ORIGINAL ARTICLE

Recovery of acetoin from *Bacillus subtilis* fermentation broth by supercritical CO_2 extraction

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Abstract
Component enrichment from fermentation broths by solvent extraction using supercritical carbon dioxide (sCO_2) has been demonstrated in the literature. This work investigates for the first time the feasibility of the enrichment of an acetoin fraction from a real fermentation broth at a pilot plant scale using sCO_2. A 4-m-tall, 28-mm-diameter, counter-current column packed with pall rings was used. The ranges of process pressure and temperature investigated were 100 to 300 bar, and 37 to 80 °C respectively. The optimum recovery of acetoin was 77.8%, with little difference between the simulated and actual broths. A modest two-fold concentration of acetoin was obtained in the extract. The results show that where a modest enrichment of the targeted product makes a significant difference in subsequent separation processes, and where the purity of the product, particularly from harmful solvents, is important, sCO_2 fluid separation is a credible option for the enrichment of such products of fermentation.

Keywords Acetoin · Bioproduct · Fermentation · Recovery · Supercritical CO_2 extraction

1 Introduction

The biological production of sweeteners and flavorants is gaining prominence due to the health benefits as a product of natural origin. While consumption of artificially produced flavorants has been linked to diseases such as obesity, heart disease, hypertension, diabetes, and cancer [1], some flavors such as 2-phenylethanol, acetoin, vanillin, and ketones, when derived from biological sources, support healthy living [1]. Consequently, a lot of effort has been directed toward developing biological sweeteners and flavorants. The economics of biologically produced acetoin may be improved by replacing the expensive nitrogen source with a cheap source, corn steep liquor [2]. More recently, it has been shown that a six-fold improvement in acetoin yield can be obtained by changing from shake flask to bioreactor in an optimization study [3].

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Although there are several studies on the synthesis of bioproducts [1, 4–6], the low concentration of the desired bioproduct makes their recovery from fermentation broth difficult, and the rheology of fermentation broth containing the products [7]. Choosing suitable separation and purification techniques significantly affects acetoin production pathways. Acetoin has a great affinity for water, which makes its recovery from fermentation broth difficult. Separation of 2,3-butanediol, an analogue of acetoin, from aqueous solution, on the other hand, has been extensively explored for a long time [6, 8, 9]. Techniques such as steam stripping, solvent extraction, pervaporation, hybrid, and vacuum membrane distillation [8, 10–12] have been used for its separation from the aqueous solute. Acetoin can be recovered from fermented broth using various techniques, as described in Table 1. Although high recovery was reported using these techniques, solvents used in acetoin recovery tend to persist in the product, which has been reported to have carcinogenic effects [13, 14]. Distillation techniques may impose some problems such as loss of the valuable component through entrainment (since acetoin is recovered in the bottoms) [7], while the thermal degradation of acetoin may occur if operated at an elevated temperature above 100 °C. Furthermore, some of these techniques necessitate complex purification steps to remove solvent residues. [15], which may impact

production costs as well as the environmental friendliness of the recovery process.

A safe substitute for conventional solvent extraction has been shown to be supercritical carbon dioxide (sCO_2) [17, 18]. The solvent power of sCO_2 is proportional to its density, which may be easily changed by varying the operating condition(s) (temperature and pressure) to allow the dissolution of different solutes/fractions. Besides being chemically inert, not flammable, and environmentally friendly, CO_2 low critical temperature allows the process to be finished at nearly ambient temperatures, avoiding thermal deterioration of the targeted product [19]. There is growing interest in using supercritical fluid extraction to fractionate bioactive materials from microbial fermentation. [18, 20].

The aim of this study was to investigate the feasibility of recovering acetoin from a typical fermentation broth in a sCO_2 pilot plant. Specifically, the objective was to investigate the recovery of acetoin from a simulated broth with a view to determine the operating parameters that could maximize acetoin recovery from a real fermentation broth at a pilot plant scale of operation. The study may provide a basis for the development of a database for downstream processing of fermentation broth of bioproducts such as acetoin which may find application in the fermentation industry.

