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Fermentative valorisation of xylose-rich hemicellulosic hydrolysates from agricultural waste residues for lactic acid production under non-sterile conditions

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ABSTRACT

Lactic acid (LA) is a platform chemical with diverse industrial applications. Presently, commercial production of LA is dominated by microbial fermentation using sugary or starch-based feedstocks. Research pursuits emphasizing towards sustainable production of LA using non-edible and renewable feedstocks have accelerated the use of lignocellulosic biomass (LCB). The present study focuses on the valorisation of xylose derived from sugarcane bagasse (SCB) and olive pits (OP) through hydrothermal and dilute acid pretreatment, respectively. The xylose-rich hydrolysate obtained was used for LA production by homo-fermentative and thermophilic *Bacillus coagulans* DSM2314 strain under non-sterile conditions. The fed-batch mode of fermentation resulted in maximum LA titers of 97.8, 52.4 and 61.3 g/L with a yield of 0.77, 0.66 and 0.71 g/g using pure xylose, xylose-rich SCB and OP hydrolysates, respectively. Further, a two-step aqueous two-phase system (ATPS) extraction technique was employed for the separation and recovery of LA accumulated on pure and crude xylose. The LA recovery was 45 – 65% in the first step and enhanced to 80–90% in the second step.The study demonstrated an efficient integrated biorefinery approach to valorising the xylose-rich stream for cost-effective LA production and recovery.

1. Introduction

Lactic acid (LA) chemically known as 2-hydroxypropionic acid, is a versatile bio-based platform chemical which finds applications in diverse sectors ranging from food, beverages, cosmetic, pharmaceutical, textile, and chemical to polymer industries as a monomer or a precursor for LA derivatives (Abdel-Rahman et al., 2016; Cubas-Cano et al., 2019; Wischral et al., 2019). The global LA market was valued at $2.9 billion in 2021 with a forecasted increase to $5.8 billion by 2030 and a compound annual growth rate of 8.0 % (Grand View Research, 2022). LA is a platform chemical and exists in form of two stereoisomers, the predominant and naturally occurring L(+) form and the rare D(−) LA. The stereochemistry of these isomers governs their physical, thermochemical and biodegradable properties. Hence, the optical purity is a decisive factor governing the physical, mechanical, rheological and functional properties of the polymer formed (Naser et al., 2021). In the last decade, the demand for optically pure LA has attracted worldwide attention due to PLA (PLLA or PDLA), a bio-based, eco-friendly, biodegradable, compostable thermoplastic and an environmentally friendly alternative to current commercially available polyethylene, polyethylene terephthalate (PET) or PET derived plastics (Abdel-Rahman et al., 2016; Filiciotto and Rothenberg, 2021).

The fossil-based production of LA is efficient and economical but results into the formation of racemic mixture, where the separation of stereoisomer is a tedious process. Additionally, the limited supply of petrochemical feedstocks and raising environmental concerns have

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triggered the search for the augmentation of substantial alternative processing techniques and processes. The biological production of LA has become indispensable in achieving the goal of moving away from petrochemical resources and providing global sustainability (Raj et al., 2022). Hence, decades of research have been focussed on developing efficient, environmentally friendly, and sustainable biobased technologies for LA production. Currently, 70% of the global LA is manufactured through fermentative route using mostly first-generation feedstocks such as sugarcane, rice, corn starch, or sweet potato (Zhang et al., 2020). To feed the exponentially growing human population, use of edible materials for biorefineries need tobe avoided and alternatives feedstocks are searched. Therefore, the use of non-edible feedstocks and renewable biowastes such as lignocellulosic biomass (LCB) not competing with sugary or starchy edible feedstocks used in the food industries, offers an attractive, green and sustainable solution for the efficient and commercial scale production of the chemical building blocks including LA. In the present time, most of such waste streams are burnt, resulting in additional CO_2 emissions. To circumvent this, there is an urgent need to effectively manage these organic carbon rich- streams by diverting them towards valorization to commercially important chemicals and materials which traditionally come through the petrochemical route.

Biomass, an inexhaustible resource obtained through the assimilation of carbon dioxide from the atmosphere, represents an interesting alternative renewable and sustainable feedstock for the continuous production of chemical building blocks. Ample amount of LCB is available on earth (~200 billion tons) with cellulose, hemicellulose, and lignin constituting its major fraction. Cellulose is the homopolysaccharide of β-D-glucose while hemicellulose is a heteropolysaccharide consisting of hexose and pentose sugars with 80–90 % of xylan (Narisetty et al., 2022; Saini et al., 2020). Though both cellulose and hemicellulose after depolymerization produce fermentable sugars, most of the studies on LCB-based feedstocks have focussed on cellulosic fraction as its hydrolysis generates glucose which is the most preferred carbon source for microbial systems. The hemicellulose fraction constitutes 20–40 % of LCB and ~~a~~ profitable LCB-based biorefineries cannot be envisaged without value addition of hemicellulosic sugars,which would generate additional revenue, strengthening economical, and environmental footprint (Tye et al., 2016; Narisetty et al. 2022). Although the feedstocks may vary in availability and composition, the process flow for extraction of fermentable sugars is similar involving two inevitable steps namely pretreatment and saccharification. For instance, if biomass is subjected to dilute acid (DA) or water treatment at high temperatures, a cellulolignin rich solid fraction is obtained, which is further processed through enzymatic hydrolysis for the production of glucose-rich hydrolysate (Guo et al., 2018; Tye et al., 2016). The liquid fraction obtained after these pre-treatments predominantly contains hydrolysed hemicellulosic rich in pentose sugars, abundantly xylose. Until recently, much attention has not been given to this fraction, as natural xylose assimilating microorganisms are scanty in nature. However, the recent review on LA production from sugarcane bagasse (SCB) emphasizes the need for complete valorisation of both the carbohydrate fractions, especially xylose, for the commercial realization of SCB-based LA biorefinery (Agrawal and Kumar, 2023). To understand the commercial competitiveness of xylose as an excellent carbon source for bioconversion to LA, we selected two different LCB's namely olive pits (OP) and SCB in this study. The primary reason for choosing these two feedstocks is their readily available nature in bulk quantities from olive oil processing and sugar industries, respectively and presently their low-grade applications.

