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High yield recovery of 2,3-butanediol from fermented broth accumulated on xylose rich sugarcane bagasse hydrolysate using aqueous two-phase extraction system

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Abstract

Downstream processing of chemicals obtained from fermentative route is challenging and cost-determining factor of any bioprocess. 2,3-Butanediol (BDO) is a promising chemical building block with myriad applications in the polymer, food, pharmaceuticals, and fuel sector. The current study focuses on the recovery and purification of BDO produced (68.2 g/L) from detoxified xylose-rich sugarcane bagasse hydrolysate by a mutant strain of *Enterobacter ludwigii*. Studies involving screening and optimization of aqueous-two phase system (ATPS) revealed that 30% w/v $(NH_4)_2SO_4$ addition to clarified fermentation broth facilitated BDO extraction in isopropanol (0.5 v/v), with maximum recovery and partition coefficient being 97.9 ± 4.6% and 45.5 ± 3.5, respectively. The optimized protocol was repeated with unfiltered broth containing 68.2 g/L BDO, cell biomass, and unspent protein, which led to the partitioning of 66.7 g/L BDO, 2.0 g/L xylose and 9.0 g/L acetic acid into organic phase with similar BDO recovery (97%) and partition coefficient (45).

Keywords: Xylose; *Enterobacter ludwigii*; 2,3-Butanediol; Aqueous two-phase system; Ammonium sulphate, Isopropanol

Highlights

- BDO accumulated on xylose-rich sugarcane bagasse hydrolysate was purified.
- Maximum extraction was recorded with 30% w/v $(NH_4)_2SO_4$ and 50% v/v isopropanol.
- BDO partition coefficient of 45.5 and recovery yield of 97.9% was achieved.
- Similar BDO recovery results were obtained with unfiltered fermented broth.Purified BDO was characterized through ^{13}C NMR and ^{1}H NMR spectroscopy.

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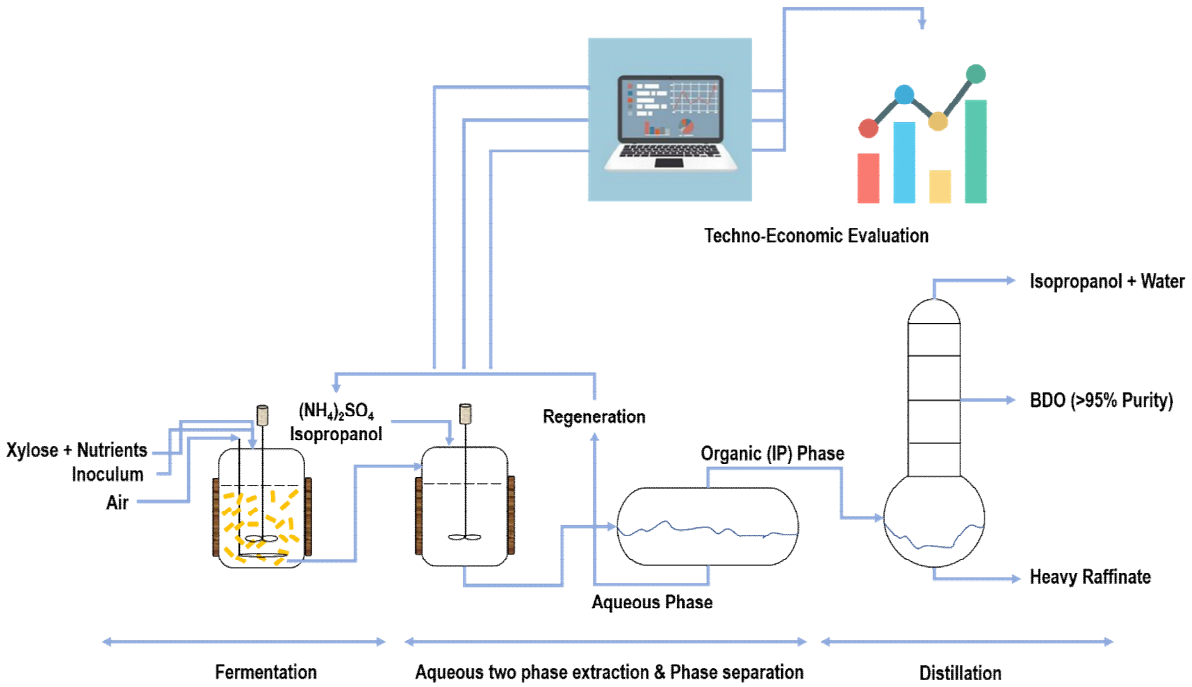
Graphical abstract



1. Introduction

Currently, energy demands, and supply of commodity chemicals is majorly fulfilled through the petrochemical route. However, the non-sustainability of fossil fuels and their adverse environmental impact has triggered the use of renewable feedstocks to produce an array of fuels, chemicals and energy. 2, 3-butanediol (BDO), is one such key chemical building block with two chiral carbons that finds vast applications in various industrial sectors like food, cosmetics, pharmaceuticals, and transportation (Vivek et al., 2021a; Wang et al., 2020). Due to its high-octane rating, BDO functions as an octane booster for gasoline and has comparable heat of combustion (27.2 kJ/g) to other biofuels such as methanol (22.1 kJ/g), ethanol (29.1 kJ/g), and n-butanol (33.1 kJ/g) (Qin et al., 2018; Lee et al., 2021; Yang and Zhang, 2019). BDO can be accumulated in large amounts (>100 g/L) from biological route whereas bioproduction of n-butanol, a C4 alcohol like BDO, suffers from low titers, yield and productivity and end-product toxicity beyond (2% v/v). BDO derivatives like 2-ethyl-2,4,5-trimethyl,1,3-dioxolanes (TMED) also exhibits promising biofuel properties with higher heat of combustion (28.3 MJ/L) and low miscibility (8 g/L) in water (Zhang et al., 2020; Zhang et al., 2021). Further BDO can be transformed into industrially viable chemicals like methyl ethyl ketone (MEK), 1,3-butadiene, and acetone-2,3-BDO-Ketal (an octane booster). The global market potential for BDO derivatives itself is ~32 million tonnes per annum, valued at $43 billion (Hazeena et al., 2020; Maina et al., 2019).