2 Materials and methods

2.1 Chemicals

Sigma-Aldrich, South Africa, provided acetoin (natural, purity 95%, CAS 513–86-0). An 88.11 g/mol crystalline dimer acetoin was procured and used in preparing the simulated broth. An air-tight dip-tube cylinder was filled with 99.98% pure liquid CO_2 from Air Liquide (Pty) Limited, South Africa. Prior to plant operation for the experiment, the pilot plant was cleaned with ethanol absolute (99.9%, B&M Scientific).

2.2 Feed preparation

2.2.1 Simulated broth

One gram of acetoin was dissolved in deionized water to equal one liter of acetoin. This study used a 25-L volume of simulated broth that contained a 1 g/L concentration. Using a magnetic stirrer, the solution was thoroughly mixed to ensure homogeneity.

2.2.2 Actual fermentation broth

Freeze-dried *Bacillus subtilis* CICC 10025 (China Center of Industrial Culture Collection) was revived in sterile *Bacillus* medium and periodically subcultured to maintain strain viability and purity. The optimized fermentation conditions (glucose concentration, corn steep liquor, and inoculum size as 78.40 g/L, 15% w/v, and 2.70% v/v, respectively) for acetoin production (10.70 g/L acetoin in the fermentation broth) as established in our previous study were used in the broth preparation [2].

2.3 Supercritical CO_2 equipment

This study was conducted using pilot plant scale sCO_2 equipment (SEPAREX SFE-5) for solids and liquid feeds (Fig. 1). An extraction section was used for solids processing, and a countercurrent fractionation section was used for liquids. In both sections, extracts and solvents were separated by using the same separating vessels. In this study, acetoin broth was recovered through counter-current fractionation. Figure 2 shows the flow diagram of the counter-current fractionation unit of the pilot plant used in this study. Detailed information about the equipment parts, code, and specifications can be found in Table 2. Using the

Table 1 Recovery of acetoin using separation techniques

S/N	Separation technique	Extractant	Recovery (%)	K	Broth source	Authors
1	Sugaring-out extraction	Different organic solvent/glucose systems	61.2	0.61*	*B. subtilis DL01*	[4]
2	Single-stage salting-out extraction	Ethanol	95.3	5.56	*B. subtilis DL01*	[16]
3	Two-stage countercurrent salting-out extraction	Ethyl acetate and dipotassium phosphate	91.3	18.7	*B. subtilis DL01*	[16]
4	Salting-out extraction	Acetone/phosphate	96.4	22.3	*Serratia marcescens H32*	[5]
5	Supercritical fluid extraction	Carbon dioxide	78	20.61	Simulated broth	Present study
6	Supercritical fluid extraction	Carbon dioxide	77	14.33	*B. subtilis CICC 10025*	Present study

*Calculated; $K = C_E/C_R$; K distribution coefficient, C_E concentration of solute in the extract, C_R concentration of solute in the raffinate respectively

Fig. 1 Pilot plant set up for recovery of acetoin broth

designed specifications (Table 3), column temperature and pressure were varied.

An internal diameter of 28 mm is present in the packed column (C42) with a height of 4 m; a viewing film is located at the base of the column below the injection nozzle. Four separate heating jackets regulate the column's temperature, which can reach 350 bar at its maximum

pressure. A double tube heat exchanger cooled carbon dioxide to 275 K before being pumped and then heated to the working temperature. At a maximum achievable pressure of 700 bars, a high-pressure piston pump delivered 300 g of liquid carbon dioxide per minute (18 kg per hour). The supercritical solvent left the column and was heated with an electrical heater to reduce its solvent power, although it was not used to fractionate the broth. By using a backpressure regulator, a pressurized cyclonic separator was used to recover the extract from the overhead current. Carbon dioxide consumption was reduced by recycling the recovered solvent and condensing it into the cooler. A feed pump with a flow rate of 0–50 ml/min and a maximum pressure of 700 bar supplied the broth.