Globally, olive cultivation is done in 41 different countries with an annual harvest of 21.6 Mt resulting in oil production of 3.2 Mt. Spain and Italy are the major olive cultivators in the world. During oil processing, OP or olive stones is the by-product obtained after stone removal from the olive pomace and constitute ~8–12 % w/w of olives. It has a chemical composition of 31.9% cellulose, 21.9% hemicellulose, 26.5% lignin, 5.5% fats, 3.2% protein, and other components (Narisetty et al., 2021; Rodríguez et al., 2008). Likewise, sugarcane is one of the largest crops in the world, cultivated in tropical and sub-tropical climatic conditions and used by sugar industries to manufacture table sugar. The global sugarcane cultivation in 2017 was 1841 million tonnes (Mt) with a major share coming from Brazil (758 Mt), India (306 Mt), China (104 Mt) and Thailand (103 Mt) (Konde et al., 2021; Meghana and Shastri, 2020; Silalertruksa and Gheewala, 2019). It is expected to reach 1960 Mt by 2030 with an increased growth rate of 1% where 65% is expected to be contributed by Brazil and India (FAO, 2021). Sugarcane bagasse (SCB) is major fibrous waste obtained after the extraction of juice from sugarcane with an estimate of 140–280 kg per tonne of sugarcane. SCB is a rich source of cellulose (26–47%), hemicellulose (19–33%), lignin (14–23%), ash (1–5%) and other components (Mahmud and Anannya, 2021).

As cellulosic fraction has endless applications in biorefineries to produce biofuels and biochemicals, the current motivation is to showcase the potential of xylose-rich hemicellulosic fraction as an efficient feedstock for the production of various value-added chemicals, specifically LA (Narisetty et al., 2022). In the present study, xylose-rich hemicellulosic hydrolysates from SCB and OP were used for LA production by *Bacillus coagulans*. The xylan fraction of SCB and OP was hydrolysed using hydrothermal (HT) and dilute acid (DA) pretreatment, respectively. All the fermentation experiments were performed in bioreactor under controlled conditions. The bioreactor operations for cultivation and LA biosynthesis were operated under non-sterile conditions and the output was compared against commercial xylose as the sole carbon source. Further, fed-batch studies were conducted to improve the LA titer and yields. Later, the LA accumulated fermented broth was recovered using an aqueous two phase (ATPS) separation technique. The downstream processing was carried out using the fermented broth obtained from the pure and crude xylose to investigate the impact and efficiency of using xylose-rich hydrolysate as feedstock for LA production. The results obtained in the present study are critically compared with the recently published state-of-the-art. The current study is a demonstration of the combined upstream and downstream process for the production and recovery of LA from xylose rich LCB hydrolysates and to the best of our knowledge this is the first study showcasing an integrated approach.

2. Materials and methods

2.1. Materials, microorganism, and cultivation maintenance

All the media components and the chemicals used were of analytical grade and purchased from Sigma-Aldrich and Fisher Scientific, unless stated otherwise. SCB hydrolysate was provided by Nova Pangaea Technologies (https://www.novapangaea.com), Redcar, UK. The crushed OP suspension consisiting of 50% w/v solids and 2% w/v sulfuric acid was incubated at 125 °C for 30 mins in an autoclave. The post incubation, solution was filtered, and pH of the liquid fraction was adjusted to 7 using NaOH pellets and stored until for further use. Detailed information on the processes and composition of xylose-rich hemicellulosic hydrolysate from SCB and OP can be obtained from previous reports (Jokodola et al., 2022; Narisetty et al., 2021). The hydrolysates were concentrated to increase the xylose concentration up to 250 g/L using vacuum distillation (Rotavapor, BUCHI UK Ltd), where the concentration was carried out for several hours at 80 °C and 100 mbar pressure. For the batch and fed-batch fermentations, the hydrolysates were diluted to obtain the desired concentrations.The *B. coagulans* DSM2314 strain used for LA production was kindly gifted from Leibniz Institute of Agricultural Engineering and Bio-economy E.V (ATB, Potsdam, Germany). The strain was cultivated using Tryptone Soya (TS g/L) [17, pancreatic digest of casein; 3, soybean meal;5, NaCl;2.5, KH_2PO_4;25, glucose] medium at pH 7.0 and temperature 50 °C. The working stocks were prepared using 30% w/v glycerol, and preserved at −80 °C. The pre-inoculum was prepared in 250 mL

Erlenmeyer flasks and incubated for 16 h at 50 °C with an agitation speed of 250 RPM on a rotary shaker (Excella 24, New Brunswick).

2.2. Effect of initial xylose levels on growth and lactic acid (LA) production

To evaluate the effect of increasing xylose concentration on cell growth and LA accumulation, *B. coagulans* DSM2314 strain was cultivated at different xylose concentrations (50, 75, 100 and 150 g/L).

2.3. Bioreactor cultivation

Generally, bacterial LA fermentation demands pH-restoration for its steady production besides controlled aeration. Both these conditions are difficult to maintain during shake flask cultivation. Hence, LA fermentations in this study were carried out in a 2.5 L bioreactor (Electrolab Bioreactors, UK). The culture conditions and bioreactor specifications can be obtained from previous report (Cox et al., 2022). The agitation speed, pH and temperature were maintained at 100 RPM, 6.0 (using 5 M NaOH) and 50 °C, respectively (Cox et al., 2022).The xylose concentration in the bioreactor during fed-batch fermentation, was maintained at ≥10 g/L using feed containing ~250 g/L xylose supplemented with 5 g/L yeast extract as a nitrogen source. The fermentations in this study were carried out under non-sterile conditions to demonstrate strain efficiency and reliability.

2.4. Downstream processing

LA was separated through a similar approach as discussed by Baral et al., (2021). The pH of fermentation broth (25 mL) containing LA was brought down to pH 2. Ammonium sulphate was added to the pH reduced broth at different concentrations of 5–70% w/v followed by vortexing for 5 mins to ensure that salt was fully dissolved. Finally, the LA was separated from the fermentation broth using isopropanol (IP) as an extractant at a concentration ratio of 1:1 v/v. The mixture was then vortexed for 1 h before leaving to stand for 15 mins at room temperature. A sample of 1 mL was taken from the lower aqueous phase of the solution to analyse the amount of remaining LA within the broth. Further to that the top organic phase was separated from the bottom aqueous fraction through a separating funnel after complete phase separation. The separation process was then repeated with the fresh solvent by adding in ratio of 1:1 v/v to the aqueous phase from the previous step as a second round of extraction. Analysis of extraction efficiency was conducted through quantification of LA. The partition coefficient (K) and recovery yield (Y) were calculated through Eqs. (1) and (2).

$$K = \frac{Concentration\ \ of\ \ LA\ \ in\ \ Organic\ \ phase}{Concentration\ \ of\ \ LAin\ \ aqueous\ \ phase} \quad (1)$$

$$Y = \frac{Concentration\ \ of\ \ LA\ \ in\ \ organic\ \ phase}{Total\ \ concentration\ \ of\ \ LA\ \ in\ \ fermented\ \ broth} \times 100 \quad (2)$$

2.5. Analytical methods

The fermentation efficiency of strain *B. coagulans* DSM2314 was evaluated by investigating its growth based on optical density, substrate assimilation and LA productivity. The optical density was measured using spectrophotometer (Jenway 6310, UK) at a wavelength of 600 nm using 1 mm cuvette. High pressure liquid chromatography (HPLC) (Agilent, 1200 Series, USA) was used to evaluatethe concentrations of xylose, AA (acetic acid), and LA. The culture supernatants collected at specific time intervals were processed (Cox et al., 2022; Narisetty et al., 2021) and the samples were loaded for evaluation (Amraoui et al., 2021; Cox et al., 2022). All the experiments were conducted in triplicates and the values were averaged. The standard deviation was not >10 %, and the same is depicted in the error bars in time series plots.