Growing awareness for environmental sustainability, the concept of biorefinery, abundance of renewable feedstocks and advantages associated with biological production of BDO such as milder operating conditions and involvement of stereo-specific enzymes in microbial systems offer lucrative technical edge compared to petrochemical route (Höfer, 2015; Song et al., 2019). In this context, various native and non-native BDO producers have been reported that can accumulate up to 150 g/L BDO using renewable edible and non-edible feedstocks (Cho et al., 2015a, 2015b). However, the high cost of feedstock and an expensive downstream processing which contribute to more than 50% of the total manufacturing cost, impedes commercial viability of fermentative BDO production. On the other hand, due to affordable upstream processes, petrochemical route dominates the current global BDO supply.

Generally, the fermented broth consists of 10 – 15% (w/v) of BDO, microbial cells, by-products (organic acids, and ethanol), protein, and excess water (80 – 90 % w/v) (Haider et al., 2018a; Li et al., 2012). Achieving a high purity BDO (≥99%) from an aqueous fermented broth containing 10-15% (w/v) BDO requires removal of large amounts of water with high heat of vaporization (Haider et al., 2018b). Although conventional distillation is well developed process for separation and purification of chemicals, it is energy intensive when implemented in for chemicals having high boiling point like BDO (182 ^{0}C).

The present investigation was undertaken to design an efficient and co-effective process for downstream processing of BDO. In the current study, we focussed on separation, purification, and characterization of BDO from the fermented broth by adapting aqueous two-phase extraction system (ATPS). The broth for this purpose was obtained by accumulating BDO on detoxified xylose-rich sugarcane bagasse hydrolysate using a mutant strain of *Enterobacter ludwigii*. Preliminary studies involved rigorous screening of different organic solvents and inorganic salts with high phase distribution and selectivity of BDO as the shortlisting criteria. Later, various parameters were optimized with the best salt and solvent combination. Further techno-economic analysis was carried out to evaluate the feasibility of aqueous two-phase extraction-assisted distillation scheme for commercialization.

2. Materials and Methods

2.1. Chemicals

The fine chemicals 2,3-butanediol, acetoin, and acetic acid as HPLC standards were purchased from Sigma Aldrich. In the fermentation studies, xylose rich bagasse hydrolysate was used as the feedstock, which was kindly provided by our industrial partner Nova Pangaea Technologies, (https://www.novapangaea.com), Redcar, UK. All the inorganic salts, and organic solvents were purchased from Fisher Scientific and Merck Millipore.

2.2. Microorganism and fermentation

The mutant *E. ludwigii* strain (Amraoui et al. 2021) was used for BDO production from xylose-rich sugarcane bagasse hydrolysate as the sole carbon source. The culture maintenance and production media composition was followed according to Maina et al., 2019. The fermentation was carried out in a fed-batch mode with initial xylose concentration of 40 g/L, with intermittent feeding using a concentrated solution of xylose (400 g/L) to maintain the residual xylose concentration above 20 g/L.

2.3. BDO separation from fermented broth using aqueous-two phase system (ATPS)

Prior to salting out, the fermented broth was clarified to remove the microbial cells via centrifugation at 8000 rpm for 10 mins, and the cell-free broth was stored at 4 ^{0}C until further use. A known concentration of inorganic salt was added to 10 ml of the cell-free broth, and vortexed until dissolved, followed by the addition of a 1:1 v/v ratio of organic solvent to the salt-saturated fermented broth. The mixture was vortexed for 3 mins, and the solution was left undisturbed at the room temperature for 2h to allow phase separation. The top organic phase was separated from the aqueous phase using a separating funnel. The organic phase was subjected to vacuum distillation at 45 ^{0}C and 150 mbar pressure to remove the solvent and concentrate the product. Previous ATPS studies for diols such as K_2HPO_4/ethanol, $(NH_4)_2SO_4$/ ethanol, and $(NH_4)_2SO_4$/isopropanol systems were found to be efficient. But most of the works reported in the literature were performed using simulated or synthetic solutions. To investigate the effect of various organic solvents on BDO partitioning into the organic phase;

isopropanol (IP), ethyl acetate (ET), chloroform (CH), acetonitrile (AN), acetone (AC), and methanol (ME) were mixed individually in 1:1 v/v with fermented broth saturated with 20% w/v $(NH4)_2SO_4$. Later the effect of different salts [K_2HPO_4, $(NH_4)_2SO_4$, $NaHCO_3$, $CaCO_3$, $CaCl_2$, Na_2HPO_4, $NaHPO_4$, $(NH_4)_2CO_3$, $MgCO_3$, and NH_4Cl] on the partitioning of BDO from aqueous to organic phase was investigated. All the experiments carried out in the study were performed in triplicates and the average values are presented, the standard error observed was $p<0.05$.

2.4. Effect of ammonium sulphate (AS) and isopropanol (IP) on the partitioning of BDO

The effect of different concentrations of AS salt (15 – 40% w/v) and isopropanol ratio (0.5 – 2.0 v/v) on partitioning of BDO from aqueous to organic phase was investigated. Further, results were validated by performing the scale-up experiments using 1-litre fermented broth containing 30% w/v AS and IP was used in the ratio of 0.5:1 v/v. Finally, the efficiency of ATPS in the separation and purification of BDO from the unfiltered fermented broth was examined.

2.5. Calculation of partition coefficient (K), recovery yield (Y) and phase ratio (P)

In the aqueous two-phase extraction of BDO from the fermented broth, three parameters partition/distribution coefficient (K), recovery percentage (Y), and phase ratio (P) was considered in evaluating the optimum concentrations of inorganic electrolyte and the organic solvent. The partition coefficient of BDO is the ratio of concentration in the top organic phase to the bottom aqueous phase (Equation 1).