2.3.1 SFE pilot plant experimental design for recovering acetoin from broth

Experimental study trends and literature surveys were used to develop the design and operating conditions for the study (one factor at a time) [21, 22]. Under these conditions, the density of sCO_2 is near the normal liquid density, which causes it to have a high solvent power [23]. The study was conducted using both simulation broth (SB) and fermentation broth (FB). Table 3 shows the experimental design showing the operation of the column and the feed that was used.
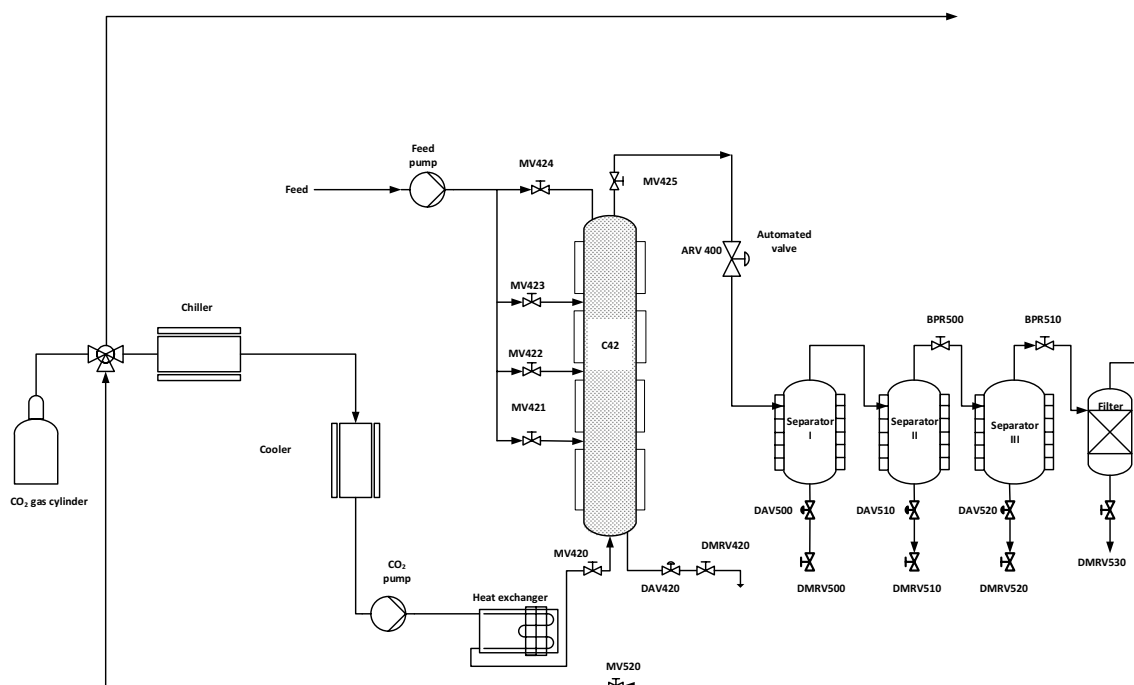

Fig. 2 Process flow sheet of the fractionation unit of the pilot plant

Table 2 Fractionating pilot plant equipment parts, code, and specifications

Equipment parts	Code	Column maximum specifications
Column	C42	350 bar, 150 °C, 4-m height, 28-mm internal diameter
Cyclonic separators	S50, S51, and S52	200 bar, 150 °C, 0.6 L
CO_2 pump	P200	300 g/min (18 kg/h), 700 bar max
Feed pump	P210	0–50 ml/min, 700 bar max
Reflux pump	P400	0–50 ml/min, 400 bar max
Cold exchangers	CE1000 and CE2000	Cooled by water and glycol mix
Heat exchangers	HE3000	250 °C
Chiller	C2000	4 kW, 20 °C

Table 3 Feed types, operating conditions, concentration factors, and acetoin recovery using sCO_2 extraction pilot plant

Runs	Feed	Total mass of feed (g)	Operating conditions			Total mass of extract (g)	Recovery % $\left(1 - \frac{E}{F} \times 100\right)$	CF $\frac{conc\ of\ E}{conc\ of\ F}$	Total Conc of E (g/L)	Conc of F (g/L)
			Temperature (°C)	Pressure (bar)	CO_2 feed rate (kg/h)					
1	SB	789.97	80	200	10	136.09	83	6.41	4.875	0.76
2	SB	603.99	37	200	10	108.23	82	2.30	1.710	0.76
3	SB	623.65	37	300	10	178.59	72	4.00	3.035	0.76
4	SB	532.73	37	300	15	118.26	78	5.04	3.832	0.76
5	FB	603.99	37	100	5	266.00	56	2.00	1.410	0.76
6	FB	603.99	37	200	10	195.75	68	1.20	0.910	0.76
7	FB	617.00	37	300	15	142.00	77	1.40	1.06	0.76

