (Supplementary Fig. 2). The smooth production of BDO from arabinose rich SBP hydrolysate indicates the possibilities of SBP as a feedstock for BDO production. The inhibitions were visible at 60 g/L where substrate uptake was slow in the beginning and ~15 g/L arabinose was consumed in 10 h as the culture took some time to adapt. Thereafter, some increment in the substrate consumption rate was observed and after 32 h of fermentation, where a residual arabinose concentration of 25.1 g/L was observed. The same was also reflected in product formation, only 6.0 g/L BDO was produced till 10 h and total a 17.7 g/L was accumulated by the end of fermentation. This retardation and low performance may be due to combined inhibition caused from the high level of substrate, AA and other phenolic and inhibitory compounds generated during pretreatment.

3.5. Batch cultivation of *E. ludwigii* on pure and crude arabinose in bioreactor

The batch culture in the bioreactor was performed with 50 g/L arabinose as this was determined to be the optimal substrate concentration during shake flask cultivations. The culture in the bioreactor was sparged with air at an aeration rate of 1.0 vvm and the pH was controlled at 7.0. *E. ludwigii* was cultivated on the pure arabinose and arabinose-rich hydrolysate from SBP. The arabinose assimilation was fast as compared to flask culture and about 35–70% of substrate carbon was metabolized within 6–12 h. The cell growth was immediate without any lag phase after inoculation and higher OD_{600} was achieved with pure arabinose in comparison to SBP hydrolysate. The final OD_{600} values attained with pure arabinose and hydrolysate were 26.1 and 13.1, respectively. The BDO production commenced at 2 h, then increased continuously, and reached 23.2 g/L for pure arabinose, while the BDO accumulation using arabinose-rich hydrolysate was 22.6 g/L at 24 h. The conversion yield with pure arabinose and arabinose-rich hydrolysate were 0.45 and 0.43 g/g, respectively. The amounts of byproducts obtained were lower in comparison to the shake flask cultivation on pure arabinose and are as follows: pure arabinose - acetoin (2.7 g/L), LA (4.1 g/L), AA (5.5 g/L), SA (3.5 g/L), ethanol (3.5 g/L); arabinose-rich hydrolysate - acetoin (2.9 g/L), LA (0 g/L), AA (8.8 g/L), SA (3.4 g/L), ethanol (2.5 g/L) (Fig. 5). The carbon balance shows that 57–60% of carbon supplemented was diverted towards BDO formation. The controlled conditions in the bioreactor channelled more carbon flux towards BDO leading to higher BDO and less byproduct formation, especially acetoin.

3.6. Fed-batch culture of *E. ludwigii* in bioreactor and recovery of BDO

After the batch cultivation, a fed-batch culture of *E. ludwigii* was carried out in a bioreactor using pure arabinose and arabinose-rich hydrolysate as carbon sources. The fermentation with pure arabinose was started with a substrate concentration of 60.1 g/L, which was actively consumed and 90% of the supplied sugar was metabolized in 28 h. The first feed was supplemented at 28 h and in the next 24 h arabinose concentration fell from 56.5 to 18.4 g/L, after which a second and final feed was added, and supplemented arabinose was completely utilized by 72 h. An active exponential cell growth was recorded in the first 24 h without any lag phase leading to a maximum OD_{600} of 28.6 which declined thereafter. BDO synthesis was concomitant with consumption of arabinose and a continuous and steady increase in production was observed with a total accumulation of 42.9 g/L with a conversion yield of 0.31 g/g. The arabinose uptake in case of SBP hydrolysate was similar to pure arabinose and an initial concentration of 54.2 was largely consumed in 24 h. The bacterial culture was supplemented with 40 g/L pure arabinose at 24 and 48 h. In the final phase of fermentation (48–72 h), arabinose assimilation slowed down and as a result 13.6 g/L residual arabinose was obtained at 72 h, which may be due to the accumulation of microbial inhibitors. The same was also reflected on BDO production which was fast and similar to pure arabinose in the initial 48 h, but a small increment of only ~6 g/L was noticed in the last 24 h with a final titer of 35.5 g/L and a yield of 0.29 g/g arabinose (Fig. 6). The cell growth was also affected and lower OD_{600} (20.6) was achieved in comparison to pure arabinose. The byproduct profile was similar to that from the batch