2.6. Statistical analysis

During batch and fed batch studies, the effect of fermentation time on the residual xylose concentration, optical density (OD_{600nm}) of the bacterium and LA formation was measured by performing One-Way ANOVA. The results obtained from this method were simultaneously validated by carrying out paired *t*-test with α being 0.05. Paired-*t*-test helped in identifying whether the correlation was positive or negative. Generally, the term "α" refers to the significance level used in the hypothesis tests and lower is the value, more statistically significant is the correlation. Likewise, during aqueous two-phase extraction, the correlation between partition coefficient and LA recovery yield was studied in both the stages.

3. Results

3.1. Influence of substrate concentration on growth and LA production using pure xylose

B. coagulans DSM2314 strain, a thermophilic and highly versatile microorganism with the ability to withstand harsh conditions like high temperatures, low aeration, and most importantly the broad substrate range and an ability to utilize inexpensive carbon sources like cellulosic fractions of LCB, and other renewable waste. The strain has standout advantages over other LA producing bacteria such as its thermophilic behaviour making it suitable for use in unsterile and/or simultaneous saccharification and fermentation processes (Jiang et al., 2019). The LA production credentials of *B. coagulans* have been demonstrated with varied types of biomass such as sugarcane bagasse, walnut shell, organic fraction of municipal solid waste, gardening residues and vine shoots (van Der Pol et al., 2016; Ahorsu et al., 2019; López-Gómez et al., 2020; Cubas-Cano et al., 2020; Garita-Cambronero et al., 2021). However, most of these studies either focussed on using glucose-rich or mixed sugar hydrolysates to assess the performance of *B. coagulans,* but rarely xylose as the lone carbon source.

Although *B. coagulans* DSM2314 can metabolise both glucose and xylose, (Maas et al., 2008), this work focuses predominantly on xylose as the main carbon source. In the current study, the initial experiments were based on cultivating *B. coagulans* at different levels of pure xylose (50, 75, 100, and 150 g/L). The batch cultivations were carried out in bench-scale bioreactors as described in section 2.3 and the parameters examined were substrate utilization rate, cell growth, and LA production. Fig. 1 provides the time course profiles for xylose utilization, optical density (OD_{600}) and LA production at specific intervals. When the initial xylose concentration was 50 g/L, it was exhausted in 30 h leading to cell OD_{600} of 3.12 and LA accumulation of 38.7 g/L with a conversion yield of 0.79 g/g (Fig. 1A). However, further increase in xylose concentration prolonged the fermentation time. When the xylose concentration was raised to 75 and 100 g/L, it was completely consumed in 48 and 96 h respectively, with concomitant cell growth and LA production. The highest cell growth achieved was OD_{600} 5.48 and 5.26 with a LA titer of 64.9 and 75.9 g/L and yield of 0.83 and 0.75 g/g, respectively (Fig. 1B and 1C). However, further enhancing the xylose concentration to 150 g/L extended the lag phase and a strong inhibition on cell growth was observed. Even after 96 h of cultivation, the maximum OD_{600} recorded was 2.68 (Fig. 1D) with a substrate utilization rate of 0.33g/L. h. About 30 g/L xylose was consumed in 48 h with a LA titer of 17.2 g/L (Fig. 1D). The substrate inhibition was very clear at high xylose levels and the reason for low performance at 150 g/L could be due to the osmotic stress caused by high substrate concentration, which results in reduced water activity and plasmolysis. One-way ANOVA study revealed that that irrespective of substrate concentration, there was significant effect of fermentation time on the optical density of the bacterium (Table S1). The analysis further showed that as the initial

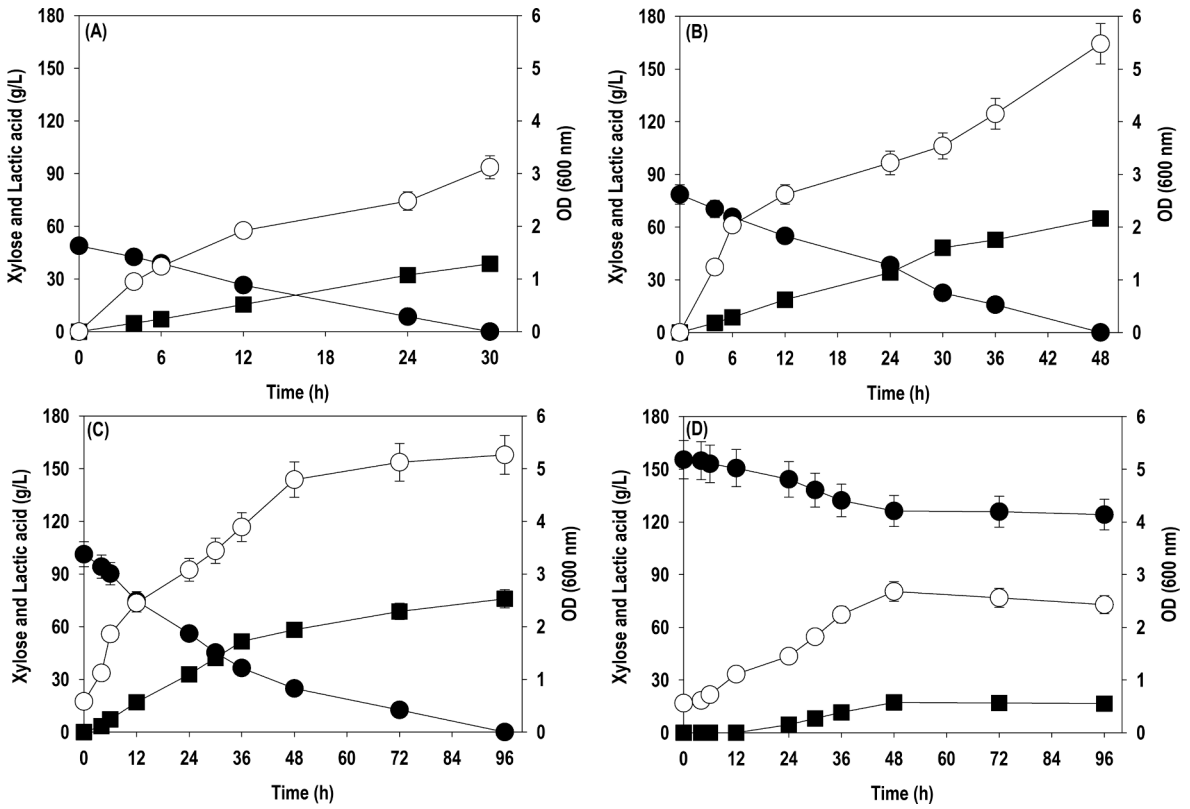


Fig. 1. Batch bioreactor cultivation of *B. coagulans* DSM2314 at different cultivations of xylose; (A) 50 g/L; (B) 75 g/L; (C) 100 g/L; (D) 150 g/L. Symbols: filled circle (residual xylose), open circle (OD_{600}), and filled square (LA).