SB simulated broth, *FB* fermentation broth, *CF* concentration factor, *Conc* concentration, *E* extract, *F* feed

2.4 SFE pilot plant operation for the recovery of acetoin from the broth

As soon as the pilot plant reached steady state, the feed pump introduced liquid feed (acetoin broth), and the CO_2 was passed through a chiller to maintain its liquid state. Pump flow rates were adjusted based on experimental design specifications using a supervisory control and data acquisition system. In order to cool the pump, the condenser was used. Before entering the column, the CO_2 was heated using a heating block so that it would have a phase change from vapor to gas. In addition to pumping up the feed (acetoin broth), CO_2 was bubbled up the column at the same time. The constant temperature was maintained along with the height of the column with the aid of heating jackets. As soon as the separator vessels were depressurized, the CO_2 solvent selectively dispersed the desired component from the liquid feed (acetoin broth). At the bottom of the column, a sump was used to collect the remaining liquid. The backpressure regulating valve causes a significant drop in temperature upon depressurization of CO_2, as it exits the column. After releasing the solutes, the solvent is recycled. A heating strip is wound around the line to prevent the solvent from freezing due to the Joule-Thompson effect.

3 Analysis

3.1 Sampling

A sample of raffinate and extract was collected from the bottom of the separation vessel's column. During the separation process, broth samples were taken every 30 min for the extract, while samples were taken every 5 min for the raffinate. A label was attached to each sample indicating its temperature, pressure, and CO_2 flow rate. Different sampling times were on the raffinate side, to allow enough sample to accumulate in the sump of the column, but not to wait long enough to flood the column with liquid, and, on the extract side, to minimize errors resulting from the collection of too small a sample. The samples were taken from the raffinate, and extracts were measured to obtain their flow rates, using a balance, and analyzed for their composition using the spectrophotometric method.

3.2 Acetoin concentration determination

Based on Westerfield's modified Voges-Proskauer reaction (VP), acetoin concentration was determined [24]. A 25-ml calibrated flask was filled with an aliquot of the sample

solution followed by 2.5 ml of 1-naphthol solution and 1.0 ml of creatine solution. The solution was kept at 30 °C after being adjusted to volume and shaking vigorously. A UV–Visible Spectrometer of 2020 GBC Cintra model was used to measure the absorbance after 40 min at 530 nm [25].

3.3 Estimation of concentration factor and percentage recovery

The concentration factor indicates how many times the feed component has been concentrated in the extract. Equation 1 shows acetoin concentration as a function of extract concentration (g/L) divided by feed concentration (g/L):

$$\text{Concentration Factor} = \frac{\text{concentration of extract}}{\text{concentration of initial feed}} \quad (1)$$

The percentage recovery was obtained by the mass (g) of extract, divided by its mass (g) in the initial feed solution relative to the mass of raffinate in the column of the plant as shown in Eq. (2) [21, 22]:

$$\text{Recovery} (\%) = 1 - \frac{\text{mass of the extract}}{\text{mass of the initial feed}} \times 100\% \quad (2)$$

4 Results and discussion

4.1 Acetoin concentration and recovery from the simulated broth

4.1.1 Effect of contact time

The first experiment, Run 1, examined the effect of contact time. Based on the equilibrium solubility data for acetoin in supercritical CO_2 reported in Effendi et al. [26], the operating parameters of a batch operation were set at 80 °C, 200 bar, and 10 kg/h of CO_2. The solubility of water in sCO_2 is also demonstrated by Wang et al. [27]. Figure 3 a and b show how contact time affects the concentration of acetoin in the raffinate and the extract, respectively. As determined using the thermodynamics handbook [28], Table 4 shows the conditions of the separators and the corresponding densities of the solvent (CO_2) based on temperature and pressure.