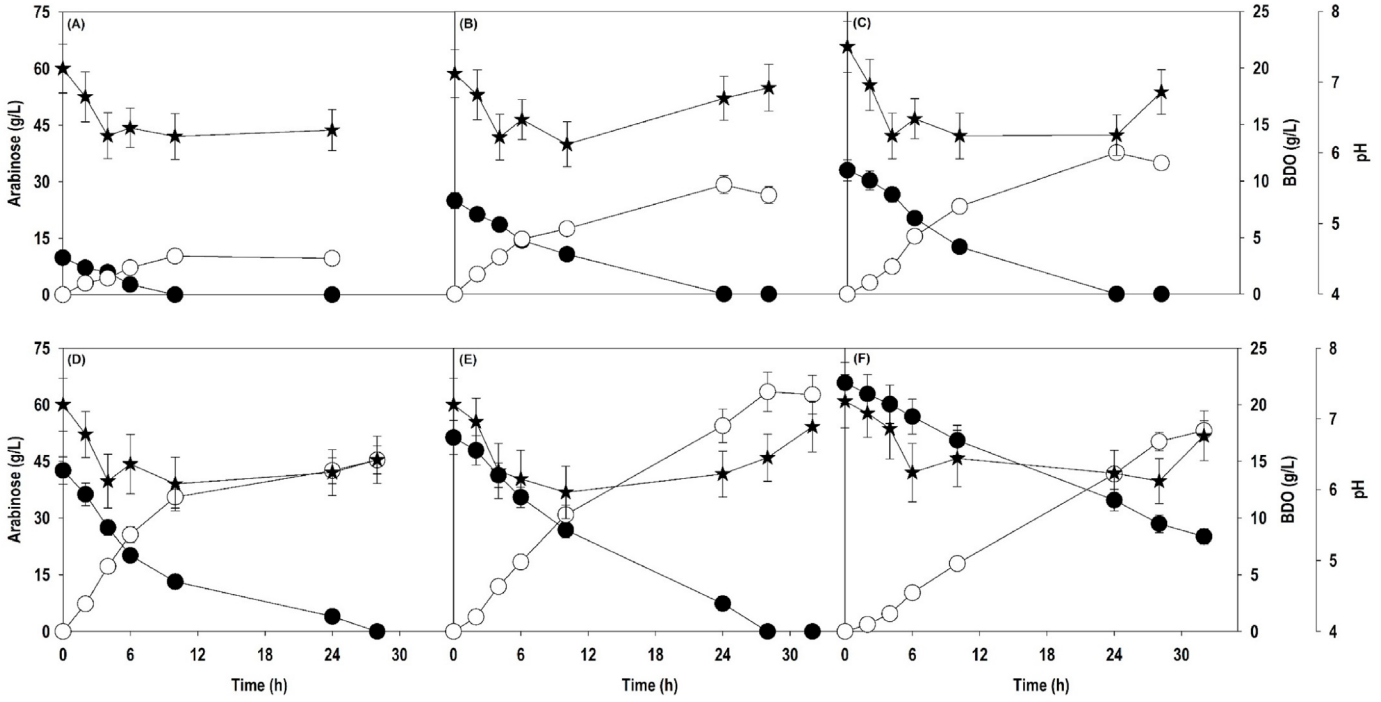

Fig. 4. BDO fermentation in batch cultures by *E. ludwigii* using SBP-derived arabinose rich hydrolysate at different levels of arabinose (A) 10 g/L; (B) 20 g/L; (C) 30 g/L; (D) 40 g/L; (E) 50 g/L; (F) 60 g/L. Symbols: filled circle (arabinose), empty circle (BDO) and filled star (pH).