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

The recovery yield of BDO from the fermented broth is calculated using the following Equation 2.

$$Y = \frac{C_T}{C_{FB}} X\ 100\% \text{..... (2)}$$

Where C_T, C_B, C_{FB} are the concentration of BDO in top organic, bottom aqueous phase, and in the fermented broth, respectively. The parameter phase ratio (P) was calculated using Equation 3.

$$P = \frac{V_T}{V_B} \text{..... (3)}$$

Where V_T and V_B are the volume of top organic phase and bottom aqueous phase obtained after the phase separation respectively.

2.6. Analytical methods

The concentration of BDO, xylose and acetic acid was analyzed using high performance liquid chromatography (HPLC Agilent 1260, Refractive index detector, 300 x 7.8 mm Rezex ROA Organic acid (H^+) column, Phenomenex) with 5.0 mM H_2SO_4 as the mobile phase with 0.4 ml/min flow rate, and 60 ^{0}C column temperature as the operating conditions. The Coomassie brilliant blue method was adapted to measure the concentrations of protein using BSA as the standard. The biomass

concentration was determined by measuring optical density at 600 nm using UV-VIS spectrophotometer. The purity of BDO confirmed using HPLC was reaffirmed by subjecting it to one dimensional (1D) NMR spectroscopy using Bruker Avance III 500 MHz spectrometer (Bruker, Milan, Italy) equipped with a 5 mm BBFO probe head operating at 500.13 MHz and 125.77 MHz resonance frequency to obtain ^{1}H and ^{13}C NMR spectra, respectively. Two standard samples of BDO and acetoin prepared in D_2O were also run to validate the predominant fermentation product.

2.7. Techno-economic analysis

A double distillation conventional base case approach reported by Lee and co-workers has been employed to study the purification of BDO from the organic phase using an academic version of ASPEN Plus software (Haider et al., 2018b). The thermodynamic properties such as vapor-liquid equilibrium (VLE) and liquid-liquid equilibrium (LLE) of the concerned molecules were calculated using a non-random two-liquid Equation (NRTL) which is a widely reported property simulator for BDO recovery (Harvianto et al., 2018). In general, economic, and binary parameters available in the ASPEN plus software was used for all calculations and no external parameters were added in software. A conventional double distillation unit flowsheet for BDO recovery is shown in Figure 1.

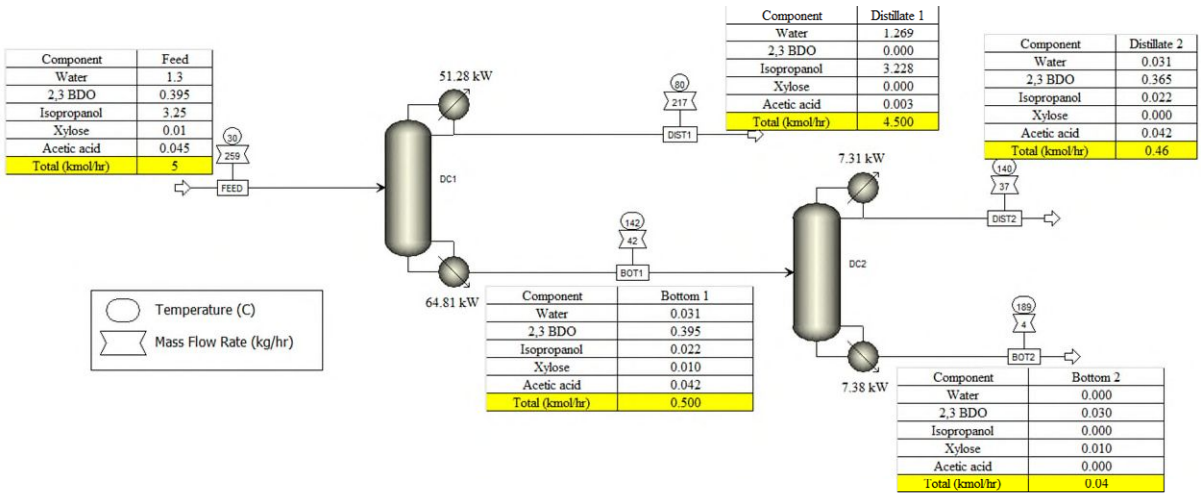


Figure 1: Flowsheet of conventional double distillation system used for recovery of BDO from organic phase.

Results and discussion

BDO accumulation through fed-batch cultivation and optimization of inorganic salt concentration for phase separation

BDO was accumulated through fed-batch fermentation of *E. ludwigii* using detoxified xylose rich SCB hydrolysate as feedstock. The fermentation was carried out in 2.5 L bioreactor with a working volume of 1.0 L and initial xylose concentration of 40 g/L, and after depletion, a feed (400 g/L xylose) was fed to

maintain xylose concentration of 20 g/L. In our previous studies, we have observed that the forced pH fluctuations tend to induce BDO accumulation by the cells. The initial pH was adjusted to 7.0, allowed to drop naturally and once it reached below pH 5.0, it was brough back to 7.0 using 5M NaOH. The BDO accumulation was observed from 6h and continued till the end of the fermentation, accumulating 68.2 ± 4.09 g/L with a yield and productivity of 0.38 ± 0.02 g/g and 0.9 ± 0.05 g/L.h, respectively. Acetic acid was obtained as major by-product and residual xylose was <10 g/L.

Initially, various concentrations of AS (5%, 10%, 15%, and 20% w/v) were investigated to observe the two-phase formation. Two distinguishable phases were observed with 15% and 20% salt concentration, whereas in case of 5 and 10%, phase separation did not happen. Hence, in subsequent experiments, a salt concentration of 20% w/v of salt was used for phase separation. However, Shaoqu Xie and associates, when performed ATPS using different phosphate salts for separation of BDO from simulated aqueous solutions, phase separation was not observed even when 28 – 30% w/w salt was added to the aqueous phase. But increase in concentration to 33% w/w resulted in phase separation (Xie et al., 2016).