substrate concentration increased, this impact became more significant. However, when paired *t*-test was performed to cross check the results obtained with one-way ANOVA, the outcome was slightly different. The analysis showed a positive correlation between fermentation time and optical density at all initial xylose concentrations. Particularly at initial xylose concentrations 75, 100 and 150 g/L, the *p* value between optical density and fermentation time was ≤0.016, indicating that the correlation was statistically significant (Table S2). Likewise, one-way ANOVA showed that when the initial xylose concentration were 50, 75 and 100 g/L there was no significant effect of fermentation time on residual xylose or increased LA titres. However, when the initial xylose concentration was raised to 150 g/L, there was a significant impact of fermentation time on residual xylose and LA accumulation titres as highlighted in Table S1. When the same parameters were studied using paired-*t*-test, fermentation duration exhibited a positive and negative correlation with LA and xylose concentrations, respectively. A very prominent and statistically significant correlation was observed at the 150 g/L initial xylose concentration, both in terms of residual xylose (*p*-0.000026) and LA accumulated (*p*-0.010), as reflected in Table S2.

3.2. Batch culture of *B. coagulans* for LA synthesis using hemicellulosic hydrolysate from OP hydrolysate

After pure xylose, xylose-rich OP hydrolysate was tested for LA production by *B. coagulans*. The OP hydrolysate with a xylose concentration of ~65 g/L was supplemented with other nutrients into the bioreactor to culture *B. coagulans* under non-sterile conditions for LA fermentation. Fig. 2 depicts time course profiles for xylose utilization, and LA production. As the hydrolysate utilized in the current study was non-detoxified, the analysis of optical density (OD_{600}) was difficult due to interference caused by particles in the suspension. The hydrolysate also had a substantial amount of acetic acid (~8.1 g/L). The xylose consumption rate was slower in comparison to pure xylose and about 20

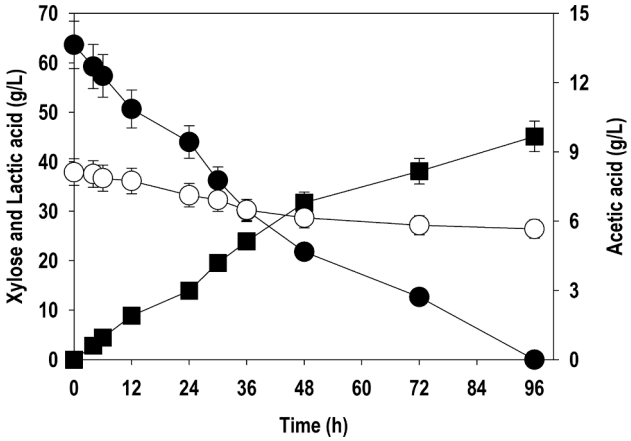


Fig. 2. Time course profiles for xylose uptake, LA production and AA (acetic acid) metabolism by *B. coagulans* DSM2314 during cultivation on OP hydrolysate. Symbols: filled circle (residual xylose), filled square (LA) and open circle (AA).

g/L of xylose was assimilated in the first 24 h. A similar rate was observed in the next 24 h which slowed down in the last 48 h where approximately 22 g/L xylose was utilized. A similar trend was also reflected in LA formation where 13.9 g/L LA was accumulated in the initial 24 h with a slight increment in the next 24 h reaching LA titer of 31.7 g/L. The final LA concentration amassed at the end of 96 h was 45.1 g/L with an overall yield of 0.71 g/g xylose. The AA level was reduced to a concentration of 5.7 g/L at the end of fermentation. Paired *t*-test analysis suggested that LA titres displayed a statistically significant positive correlation with the fermentation time (Table S3).

3.3. Batch LA production using xylose-rich hydrolysate from SCB

The xylose-rich hemicellulosic hydrolysate from SCB was employed for the biosynthesis of LA by *B. coagulans*. The variation in xylose assimilation, LA formation and acetate concentration during fermentation are shown in Fig. 3. The batch cultivation was initiated with an initial xylose concentration of 60.6 g/L in the SCB hydrolysate. The time course profile was similar to cultivation on OP hydrolysate. The xylose consumption was slow from the beginning and even after 96 h, xylose was not fully metabolized. Unlike pure xylose and OP hydrolysate, LA production commenced quite late in the case of SCB hydrolysate. The LA synthesis first appeared at 12th h and was very slow until 30th h when it reached 6.3 g/L. Thereafter, it was increased slightly, and the LA titer achieved at the end of 96 h was 36.9 g/L with a yield of 0.61 g/g. The LA titer and yield obtained with SCB hydrolysate were lower as compared to pure xylose and OP hydrolysate. When the statistical analysis was performed, the pattern obtained was identical to that observed with hemicellulosic hydrolysates of OP (Table S3). Rather in the present case, the positive correlation between the duration of fermentation and LA titre was more significant (p:0.004) compared to OP derived hemicellulosic hydrolysate (p:0.025). The AA concentration in the beginning was 7.6 g/L and reduced to 5.5 g/L at the end of the process. This substantial drop in AA level during LA fermentation in both OP and SCB hydrolysates, indicates its participation in the metabolism. The presence of AA and other inhibitors coming from biomass hydrolysate might be responsible for the low performance witnessed with SCB hydrolysate.