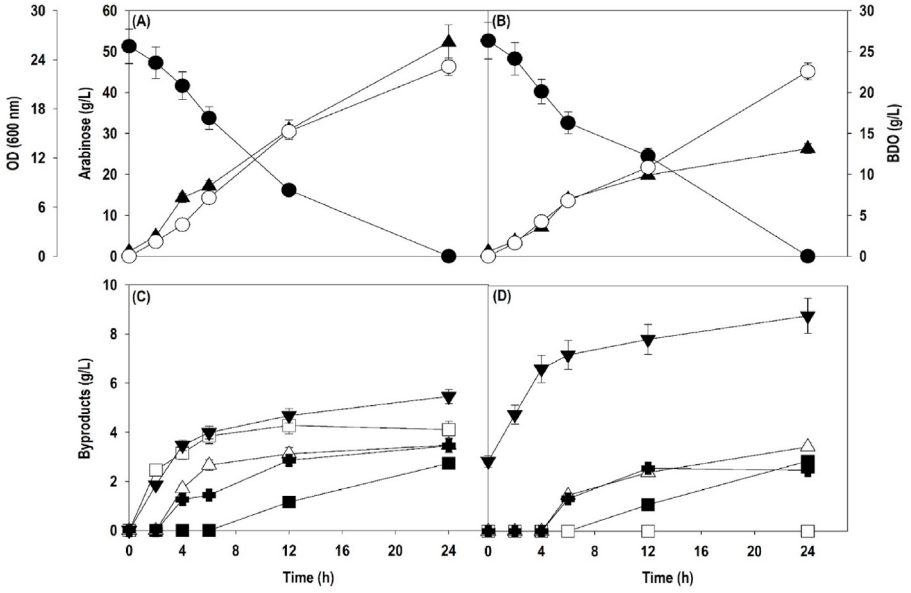

Fig. 5. Kinetics of arabinose consumption, OD_{600}, BDO and byproducts formation by *E. ludwigii* during batch cultivation in bioreactor using pure arabinose and crude arabinose: (A) & (B) residual arabinose, OD_{600} and BDO; (C) & (D) byproducts (acetoin, ethanol, acetic, lactic and succinic acid). Symbols: filled circle (arabinose), empty circle (BDO), filled triangle up (OD_{600}), filled square (acetoin), empty square (LA), filled triangle down (AA), empty triangle up (SA) and filled cross (ethanol).

cultivation in the shake flask and bioreactor where acetoin, SA and AA were obtained as major byproducts. Table 3 shows the carbon balance for BDO production from pure arabinose and arabinose-rich hydrolysate. Similar results were obtained in both the cases and analysis of the carbon material balance revealed that the carbon recovery was more than 100%. The extra carbon of 5–7% might have originated from the yeast extract which was not considered for carbon analysis. Approximately, 50% of arabinose carbon was directed through the metabolic pathway for BDO and acetoin production and 4.5% towards cell mass synthesis. The carbon loss in the form of CO_2 was ~30%, largely generated during biosynthesis of BDO and acetoin, and 22–24% was lost in the form of byproducts including AA, SA, LA, and ethanol. The BDO that was accumulated on pure arabinose and arabinose-rich hydrolysate was recovered via aqueous two-phase extraction system using isopropanol and $(NH_4)_2SO_4$ as solvent and electrolyte [17]. BDO along with acetoin,