3.2. Screening of various organic solvent for extraction of BDO from fermented broth

BDO is highly soluble in water, therefore, fermented broth containing BDO is a homogeneous mixture. Currently LanzaTech commercialized the biological production of ethanol and BDO, in which the product separation was carried out using simulated moving bed (SMB) process, but the process is still energy and cost intensive (Haider et al., 2020), and other promising processes investigated for BDO separation are reactive extraction (Li et al., 2012), liquid-liquid extraction (Wu et al., 2012), sugaring-out (Dai et al., 2015) and salting-out or aqueous two phase extraction (Dai et al., 2011). Initially liquid-liquid extraction was understood to be commercially viable, for which various solvents like n-butyl acetate, 1-butanool, ethyl acetate, oleyl alcohol, 1-decanol, and tetraoctyl ammonium 2-methyl 1-napthoate were reported as an extractant in solvent mediated BDO separation (Birajdar et al., 2014; Garcia-Chavez et al., 2012; Li et al., 2012; Wu et al., 2012; Xie et al., 2017). The liquid – liquid extraction has various advantages like simple unit procedures, low energy consumption in separation and purification of the metabolite. However, the BDO separation suffers from many problems such as lower partition coefficient and poor recovery leading to requirement of large amounts of solvent (Birajdar et al., 2014). Hence, a combination of salting-out and solvent extraction techniques was adapted to improvise the efficacy of the method. In ATPS method, hydrophilic/ hydrophobic aldehydes or alcohols are usually used as organic solvents based on the properties of the end product (Iqbal et al., 2016; Vivek et al., 2018).

In this study as described in the section 2.3 different solvents were screened to evaluate the partition behaviour of BDO. With 20% w/v AS, the partitioning of BDO into isopropanol was 6 times (K =

6.5) higher in comparison to other solvents (Table 1). The better separation of BDO with polar solvents may be due to its hydrophilic behaviour. Higher the partition, greater the recovery rate. About 87% BDO recovery yield was obtained with isopropanol which is ~54% higher than using acetone or methanol as the extractant (Table 1). In general, the partition of hydrophilic metabolites like BDO is maximum with hydrophilic solvent-salt system. In current study, IP – AS system provided better partition and recovery yields than the ET – AS system. Earlier, 91.4% BDO recovery was reported using 34% (w/w) IP and 20% (w/w) AS from glucose-fed fermentation broth obtained using *Klebsiella pneumoniae* CICC 10011 (Bo and Xiu, 2009). Contrary to our results, Li et al. 2010 reported highest recovery yield of 99.1% with ET – AS, which was significantly higher than IP - AS system. They also observed that partition coefficient of BDO in methanol, ethanol and isopropanol is significantly higher (K ~30) compared to hydrophobic solvents like ethyl ether, hexane, and acetic ester.

Another energy consuming step in the ATPS is the distillation of the organic solvent to recover and purify BDO. The volume of the organic phase that needs to be distilled also adds to the cost of the process. Therefore, we calculated the phase ratio (P) to quantify the volume of the organic phase. When using AS and IP for extraction, the organic and aqueous phases were distributed evenly (P = 1), while with acetone and methanol, P values of 3 and 7 were observed, respectively, indicating that a large amount of water molecules along with BDO, were partitioned into the organic phase (Table 1). The presence of water molecules decreases the final titers and purity of BDO.

Solvents	Partition Co-efficient (K)	Recovery Yield (%)	Phase ratio (Vol_{org}/Vol_{aq})
Isopropanol (IP)	6.5 ± 0.35	86.66 ± 3.12	1 ± 0.01
Ethanol (ET)	1.4 ± 0.05	59.06 ± 2.45	3 ± 0.02
Chloroform (CH)	0.17 ± 0.007	14.61 ± 0.72	1 ± 0.009
Acetonitrile (AN)	1.06 ± 0.06	51.66 ± 2.36	1 ± 0.009
Acetone (AC)	1.28 ± 0.04	56.33 ± 2.58	3 ± 0.03
Methanol (ME)	1.21 ± 0.05	54.86 ± 1.95	7 ± 0.08

Table 1: Effect of various organic solvents on the partition co-efficient (K), recovery yield (%) of BDO and phase ratio.

In the ATPS, addition of inorganic salts to the aqueous phase, would reduce the availability of water molecules for BDO, thereby BDO partitions into the organic solvent (extractant) leaving the extracellular proteins, by-products, and macromolecules as raffinate (aqueous solution). Then the solvent is distilled to discrete BDO with high purity and concentration. The solvent obtained after the distillation can be re-used for the next batch of separation. The process is energy efficient, as the separation of BDO from the organic solvent (low heat of vaporization) is easier than from water (high heat of vaporization) with ease of recovery, and reuse of solvents. Most of the processes investigated

in the literature are either model or synthetic solutions, not much attention has been paid on fermentation broth consisting of by-products, proteins, salts, sugars, and other complex nutrients.

Screening of inorganic salts with IP as an extractant

According to the results obtained in the previous section, the inorganic salts play a significant role in salting out for distinguishable phase separation to occur and impacts efficiency of separation. The partitioning order of BDO from aqueous phase to organic (IP) with different salts at 20% w/v was as follows: $(NH_4)_2SO_4$ > $CaCO_3$ > KH_2PO_4 > Na_2HPO_4 > NaH_2PO_4 > $(NH_4)_2CO_3$ > $MgCO_3$., In case of $CaCl_2$ and NH_4Cl, no visible phase separation was observed (Table 2). According to Xie et al. (2017) the partition coefficient increased with increase in net charge on anion: $K_4P_2O_7$ > K_3PO_4 > K_2HPO_4 > K_2CO_3. Similar observation was made in other studies conducted on separation of diols like 1,3-propanediol and BDO from the synthetic solutions or fermented broth (Li et al., 2010; Vivek et al., 2018).