3.4. Fed-batch cultivation for LA production using pure xylose and crude xylose-rich hydrolysates

To achieve commercial viability, product titers should be comparable to market size and potential, but higher titers require high concentrations of substrate, which often leads to substrate mediated inhibition. This limitation can be addressed through the fed-batch process by constantly supplementing the substrate as feed to the microbial strain by maintaining the substrate concentration lower than the inhibitory levels . Following batch fermentation, fed-batch mode of cultivation was carried out to further enhance the LA titers using SCB and OP hydrolysates and compared the process efficiency with pure xylose. In batch cultivation, a decrease in substrate utilisation rate and LA productivity was observed as xylose concentrations exceeded 75 g/L. As a result, in the fed-batch mode, the initial batch cultivation was started with 50 g/L, and concentrated pure and crude feed was supplemented to adjust the xylose levels to 50–60 g/L when the concentration in the reactor was reduced to <10 g/L. In the case of fed-batch fermentation with pure xylose, when the initial substrate concentration was 51.2 g/L, ~80% xylose was utilized in 24 h resulting in OD_{600} and LA titer of 2.51 and 39.6 g/L, respectively (Fig. 4). The first feeding was done at 24 h when the xylose concentration was reduced to 8.7 g/L. As a result of it, xylose concentration enhanced to 61.3 g/L and 82% of substrate utilization was observed at the end of 72 h (48 h after Feed I) with a total LA accumulation of 79.3 g/L. Both xylose uptake and LA production rate in this phase were lower than in the previous one. The last feed of 45.3 g/L xylose was given at 72 h, increasing the residual xylose concentration to 55.8 g/L. In the last phase, xylose consumption and LA formation rates were significantly reduced. After 168 h, a residual xylose concentration of 22.5 g/L was observed and a final LA concentration of 97.8 g/L with an overall yield of 0.77 g/g was recorded. The cell growth (OD_{600}) was faster in the initial 24 h followed by a slow and steady increase until 96 h where it reached 4.12 and declined thereafter.

After pure xylose, *B. coagulans* was cultivated on OP & SCB hydrolysate in fed-batch mode. Fig. 5 shows the time course profiles for xylose consumption and LA accumulation from OP & SCB hydrolysate during the fed-batch mode of cultivation. The initial batch with 53.6 g/L xylose from OP hydrolysate was metabolized very slowly as compared to pure xylose and it took 72 h for xylose to drop below 10 g/L. At 72 h, the culture was supplemented with xylose feed increasing the xylose level to 58.2 g/L which was depleted in the next 96 h making the total fermentation time 168 h. The LA production started at 6th h and maximum production was observed between 24 and 72 h followed by a slow and steady increase until the end of fermentation when the total LA amassed was 61.3 g/L with a yield of 0.71 g/g. However, xylose was not completely utilized and even after 168 h, the residual xylose concentration observed was 20.0 g/L. The pattern of fermentation using xylose rich SCB hydrolysate in terms of xylose assimilation and LA biosynthesis was similar to OP hydrolysate, however, the fermentation rate was slower. Like batch fermentation, LA production was observed after 24 h and accumulated 18.1 g/L after 72 h. After the first feed, the concentration of LA was increased to 52.4 g/L at 168 h, with a yield of 0.66 g/g. At the end of cultivation, 21.6 g/L of xylose remained unutilized, as observed with pure xylose and OP hydrolysate. In comparison to the pure xylose, *B. coagulans* showed > 30% reduced substrate utilization rate, LA accumulation and productivity in crude hydrolysates, as shown in Table 1. It could be attributed to unidentified inhibitors such as AA, lignin-derived water-soluble inhibitors and sugar-degradation products like 5-HMF and furfural. Surprisingly, irrespective of source of xylose, the correlation between xylose consumption rate and LA yield was statistically insignificant (*Pearson correlation coefficient*-0.958; p (two tailed) – 0.147). However, LA productivity and its yield displayed statistically significant positive correlation (*Pearson correlation coefficient*-0.97; p (two tailed) – 0.03).

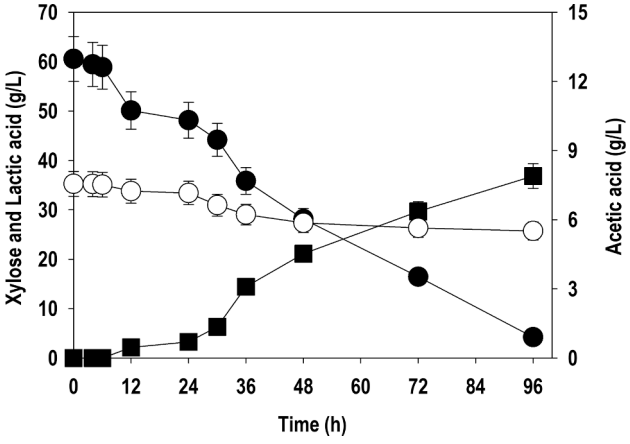


Fig. 3. Kinetics of xylose uptake, LA production and AA (acetic acid) metabolism by *B. coagulans* DSM2314 during cultivation on SCB hydrolysate. Symbols: filled circle (residual xylose), filled square (LA) and open circle (AA).

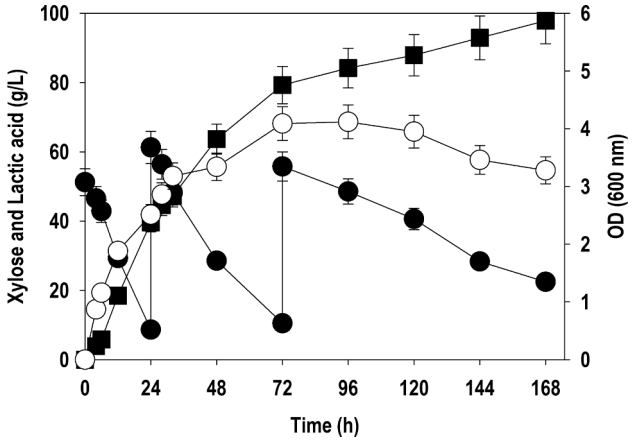


Fig. 4. Fed-batch culture of *B. coagulans* DSM2314 on pure xylose. Symbols: filled circle (residual xylose), open circle (OD_{600}), and filled square (LA).

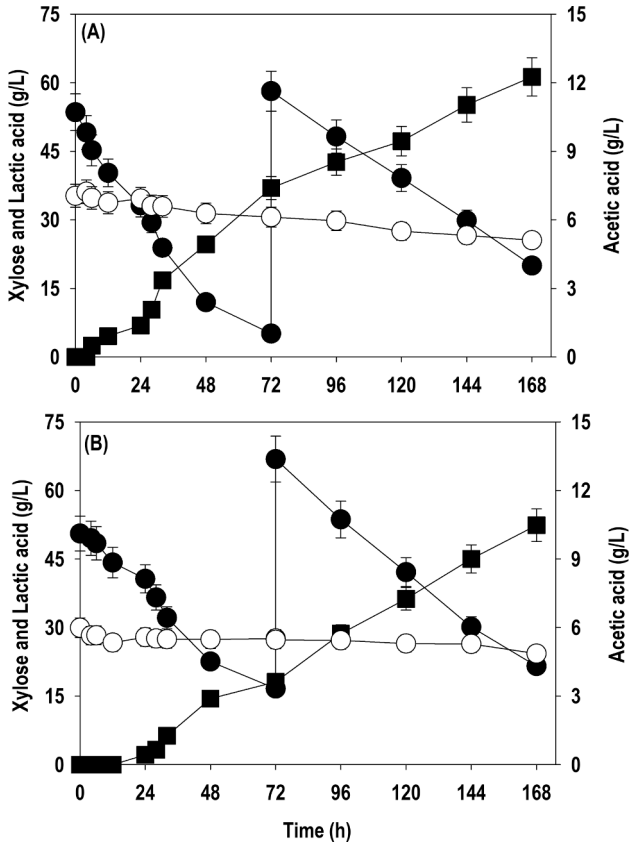


Fig. 5. Bioreactor cultivation of *B. coagulans* DSM2314 on xylose-rich (A) OP and (B) SCB hydrolysates in fed-batch mode. Symbols: filled circle (residual xylose), filled square (LA) and open circle (acetic acid).