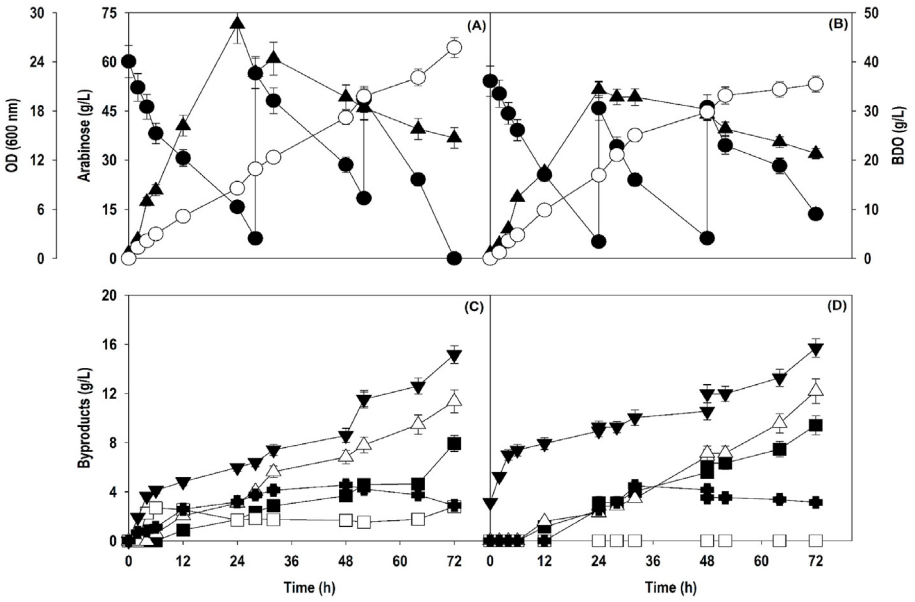

Fig. 6. Variation in arabinose consumption, OD_{600}, BDO and byproducts (acetoin, ethanol, acetic, lactic, and succinic acid) production by *E. ludwigii* during fed-batch cultivation of *E. ludwigii* in bioreactor on: (A) & (C) pure arabinose; (B) & (D) SPB-derived arabinose rich hydrolysate. Symbols: filled circle (arabinose), empty circle (BDO), filled triangle up (OD_{600}), filled square (acetoin), empty square (LA), filled triangle down (AA), empty triangle up (SA) and filled cross (ethanol).

Table 3
Carbon balance for BDO production using pure arabinose and arabinose-rich hydrolysate from SBP by E. ludwigii. during fed-batch culture in bioreactor[a,b].

	Pure arabinose			Arabinose-rich hydrolysate		
Substrate and Metabolites	mM	C (mM)	C (%)	mM	C (mM)	C (%)
Arabinose ($C_5H_{10}O_5$)	933.3	4666.7	100.0	803.9	4019.7	100.0
Cell dry weight ($CH_{1.8}O_{0.5}N_{0.2}$)	209.1	209.1	4.5	182.1	182.1	4.5
BDO ($C_4H_{10}O_2$)	476.9	1907.7	40.9	393.9	1575.5	39.2
Acetoin ($C_4H_8O_2$)	90.2	360.9	7.7	106.9	427.7	10.6
LA ($C_3H_6O_3$)	31.0	93.0	2.0	0.0	0.0	0.0
AA ($C_2H_4O_2$)	252.8	505.7	10.8	209.5	419.0	10.4
SA ($C_4H_6O_4$)	96.3	385.1	8.3	103.2	412.9	10.3
Ethanol (C_2H_5O)	62.8	125.7	2.7	67.8	135.7	3.4
Carbon dioxide (CO_2)[c]	1353.7	1353.7	29.0	1175.7	1175.7	29.2
Total Products		4940.9			4328.5	
Carbon recovery %			105.9			107.7

[a] The calculation for carbon balance was made without taking into account the carbon coming from yeast extract.
[b] The data correspond to the 72 h sample for the culture shown in Fig. 6.
[c] The CO_2 (mM) was calculated according to following formula: 2*(BDO + Acetoin) + AA + Ethanol − SA. The calculation did not consider CO_2 in the liquid and head space.

AA and SA was extracted from aqueous to organic phase with a partition coefficient of 44.6, 1.5, 0.58, 0.63 for the fermented broth on pure arabinose and 38.5, 1.6, 0.26, 0.29 for the arabinose-rich hydrolysate. The recovery yield of BDO on pure and SBP arabinose were 97.8 and 97.5, respectively (Table 4).