Inorganic salts	Partition Co-efficient (K)	Recovery Yield (%)	Phase ratio (Vol_{org}/Vol_{aq})
KH_2PO_4	1.38 ± 0.05	58.07 ± 2.65	3 ± 0.00
$(NH_4)_2SO_4$	6.5 ± 0.36	86.66 ± 3.52	1 ± 0.05
$NaHCO_3$	0.0	0.0	3 ± 0.00
$CaCO_3$	1.83 ± 0.06	64.77 ± 3.12	1 ± 0.00
$CaCl_2$	0.0	0.0	3 ± 0.03
Na_2HPO_4	1.3 ± 0.04	57.11 ± 2.56	1 ± 0.009
NaH_2PO_4	1.28 ± 0.04	56.24 ± 2.42	3 ± 0.03
NH_3CO_3	0.98 ± 0.05	49.56 ± 1.89	3 ± 0.03
$MgCO_3$	0.92 ± 0.05	48.02 ± 1.65	3 ± 0.03
NH_4Cl	0.0	0.0	3 ± 0.03

Table 2: Screening of inorganic electrolytes (20% w/v) on the partition co-efficient (K), recovery yield (%) of BDO using isopropanol as the organic solvent for extraction**.**

Though these studies contradict the current work where better partition coefficient and recovery of BDO from the fermented broth were obtained with AS-IP system against phosphates and carbonates salts. The partition coefficient (6.5) and recovery yield (86.7%) obtained with combination of AS and IP in current work (Table 2) are comparable to results achieved using $(NH_4)_2SO_4$/ethanol (K = 5, R < 90%) and K_2HPO_4/ethanol (K > 10, R > 90%) systems by Dai et al., 2011, 2014. The phase ratio obtained from salts other than AS is about 3 times higher (Table 2). Considering the facts that AS enriched bottom aqueous phase can either be reused as nitrogen supplement for the next batch of fermentation or recycled for further downstream processing. IP-AS system as the best possible combination for BDO partition and recovery from the fermented broth was further investigated.

3.4. Optimization of AS concentration, and ratio of IP to fermented broth

Like salt concentration, the amount of organic solvent also influences the partition behaviour and recovery of BDO. To this end, various concentrations of AS (15, 20, 25, 30, 35 and 40% w/v) and different IP ratios (0.5, 1.0, 1.5, and 2.0 v/v) were investigated for separation of BDO to determine an optimal ratio of AS and IP. As the concentration of AS was enhanced from 15 to 30%, there was remarkable improvement in the partition coefficient from 3.2 to 45.5 (Figure 2a), due to increased salting-out of BDO.

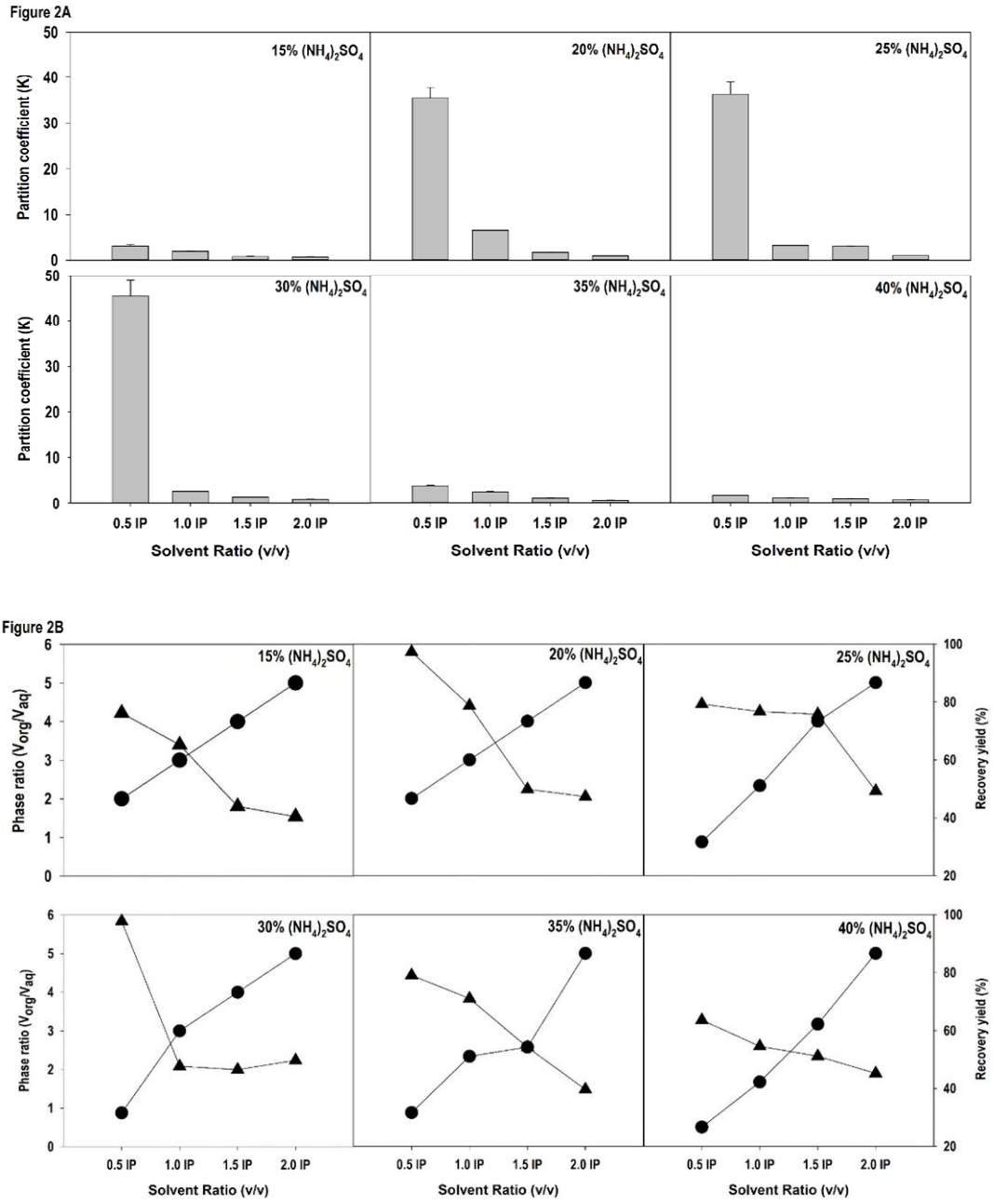


Figure 2: Impact of different levels of $(NH_4)_2SO_4$ (15 – 40% w/v) and isopropanol (50 – 200% v/v) on (A) partition coefficient (K), (B) recovery yield and phase ratio, of BDO from the fermented broth.