3.5. Separation of LA from the fermented broth

Separation of LA from the fermented broth was carried out by adopting a two-stage aqueous two-phase extraction technique using 5–70% w/v ammonium sulphate as the salting-out agent and 1:1 (v/v) isopropanol as an extractant (supplementary Fig. 1). With the increase in ammonium sulphate concentration from 5 to 20% w/v, the partition co-efficient (K) and recovery yield (Y%) were increased from 0.9 to 1.75, and 50 to 68%, respectively. To maximize the recovery, yield the ammonium sulphate concentration was further increased from 20 to 70% w/v, which had no significant effect on recovery instead a negative impact was observed on K and Y values reducing the recovery to 54%. Hence, 20% w/v ammonium sulphate was considered as the optimal salt concentration for maximum partition and recovery of LA into the organic phase. Further, when the fermented broth from the fed-batch fermentation was used for the separation of LA, a partition co-efficient (K) of 1.92, 0.89, and 0.84, and recovery yield (Y) of 65.7, 48.6, and 45.8% was obtained on pure xylose, OP and SCB xylose-rich hydrolysates, respectively. It was observed that an additional 40–50% of LA was observed in the aqueous phase, therefore, after the separation of the top organic layer, fresh isopropanol was added to the aqueous phase in the second round of extraction and the process was repeated as described in section 2.4. During the second stage of extraction, the K values observed were 9.93, 5.89, 5.01 with a total LA yield of 90.8, 85.9, and 83.4% using pure xylose, OP and SCB xylose-rich hydrolysates (Table 2), respectively. When the statistical analysis was performed for both the stages of extraction, irrespective of origin of LA (pure or crude xylose), there was a positive significant correlation between partition coefficient and recovery yield of LA (%). The statistical significance was more pronounced in the Stage II (p-0.000083) compared to Stage I (p-0.012) as shown in Table S4.

Although fresh isopropanol was used in this study, distilled isopropanol from the first round can be reused in a larger scale or commercial scale applications. After two-stage extraction the aqueous phase or the fermented broth was left with <1 g/L LA in all three cases, which describes the efficiency of the process extracting >95% of the LA from the fermented broth. As the organic solvent (isopropanol) is easily vaporised and distilled compared to water, LA can be efficiently separated and purified from the organic phase. Further to that no additional by-products were observed other than LA as the *B. coagulans* DSM2314 strain is a homofermentative organism. Hence the process described here in two-stage ATPS extraction is an energy and cost-effective downstream process for the separation and purification of LA from the fermented broth.

4. Discussion

The major obstacles or limitations hindering the commercial viability of biological processes are expensive raw materials, nutrient sources, production processes and product separation. To meet the growing demand, these challenges must be addressed. It is said that the cost of LA must reduce by 50% for PLA to compete with commercially available plastics (López-Gómez et al., 2019). In the past few decades, studies revealed the tackling of the raw material problem by looking into affordable substrates from inexpensive renewable feedstocks like LCB such as agricultural or forest residues (Ma et al., 2022). In this study, two of the limitations of the LA production process are addressed, one utilization of cost-effective xylose rich SCB and OP hydrolysates as the sole carbon sources, and the second a simple separation and purification techniques for the recovery of LA from the fermented broth which usually totals to between 40 and 70% of production costs (Baral et al., 2021). Further, all the experiments were carried out under non-sterile conditions which would also contribute towards cutting down the operational cost as sterilization is an energy intensive unit operation.

Ye et al. (2013) investigated a newly isolated *B. coagulans* C106 for LA accumulation from xylose and found that increasing the xylose concentration from 85 to 117 g/L, reduced the LA productivity from 7.5 to 3.7 g/L. h. The study also revealed that neutralization with $CaCO_3$ was more effective than NaOH and improved the TYP metrics. When NaOH was used as neutralizing agent, 151 g/L of xylose was converted

Table 2
Partition co-efficient (K) and recovery yield (Y) of LA obtained using aqueous two-phase system (ATPS) extraction method.

Fermented Broth	1st Stage		2nd Stage	
	Partition Co-efficient (K)	Recovery Yield (Y)	Partition Co-efficient (K)	Recovery Yield (Y)
Pure Xylose	1.92 ± 0.11	65.7 ± 2.8	9.93 ± 0.6	90.8 ± 3.9
SCB	0.84 ± 0.05	45.8 ± 1.7	5.01 ± 0.2	83.4 ± 2.7
OP	0.89 ± 0.06	48.6 ± 1.5	5.89 ± 0.2	85.9 ± 2.5

Table 1
Comparison of results from fed-batch fermentation after 168 h using pure xylose, xylose-rich OP and SCB hydrolysates.

Source of Xylose	Xylose consumed (g/L)	LA (g/L)	Xylose consumption rate (g/L/h)	LA productivity (g/L/h)	LA Yield (g/g)
Pure xylose	126.7 ± 4.0	97.8 ± 2.1	0.75	0.58	0.77
OP-derived xylose-rich hydrolysate	86.6 ± 2.1	61.3 ± 1.1	0.52	0.36	0.71
SCB-derived xylose-rich hydrolysate	79.3 ± 1.7	52.4 ± 1.0	0.47	0.31	0.66