4. Discussion

Waste can be an attractive renewable source if utilized intelligently. Due to the intense global pressure towards green environmental technology, scientists are now investing more efforts to innovate alternative uses that reduce the amount of waste materials rich in renewable carbon. The use of agro-industrial waste and side streams for fermentative production of chemicals would maximize the profit of integrated biorefineries and enhance the commercial viability of industries generating such residues. SBP, a high volume low-cost major by-product from the sugar industries which is readily available and generated in huge quantities. Sugar beet crop provides ~20% of the sugar demand of the world. The EU and USA are the global leaders in sugar production using sugar beet and every year Europe alone produces about 20 million tonnes SBP [10]. Currently, SBP is dried and pelletized, an energy intensive process and sold as animal feed and therefore, does not harness its full potential. The valorisation of SBP into chemical building blocks will incur multiple benefits; enhancing the profitability of the sugar industries, and reducing the energy costs and greenhouse gas emissions arising from the drying process [9,19]. SBP is rich in cellulose and non-cellulosic polysaccharides such as arabinans. The cellulosic fraction can be easily hydrolysed via enzymatic routes yielding a glucose-rich solution which is devoid of inhibitors. Glucose being the primary choice for the majority of microorganisms, can be easily valorised into high value products. The main challenge lies in the conversion of non-cellulosic sugars such as xylose, arabinose via a biochemical route as a large number of cell factories cannot metabolize these sugars. In the last two decades, significant work has been done in finding diversified native and developing non-native microorganisms which can utilise xylose as a feedstock for bioproduction, but literature is quite scarce in the

Table 4
Separation of BDO from fermented broth accumulated on pure arabinose and arabinose-rich SBP hydrolysate using aqueous two-phase extraction system.

Metabolites	Pure arabinose		Arabinose-rich SBP hydrolysate	
	Partition coefficient	Recovery yield (%)	Partition coefficient	Recovery yield (%)
BDO	44.61	97.81	38.52	97.47
Acetoin	1.51	60.24	1.58	61.28
LA	0.0	0.0	0.0	0.0
AA	0.58	36.70	0.26	20.80
SA	0.63	38.80	0.29	22.69
Ethanol	0.0	0.0	0.0	0.0

case of arabinose [11,20]. Biological production of bulk chemicals has garnered increasing attention in recent years and one example is the microbial production of BDO [12]. To date little information is available on the biosynthesis of BDO from arabinose and in the current work, we have investigated BDO production using pure arabinose and crude arabinose from SBP by *E. ludwigii*. The bacterium is not only versatile in metabolizing a wide range of substrates to BDO, but has also successfully attained a high BDO level (>100 g/L) using pure and crude carbon sources [13,21]. The metabolic pathway for production of BDO from arabinose is shown in Fig. 7. The stochiometric reaction for bioconversion of arabinose to BDO is as follows: 6 Arabinose +10 NAD^+ + 4 ADP +4 Pi → 5 BDO +10 CO_2+10 NADH +4 ATP. The equation indicates that a theoretical yield of BDO on arabinose would be 0.50 g/g.

The work was started with BDO production by *E. ludwigii* from pure arabinose at different levels (Fig. 1). Among the different concentration used, 50 g/L was identified as the optimal level in terms of cell growth, BDO titer and yield. To eliminate the impact of pH drop on cell metabolism and BDO formation during fermentation, it was manually controlled and kept in the range of 6.0–7.0. However, at 60 g/L a marginal drop in OD_{600}, BDO titer and yield was noticed along with the presence of a little amount of residual sugar. Similar results were obtained during co-fermentation experiments where best results were achieved with 50 g/L arabinose +5 g/L Glucose (Fig. 3). Saha and Bothast (1999) screened, isolated, and identified an arabinose metabolizing and BDO accumulating *Enterobacter cloacae* strain [22]. The strain was cultured at different concentrations of arabinose (2–10% w/v). The BDO concentration was found to be enhanced with an increase in arabinose level. The BDO titers of 7.0, 11.5, 15.2, 17.8 and 34.4 g/L were achieved with arabinose concentrations of 20, 30, 40, 50 and 100 g/L, respectively. Although the yield was almost unaffected (0.34–0.38 g/g), the fermentation was slow at 100 g/L and took longer time (72 h) to reach the maximum titer. The titers and yields