Similarly, increasing the amount of organic solvent improves partitioning and recovery of BDO, but exceeding 0.5 v/v ratio reduced the recovery yield. To better understand the limits, a wider range of solvent concentrations was screened. This is due to fact that the recovery yield is determined by the partition co-efficient and the phase ratio. The higher the salting-out of BDO from the aqueous phase, more is the partition into the organic phase (Li et al., 2010). The partition coefficient of 45.5 and recovery yield of 97.9% with 30% w/v AS and 0.5 v/v IP ratio was found to be the optimal for separation of BDO from the fermented broth (Figure 2a and 2b). Bo and Xiu (2009) reported highest partition coefficient of 9.9 and recovery yield of 93.7% using a similar IP – AS system (34 % v/v IP and 20% w/v AS).

3.5. Impact of BDO concentrations on partition coefficient and recover yield

The perusal of literature shows that the high titer (100-150 g/L) has been reported during biological synthesis of BDO from various feedstocks like crude glycerol, lignocellulosic sugars or pure sugars. In our previous studies, we have also reported BDO concentration of 118.5 g/L from brewers spent grain hydrolysate by *E. ludwigii* (Amraoui et al. 2021). Hence the validation of the optimized concentrations of the inorganic salt (30% w/v AS) and organic solvent (0.5 v/v ratio IP) was carried out using fermented broth adjusted to different concentrations of BDO (50 – 150 g/L). The partition coefficient decreased to 12.1 with an increase in the BDO concentrations, but recovery yield obtained was similar (~ 95%) at all the BDO levels (Figure 3a and 3b). At BDO concentration of 150 g/L, 138.6 g/L BDO was partitioned into the top organic phase with a partition coefficient and recovery yield of 12.1 and 92.4%, respectively. Xie et al., 2016 observed that an increase in the inorganic salt concentration is required to enhance the partitioning of BDO into the organic phase. With 15% w/v BDO in the aqueous phase, 100% recovery was obtained with $K_4P_2O_7$, and K_2HPO_4, when their initial concentration was increased from 33% w/v, was increased to 63% and 60% w/v respectively.