to 118.4 g/L LA with 0.78 g/g yield. However, the replacement of NaOH with $CaCO_3$ has marked effect, enhancing LA titre and yield to 140.9 g/L and 0.98 g/g respectively (Ye et al., 2013). These results affirm that the role of neutralizing agents also cannot be undermined in LA fermentation studies, which mandatorily required pH restoration. Though the trend was similar to our study, Ye et al., (2013) achieved higher volumetric productivities and titre, especially at elevated xylose levels which are contradictory to the current study, as the DSM2314 strain displayed significant inhibition at high xylose concentration (150 g/L) resulting in low cell density and LA titre (Fig. 1D). Likewise, Ma et al. (2016) achieved higher production using *B. coagulans* NBRC 12714 strain, where a total of 100 g/L xylose was completely consumed within 32 h resulting in a titre, yield, and productivity of 92.8 g/L, 0.93 g/g and 2.90 g/L. h, respectively. The results obtained in the present work are in consensus with the findings of Ouyang et al., (2012) wherein *B. coagulans* NL01 strain under non-sterile fermentation conditions produced 75 g/L LA from 100 g/L xylose with yield and productivity of 0.75 g/g and 1.04 g/L. h., respectively. However, recently a more robust *B. coagulans* DSM IS 14–300 strain isolated from hemp leaves was tested for LA production. The said strain was able to assimilate 117.7 g/L xylose in merely 25 h and accumulated 84.9 g/L of LA with a productivity of 3.4 g/L.h (de Oliveira et al., 2019). However, in the present study, further enhancing the xylose concentration to 150 g/L extended the lag phase and a strong inhibition on cell growth was observed. All these states of the art furnish strong supporting evidence that initial xylose concentrations in the range of 50–100 g/L can be efficiently transformed to LA by *B. coagulans* in a batch mode and also confirms wide diversity of xylose assimilation and its valorisation to LA among species level.

After pure xylose, non-detoxified SCB and OP hydrolysates were employed for LA production. The batch cultivations were started with an initial xylose concentration of 50 g/L containing substantial quantities of AA (8.1–7.5 g/L) with no other detectable inhibitors like furfural and 5-hydroxymethyl furfural (HMF). The xylose utilization rate in hydrolysate during batch fermentations was considerably lower when compared with pure xylose with an initial lag phase of 4–6 h resulting in LA titres of 36.9 and 45.1 g/L with 0.61 and 0.71 g/g yield from SCB and OP hydrolysates, respectively (Figs. 2 and 3). Noticeable inhibitory effects were experienced with both hydrolysates owing to the initial AA concentrations. Hydrolysis of lignocellulosic biomasses often creates inhibitory by-products to the fermentative process with detrimental effects and is a major limitation for LCB to be commercially used as a feedstock (Kawaguchi et al., 2022). To the best of our knowledge this is the first study where xylose-rich hydrolysate derived from OP has been valorised to LA. Similar to the current study, Ouyang and associates demonstrated a non-sterile LA fermentation using hemicellulosic hydrolysate obtained from corn cobs. The strain *B. coagulans* NL01 accumulated 18.2 g/L LA from xylose-rich hydrolysate with 0.73 g/g yield (Ouyang et al., 2012). Abdel-Rahman and associates evaluated the effect of inhibitors on growth and LA production efficiency of the *B. coagulans* Azu-10 strain. They found that increase in furfural (0–6 g/L) and HMF (1–5 g/L) concentrations decreased the cell growth (μ_{max}) from 0.80 to 0.16 h^{-1}, which was reflected in LA titres and yield. With an increase in furfural concentrations from 0 to 5 g/L, positive effects on LA titres were observed until 4 g/L (19 g/L versus 22.1 g/L), decreased to 15.9 g/L at 5 g/L furfural and further at 6 g/L furfural, LA production was completely inhibited. The cell growth was negatively affected with increase in HMF concentrations; however, LA titres and yields remain unaffected, which explains furfural had more inhibitory effect than HMF. In case of carboxylic acids AA concentrations between 5 and 15 g/L, 5 g/L formic acid, and 1–7 g/L levulinic acid was observed to have insignificant inhibitory effect on cell growth and LA production, but a further increase of AA and formic acid concentrations at 20 and 10 g/L, respectively, resulted in a drastic decrease in cell growth rate and almost 100% inhibition of LA production (Abdel-Rahman et al., 2021). These works align with the current study identifying AA as an important carboxylic acid inhibitor to both cell growth and LA production. Inhibitor tolerance reported was observed to be strain specific and depends on various conditions like the source of strain isolation, conditions of maintenance, pre-culture conditions etc. There is one interesting report by de Oliveira et al., 2019 where SCB-derived hemicellulosic hydrolysate was used for LA production, obtained by dilute hydrochloric acid hydrolysis. In the said study, *B. coagulans* DSM ID 14–300 strain o could detoxify hemicellulosic hydrolysate by assimilating furfural and HMF completely. A batch study with this homo-fermentative and thermophilic bacterium resulted in nearly complete consumption of all the sugars present in hydrolysate (xylose, glucose and arabinose- 48, 7.9 and 6.6 g/L) within 49 h. The maximum LA produced was 56.0 g/L, with yield and productivity being 0.87 g/g and 1.7 g/L. h, respectively. This study and the present investigation both confirm that besides AA, there are soluble inhibitors derived from sugars and lignin which are present in extremely low concentrations but may have a detrimental impact on the fermentative ability of microbes (de Oliveira et al., 2019).

The batch study also clearly revealed that after a threshold concentration, substrate inhibition was prominently exhibited. To further improve the LA titres for addressing the substrate mediated inhibition, a fed-batch mode of cultivation was performed using pure xylose, and xylose-rich SCB and OP hydrolysates resulting in a maximum LA titer of 97.8, 52.4, and 61.3 g/L with 0.77, 0.66, and 0.71 g/g yield, respectively (Figs. 4 and 5). In a study by Wang and associates, pure xylose and corncob molasses containing 9% w/v glucose, 45% w/v xylose, and 14.6% w/v arabinose was used as a substrate for *Bacillus* sp., XZL9 strain. Fed-batch fermentation using pure xylose resulted in a high LA concentration of 80.8 g/L with 0.98 g/g yield while a maximum LA concentration of 74.7 g/L with a yield of 0.82 g/g was achieved with corn cob molasses (Wang et al., 2010). Similar to the present work, Ye et al., (2013) were also able to attain high LA titres by shifting fermentation from batch to fed-batch mode. In their study, the initial xylose concentration was 120 g/L which was later fed with 80 and 60 g/L xylose in two stages. The maximum LA titre achieved was 215.7 g/L with productivity being 4.0 g/L. h. However, it should be noted that this LA concentration was obtained at the cost of extra yeast extract addition (10 to 20 g/L). Table 3 comparatively assesses the performance of *B. coagulans* DSM2314 against other potential xylose assimilating bacterial strains, which can biotransform xylose to LA and shows that a handful of examples of LA production from LCB-derived xylose sugar exist in the state of the art. The results accumulated on pure xylose in terms of titre, yield and productivity are comparable to most of the reports. The literature is scarce on the use of crude xylose for LA synthesis and comparing our result with these reports indicate that the *B. coagulans* DSM2314 used in the present work outperformed and exhibited robustness in terms of LA titre. Further, LA was amassed under non-sterile conditions using a meagre amount of complex nitrogen sources with a yeast extract concentration of 2.0 g/L. Besides glucose, the efficacy of *B. coagulans* in translating pure as well as crude xylose into LA provides an opportunity for biorefineries to utilize LCB-based feedstocks as alternative substrates at industrial levels. Most of the studies reported in the literature to improve the LA titres made use of glucose and xylose mixtures obtained after the enzymatic hydrolysis of lignocellulosic feedstocks. In the past five years, there has been practically no study on the valorisation of xylose-rich lignocellulosic hydrolysates to LA in a fed-batch mode. Conventionally, LA fermentation at an industrial scale is also run in batch mode, especially due to ease of operation. As a result, most of the researchers focus primarily on attaining high LA concentrations using a batch process. But authors are of the opinion that if LA titres are significantly enhanced by shifting from batch to fed-batch or continuous without compromising on yields and productivity, these modes should also be evaluated at a large-scale.