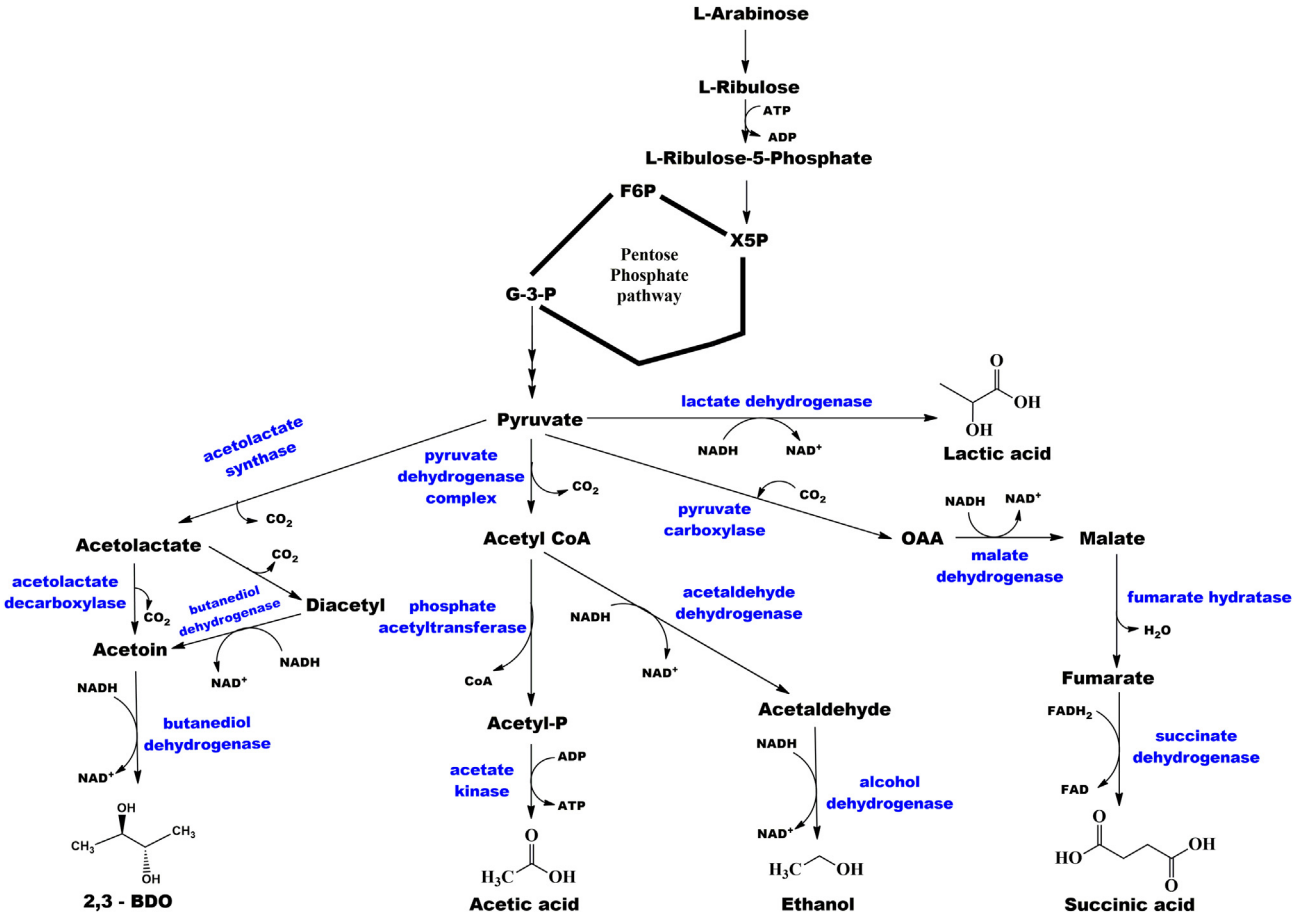

Fig. 7. Metabolic pathway for BDO and byproduct formation from arabinose.

obtained show congruence to the current work with 50 g/L arabinose as an optimal level for BDO production. In another report, Liakou et al. (2018) cultivated five different bacterial strains on a variety of sugars including arabinose in a shake flask. The BDO accumulated on arabinose by *E. ludwigii* FMCC 204, *Enterobacter aerogenes* FMCC 9, *E. aerogenes* FMCC 10, *Enterobacter* sp. FMCC 208 and *Citrobacter freundii* FMCC 207 were 6.9, 7.3, 7.4, 6.9 and 7.6 g/L with yields of 0.31, 0.33, 0.34, 0.38 and 0.36 g/g, respectively [23]. These results are similar to the shake flask data in the current work where BDO titer of 7.5 and 9.7 g/L were achieved for arabinose concentrations of 22.5 and 24.8 g/L, respectively.