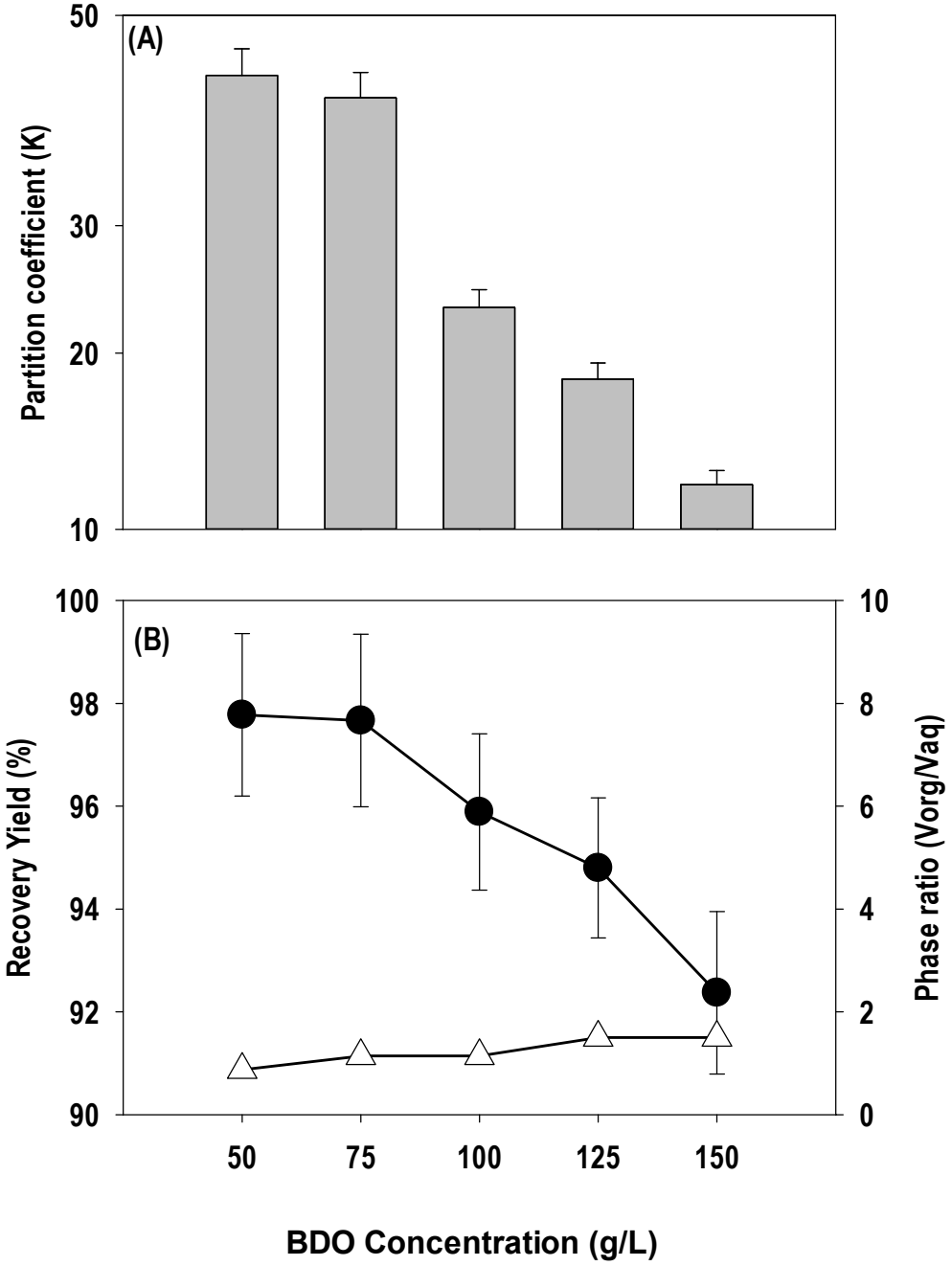


Figure 3: Effect of different levels of accumulated BDO on (A) partition coefficient (K), (B) recovery yield and phase ratio using optimal combination of $(NH_4)_2SO_4$ (30% w/v) and isopropanol (50% v/v).

BDO Separation from unfiltered fermentation

In all the experiments above, cell-free fermented broth was used, where the microbial cells were separated through centrifugation and the results obtained were promising with 95% recovery yields. To enhance the economic aspect of BDO separation with ATPS, unfiltered fermented broth (broth directly after fermentation) was used for separation and purification of BDO. The main aim of this experiment

was to eliminate two-unit procedures involving removal of microbial cells and proteins by activated charcoal or ion-exchange resin treatment and cut-down the operational cost. The fermented broth with 68.2 g/L BDO, cell biomass, and unspent protein was subjected to ATPS with optimized IP – AS system. After the addition of AS and IP to the fermented broth and vertexing it for 5 minutes, a distinguishable transition phase was formed between the aqueous and organic phase. We found that the aqueous phase contained 99 and 94% of cells and protein, the organic phase consisted of 66.7 g/L BDO, 2.0 g/L xylose and 9.0 g/L acetic acid with BDO partition co-efficient and recovery yield of 45 and 97%, respectively. The results obtained are better in comparison to K_2HPO_4/ethanol (Dai et al., 2011), and $(NH_4)_2SO_4$/ethanol (Li et al., 2010) system where 89% and 91.2% protein was accumulated in the aqueous phase, respectively, with 7.1 K_{BDO}, and 91.7% recovery yield. Similar results obtained with treated and untreated fermentation broth suggest that a high recovery of BDO is possible even without removing cell mass and unspent proteins which will lead to substantial reduction in operating cost for BDO separation. The concentrated aqueous phase rich in AS can be used as supplement of nitrogen source for the next batch of fermentation. The other possibility could be precipitation of salt by addition of 2 volumes of methanol or chilled acetone (Baral et al., 2021) and recycling of it for next round of downstream processing.

Techno-economic analysis

The organic fraction after the ATPS extraction was considered as the feed for the TEA analysis. The initial feed flow rate was fixed to 5 kmol/hr at 30 ^oC, and 1.013 bar pressure which enters directly into DC-1 at the 10^{th} stage, whereas reflux ratio and distillate to feed ratio were fixed at 0.001 and 0.9 respectively. Also, the feed, top, and bottom stage temperatures were calculated as 54.10, 79.80 and 141.90 °C, respectively. Notably, the feed composition was defined based on the experimental data and consisted of 0.260, 0.079, 0.650, 0.002, and 0.009 mole fractions of water, BDO, isopropanol, xylose, and acetic acid, respectively. The overall input and out parameters for feed, DC-1 and DC-2 are shown in Table 3. It was found that the DC-1 removed 97.6% water (100 ^oC) and 99.3% isopropanol (82.15 ^oC) as the top product. A 100% (mol) recovery of BDO (180.7 ^oC) was measured in DC-1 with 85.7% (by mass) purity along with acetic acid, xylose (404.8 ^oC) and isopropanol in minor quantities which became feed for the DC-2 at 9^{th} stage for further purification. The reflux ratio and distillate to feed ratio were fixed to 0.009 and 0.92, respectively, after regression analysis. The feed, top and bottom stage temperature were calculated to be 173.70, 139.50, 188.80 °C, respectively. As a result, the purity of BDO improved to 88.1% (by mass) albeit the recovery rate reduced to 92.4% (mol). It indicates a higher recovery of BDO can be achieved in DC-1 with low purity. On the contrary, the purity of BDO can be slightly improved at the cost of loss in recovery. Thus, a trade-off between the purity and recovery needs to consider while designing BDO purification units.

	Distillation Column-1 (DC-1)			Distillation Column-2 (DC-2)		
Component	**Feed-1**	**Distillate-1**	**Bottom-1**	**Feed-2**	**Distillate-2**	**Bottom-2**
Water	1.3	1.269	0.031	0.031	0.031	0.000
2,3 BDO	0.395	0.000	0.395	0.395	0.365	0.030
Isopropanol	3.25	3.228	0.022	0.022	0.022	0.000
Xylose	0.01	0.000	0.010	0.010	0.000	0.010
Acetic acid	0.045	0.003	0.042	0.042	0.042	0.000
Total (kmol/hr)	5.00	4.50	0.50	0.50	0.46	0.04

Table 3 Overall inlet and outlet compositions in conventional double distillation system for 2,3 BDO recovery

It is evident that 90% of the fermentation broth was separated from the product mixture in DC-1. Thus, it is obvious to hypothesize that DC-1 would consume maximum energy during the process. The overall operating conditions, energy consumption, and economic parameters are given in Table 4. Accordingly, the software has calculated the exceptionally high energy consumption of 51.28 kW condenser duty and 64.81 kW reboiler duty. On the contrary, the energy consumption by the condenser and reboiler of the second column was measured only 7.31 kW and 7.38 kW respectively. The apparent reason for low duty in the DC-2 column is less flow rate (0.5 kmol/hr) compared to 5 kmol/hr flow rate in the DC-2. The basic economic analysis of the entire purification scheme was performed on the ASPEN plus economic analyser for a project duration of 10 years. The desired rate of return (20%), tax rate (40%), and straight-line depreciation method were chosen to analyze project performance. The total project and operating cost of the project found to be $2.38 and $0.98 million per year, respectively. Notably, BDO purity in the DC-2 improves by only 2% whereas it contributes to 38% of the total annualized cost out of which 35% comes from capital cost. It indicates that the operating cost of DC-2 is not very high due to low heat duty and merely accounts for 3% of the total annualized cost. Therefore, if BDO purity can be compromised to 85.7%, a one-column system would be appropriate whereas a double distillation system would be needed for higher purity. The overall annualized cost for a conventional double distillation unit was calculated to be $3.36 million per year. The results obtained are in line with the similar TAC reported by Lee and co-workers for two distillation column setups, which further validates our results (Haider et al., 2018a).