Separation of LA from fermentation broth is a significant cost associated step, accounting for up to 70% of the total production costs (Prado-Rubio et al., 2020). In the current work, an ATPS method was employed to recover accumulated LA and synthetic medium containing pure xylose displayed - higher LA recovery compared with both

Table 3
Summary of microbial lactic acid production from xylose.

Organism	Feedstock	Fermentation mode	Nitrogen source (g/L)	Operating condition	Lactic acid			Reference
					Titer (g/L)	Yield (g/g)	Productivity (g/L. h)	
Bacillus sp. strain XZL9	Pure xylose	Fed-batch	YE (10)	Sterile	133.8	–	0.61	Wang et al. 2010
	Xylose-rich corncob molasses				74.7	0.50	0.25	
Enterococcus mundtii QU 25	Pure xylose	Batch	YE (4); P (10); BE (8)	Sterile	86.7	0.85	0.90	Abdel-Rahman et al., 2011
B. coagulans 36D1	Pure xylose	Fed-batch	YE (5); P (10)	Sterile	163.0	0.87	0.75	Ou et al. 2011
B. coagulans NL01	Pure xylose	Batch	YE (2.5)	Non-sterile	75.0	0.88	1.04	Ouyang et al. 2012
	Corn stover prehydrolysate				18.2	0.73	0.38	
B. coagulans C106	Pure xylose	Fed-batch	YE (20)	Non-sterile	215.7	0.95	4.0	Ye et al. 2013
B. coagulans NL-CC-17	Pure xylose	Batch	YE (9.3); CSL (4.5); T (3)	Non-sterile	90.3	0.90	0.75	Zheng et al., 2014
	Corn stover prehydrolysate				23.5	0.83	0.65	
Enterococcus mundtii QU 25	Pure xylose	Continuous with cell recycling	YE (12.5); CSL (12.5)	Sterile	41.0	1.01	6.15	Abdel-Rahman et al. 2016
B. coagulans IPE22	Pure xylose	Batch	YE (5); P (10); BE (10)	Sterile	49.8	0.90	2.77	Wang et al., 2018
	Corn cob hydrolysate				53.5	0.92	2.97	
B. coagulans DSM ID 14–300	Pure xylose	Batch	YE (20)	Sterile	84.9	0.72	3.40	de Oliveira et al., 2019
	SCB hemicellulosic hydrolysate			Sterile	56.0	0.87	1.70	
P. acidilactici XH11	Corn cob hydrolysate	Batch	YE (10); P (10)	Sterile	61.9	0.48	0.64	Qiu et al., 2022
*B. coagulans*DSM2314	Pure xylose	Fed-batch	YE (2)	Non-sterile	97.8	0.77	0.58	This study
	Xylose-rich OP hydrolysate				61.3	0.71	0.36	
	Xylose-rich SCB hydrolysate				52.4	0.66	0.31	

BE: Beef extract; CSL: Corn steep liquor; P: Peptone; T: Tryptone; YE: Yeast extract.

hydrolysate-based fermentation broths (Table 2 and supplementary Fig. 1). This is most likely attributed to the residual and particulate matter in the fermentation broth from both the hydrolysates. Most other downstream processing studies use either pure media or synthetic media designed to mimic the real fermentation broth, which limits the validity when excluding chemicals and suspended solids which may otherwise interfere with the separation process. The study by Baral et al. (2021) used fermentation broth from SCB and was able to reach a separation efficiency of 86% using 60% w/v ammonium sulphate and ethyl acetate as the extractant. In another study by Aydoğan and associates, ethanol and dipotassium hydrogen based ATPS system was evaluated at different concentrations for the separation of LA accumlated on whey-based fermentation medium. The study successfully separated LA from a synthetic fermentation broth with a separation yield of 80% and partition coefficient of 2.06. (Aydoğan et al., 2011). Yan and associates conducted LA separation using a sugaring out technique. The simulated fermentation broth was used to assess different solvents and sugars. Isopropanol as an extractant achieved the highest separation yield along with glucose as an optimal sugaring agent . A separation mixture of isopropanol (40% w/w) and glucose (12% w/w) was utilised for the successful separation of LA from the fermentation broth with a yield of 84.3% and a partition coefficient value of 1.39 (Yan et al., 2016). Although various reports are available on the successful separation of LA, most of the studies used simulated fermented broths, however, the results may not be replicated when real fermented broths are used due to the presence of unknown contaminants, proteins etc.

5. Conclusion

The maximal utilization of fermentable sugars from LCB feedstocks is vital to the sustainable economic production for green materials. The current study evaluated the use of pure xylose and two xylose-rich SCB and OP hydrolysates for LA production using thermophilic *B. coagulans* DSM2314 strain under non-sterile conditions with a circular biorefining approach. The novelty of the study lies in exploiting the potential of *B. coagulans* DSM2314, which produced appreciable titers of LA from xylose and xylose rich hydrolysates of OP and SCB under non-sterile condition. Furthermore, LA was successfully recovered from the fermented broth using a two-stage ammonium sulphate and isopropanol based ATPS extraction. This study showcases that besides glucose, the efficacy of *B. coagulans* in translating pure as well as crude xylose into LA provides an opportunity to completely utilize the renewable carbohydrate fraction in LCB-based biorefineries. The biorefinery approach of using abundant lignocellulosic waste and low-cost purification and separation methodologies can spearhead LA production in an environmentally and cost-effective manner. Further to this work, a technoeconomic assessment and LCA studies are underway to evaluate the potential of *B. coagulans* DSM 2134 strain in the valorisation of hexose and pentose sugars from LCB feedstocks to showcase the economic feasibility and commercial potential.

Contributions

RC, VN and EC carried out all the experimental work. RC, VN, and VK analysed the data and wrote the Manuscript. DA provided useful suggestions for experimental design and revised the Manuscript critically. SJ, GK and DK were involved in proofreading and revised the Manuscript critically. All authors have read and approved the final Manuscript.

Declaration of Competing Interest

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

Data availability

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.05.015.

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