The authors did not find any full length or detailed study on microbial production of BDO from SBP. The presence of a large amount of arabinose makes SBP different from other lignocellulosic feedstocks where arabinose is replaced by xylose. Furthermore, being a low lignin biomass, the deconstruction of SBP can be achieved by a mild fractionation method which minimizes the formation of toxic inhibitory products arising from the degradation of sugars such as furfural and HMF [9]. In the current study, we employed a dilute acid (H_2SO_4) pretreatment method to recover arabinose in the hydrolysate. The acid and solid loading was optimized to maximize the arabinose recovery while minimizing/eliminating the sugar loss and formation of fermentation inhibitors (Tables 1 and 2). The hydrolysates obtained with 1–2% acid loading and 5–15% solid loading were free from furan derivates furfural and HMF. The maximum arabinose yield (0.18 g/g) was recorded with 2% acid loading and 10% solid loading. The results were comparable to the work of Alexandri et al. (2019) who pretreated SBP via autohydrolysis and dilute acid pretreatment (H_2SO_4 and HCl) and found hydrolysis with H_2SO_4 at 0.5% as the optimum [19]. Further, they optimized the solid loading (6, 7.5, 10 and 15%) and the duration of pretreatment (15 and 30 min) and in all the cases arabinose was obtained as the major sugar in the range of 10.6–23.2 g/L. The arabinose concentrations at 10 and 15% solid loadings with 15-min autoclaving were 18.2 and 23.2 g/L [19] which were close to the values achieved in the present study i.e. 17.9 and 25.0 g/L at 10% and 15% solid loadings, respectively, at 2% v/v acid loading. Similar to fermentation and co-fermentation with pure sugars, *E. ludwigii* was grown on SBP hydrolysate containing various arabinose levels (10–60 g/L). In the case of SBP hydrolysates, the behaviour was the same and the highest BDO titer (21.1 g/L) was achieved at 50 g/L arabinose, but the degree of inhibition was significant at 60 g/L arabinose leading to a slow metabolism and low product formation (Fig. 4). This could be attributed to presence of AA and other unknown inhibitors in the SBP hydrolysate at significant levels.