Parameter	**DC-1**	**DC-2**
Nos. of stages	20	20
Feed stage	9	9
Reflux ratio	0.001	0.09
Distillate to feed ratio	0.90	0.92

Qc (kW)	51.28	7.31
Qr (kW)	64.81	7.38
Capital Cost ($ 10^6)/yr	1.19	1.19
Operating cost ($ 10^6)/yr	0.872	0.108
Total annualized cost ($ 10^6)	2.062	1.298

Table 4 Input-output parameters and economic indicators for Distillation Column 1 (DC-1) and Distillation Column 2 (DC-2).

Further to that a two-column analysis was carried out. It is evident from a two-column analysis that BDO with similar purity can be obtained from a single column system itself which closer to experimental conditions. Thus, a further TEA study on single column purification system at different vacuum pressures (absolute) was performed. It was observed that irrespective of vacuum (absolute) pressure, the recovery and purity of BDO remained nearly constant to 100% and 85.7% by weight, respectively, as observed in previous section for DC-1 in a two-column system. It was also found that a decrease in operating pressure (increase in vacuum) led to decrease in operating temperature which was self-adjusted by the software. Nevertheless, since yearly operating cost contribution to the total annualized cost is significantly less, thus the overall change in TAC was insignificant as shown in Table 5.

Pressure (absolute) mbar	**BDO Purity, wt.%**	**Capital Cost ($ 10^6)/yr**	**Operating cost ($ 10^6)/yr**	**Total annualized cost ($ x 10^6)**
1000	85.7	1.80502	0.9655	2.77052
750	85.6	1.80502	0.9655	2.77052
500	85.7	1.80077	0.96539	2.76616
250	85.7	1.79976	0.96539	2.76515
150	85.6	1.7979	0.96539	2.76329
100	85.7	1.79766	0.96539	2.76305

Table 5 Effect of vacuum (column operating pressure) on BDO recovery

It is hypothesized that the increase in operating cost for maintaining a higher vacuum is compensated by the decrease in operating cost due to lowering of operating temperature of the column. Overall, it is observed that the change in total operating cost is insignificant even if the distillation is carried out in vacuum because of compensation by the decrease in energy cost associated with the operating cost of the column. Nevertheless, a further detailed study on the whole process is needed to analyze that cost of BDO which could be an interesting area of research and has

not been considered in the present study. It is to be noted that minor variations in the results can be expected due to change in base year and different version of the software.

3.8. Purification and characterization of purified BDO using ^{13}C NMR and ^{1}H NMR spectroscopy

The organic phase obtained after the separation was enriched with BDO and subjected to vacuum distillation to separate BDO from IP. The concentrated BDO obtained contained little amount of xylose and acetic acid (< 2 g/L). As fractional distillation cannot be performed in a laboratory scale, silica gel column chromatography was carried out with chloroform and methanol as mobile phase. After loading the silica column with concentrated BDO sample, the mobile phase was eluted in a gradient (90:10 to 70:30) by increasing the polarity. After chromatographic separation, the purity of BDO was verified using NMR spectroscopy. The structural characterization was carried out using ^{13}C and ^{1}H NMR using commercial (Sigma) BDO as the reference. The ^{13}C NMR spectrum of purified BDO and commercial grade BDO along with ^{1}H NMR were performed. In ^{13}C NMR, the two carbon signals at 16.7 and 70.9 ppm represented the methyl ($-CH_3$) and methyne (-CH) carbon, respectively. Similarly, in ^{1}H spectrum the proton signal obtained at 0.966 and 0.978 ppm as doublet corresponded to the methyl proton. On the contrary, the signal at 3.53 ppm represented the –CH proton of the BDO having a quartet structure. Besides that, a strong signal was observed at 4.71 ppm, which internally referred to as the proton of D_2O.

4. Conclusion

In the current study, separation, and purification of BDO accumulated on xylose-rich sugarcane bagasse hydrolysate by *E. ludwigii* was performed by using ATPS. The highest extraction efficacy for BDO was obtained with combination of AS and IP. The high partition coefficient and recover yield (>95%) of BDO achieved demonstrated potential of the process. The study shows that a high recovery of BDO is possible even without removal of microbial biomass and unspent proteins. The coupling of cost-effective downstream processing with low-cost upstream may have great potential for commercial production of BDO from lignocellulosic feedstocks. Further work on technoeconomic analysis is underway.

Note: E-Supplementary data of this work can be found in e-version of this paper online.

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Authors' contributions: NV, YA, AA, EA and DA carried out all the experimental work. NV, EA and VK analyzed the data and wrote the manuscript. DA, BP, AP, SG, NV and VK were involved in

proofreading the manuscript and revised the manuscript critically. All authors read and approved the final manuscript.

Declaration from authors:

As Ashok Pandey, a co-author on this paper, is the Editor-in-Chief of Bioresource Technology, he was blinded to this paper during review, and the paper was independently handled by Christian Larroche as editor.

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High yield recovery of 2,3-butanediol from fermented broth accumulated on xylose rich sugarcane bagasse hydrolysate using aqueous two-phase extraction system

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