After the shake flask cultivation, BDO fermentation was carried out in bioreactor to further improve the production parameters. The titers obtained with pure arabinose and SBP hydrolysate were similar to that of shake flask experiments except with higher yields and productivities (Fig. 5). In the fed-batch culture, titers were improved, however, yields were lower (pure arabinose: titer – 42.9 g/L, yield – 0.31 g/g; SBP arabinose-rich hydrolysate: titer – 35.5 g/L, yield – 0.29 g/g) (Fig. 6). One of the reasons for the low production, especially in the later phase could be due to presence of AA in substantial levels (15–20 g/L). AA was one of the main byproducts accumulated during BDO biosynthesis and it was also present in the SBP arabinose-rich hydrolysate produced by hydrolysis of acetyl groups in hemicellulosic components of SBP. AA is a toxic organic acid, and its toxicity starts at concentrations as low as 10 mM. The toxicity of AA is two-fold, pH-based growth inhibition and a negative impact of the acetate ion on metabolism [24,25]. Similarly, LA, SA and ethanol were the major by-products observed during the biosynthesis of BDO regardless of the substrate either hexoses or pentoses. Besides their toxicity, metabolic pathways leading to byproducts competes with BDO formation for the reducing equivalents. Accepting assumptions and elucidations on effect of these by-products on BDO accumulation, our total carbon flux shows that 50% substrate was channelled towards the desired product pathway with ~40 and 10% recoveries for BDO and acetoin, respectively, and the rest of the carbon towards CO_2, SA, LA, AA, and ethanol, respectively. Carbon dioxide is the byproduct during BDO/acetoin formation and one-third of carbon is lost in the form of CO_2 which is inevitable and cannot be avoided. During the fed-batch culture, about 30% CO_2 was lost which can utilized for many purposes. For example, CO_2 is a substrate for many reactions leading to high value products e.g., biosynthesis of SA. It can also be pumped into glasshouses to support horticulture which would otherwise make use of fuels to generate CO_2 [9]. The yield can be significantly improved if carbon lost in the form of byproducts (30–35%) can be diverted towards BDO formation. Various reports are available in literature where byproduct formation has been abolished via gene deletions to improve the final BDO titers. For example, Thapa and associates constructed a mutant of *Enterobacter aerogenes* deleting the d-lactate dehydrogenase (ldhA), phosphate acetyltransferase (pta), malate dehydrogenase (mdh), and acetaldehyde dehydrogenase (acdh) genes responsible for LA, AA, SA and ethanol production, resulting in 8-fold increase in the BDO accumulation [26]. Hence further investigation on genetic engineering strategies to be evaluated to increase BDO titers. Since there are only limited reports on pure arabinose, xylose-based BDO production is used for our current discussion [14] as like arabinose, xylose is a pentose sugar and has been employed for biomanufacturing of BDO. In our previous work, we made use of pure and crude xylose (detoxified and non-detoxified hemicellulosic hydrolysate from sugarcane bagasse) for BDO production by *E. ludwigii* [14]. The BDO titers and yields obtained from pure xylose, detoxified and non-detoxified hydrolysate were 71.1, 63.5 and 32.7 g/L with yields of 0.40, 0.36 and 0.33 g/g, respectively. It was evident there was a large difference between BDO titers and yields accumulated on pure xylose and non-detoxified hydrolysate. Contrary to this, the difference is much smaller in the current work and could be due to the absence/low amounts of toxic inhibitors. However, the difference is still quite significant in the cases of pure arabinose and xylose and the reasons for this are unclear. More studies are required to further understandings and optimize process parameters to improve production.

5. Conclusion

The current study describes the promising application of arabinose from SBP, an inexpensive substrate, for BDO production by *E. ludwigii* within an integrated biorefinery concept. The work has demonstrated the technical feasibility of integrated fermentative production and downstream processing of BDO on arabinose. In a fed-batch mode of cultivation, final BDO titers of 42.9 and 35.5 g/L from pure arabinose and SBP hydrolysate with conversion yields of 0.31 and 0.29 g/g, respectively, were achieved. Finally, BDO accumulated on pure arabinose and SBP hydrolysate were recovered using an aqueous two-phase extraction system with a recovery yield of ~97%. *E. ludwigii* exhibits immense potential to serve as a biocatalyst for industrial BDO production as the bacterium can efficiently ferment all the major sugars in a biowaste stream including, glucose, xylose, and arabinose to BDO with a high yield. The valorisation of arabinose along with cellulosic glucose will enhance the sustainability and economic profitability of SBP-based biorefineries. The exploitation of SBP could be extended for microbial production of other chemical building blocks. Further work in process optimization and pathway engineering is needed to achieve higher titers, yields and therefore productivity for the industrial manufacture of BDO.

Availability of data and materials

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

CRediT authorship contribution statement

Narisetty Vivek: Conceptualization, Methodology. **Sudheera Narisetty:** Writing – review & editing. **Samuel Jacob:** Conceptualization, Writing – review & editing. **Deepak Kumar:** Conceptualization, Writing – review & editing. **Gary A. Leeke:** Conceptualization, Writing – review & editing. **Anuj Kumar Chandel:** Writing – review & editing. **Vijai Singh:** Writing – review & editing. **Vimal Chandra Srivastava:** Writing – review & editing. **Vinod Kumar:** Supervision, Conceptualization, Writing – original draft, Data curation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2022.04.024.

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