The concentration profile of the raffinate can be divided into two sections. It can be seen that acetoin concentration in the raffinate decreased exponentially with time from 1 to 0.26 g/L (Fig. 3a) in the first 30 min in the first section of the plot, and the second section characterized by somewhat constant solute concentration, and an extended period of operation of 270 min, the value remained approximately constant at 0.3 ± 0.23 g/L. The concentrations of acetoin in the extract, 4 g/L, in separators 2 were about four times the
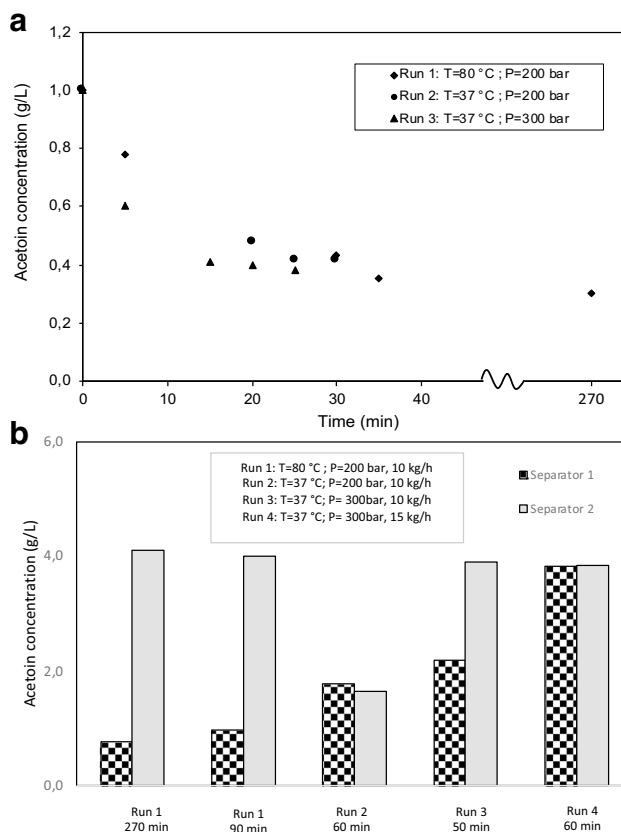
Fig. 3 **a** Effect of contact time on acetoin concentration in the raffinate and **b** concentration of acetoin in the extract tapped from extractors (1 and 2) at selected sampling time

Table 4 Separator conditions and corresponding densities

Separator	Temperature (°C)	Pressure (bar)	Density (kg/m^3)
1	18	54	794.8
2	29	45	147.3

concentration in separator 1 (Fig. 3b) when both were tapped at 90 and 270 min at their respective conditions referenced in Table 3. It is important to note that there is no significant difference between the acetoin concentration of extract tapped at 90 and 270 min (Fig. 3b). Given that 4.875 g/L and 0.761 g/L acetoin concentration were obtained in the extract and the feed respectively, a concentration factor of 6.41 was achieved. Our results also show that a recovery of 83% can be obtained from the recorded mass of extract relative to 789.97 g mass of the feed. While noting the significance of fractionation time on the technical and economics of acetoin concentration using sCO_2, our data suggests that there is no significant difference between the concentration factor beyond 30 min. Thus, it may not be necessary to

extend the fractionating time for acetoin concentration and recovery beyond this period since the system has reached a steady state. It is important to note that with the scale of our operation being at the pilot plant, the sampling time interval (about 60 min) was chosen carefully in order not to disturb the equilibrium of the system.

4.2 Effect of temperature

In an attempt to integrate bioprocess with high-pressure technology [13], an additional experiment was conducted at fermentation temperatures to avoid possible thermal damage to the product. This may explain why it is desirable, especially in food-related industries, that solvent temperature be kept low [29], as Eller et al. [30] reported the proportional relationship between capital costs or energy inputs and pressure and/or temperature requirements of supercritical fluid separation. The effect of temperature was investigated in the second experiment, Run 2, by setting the column temperature to 37 °C, the fermentation temperature, while pressure and CO_2 feed rate were kept constant at 200 bar and 10 kg/h and operated in a batch mode. The extraction/contact time was limited to 30–90 min (Fig. 3a) based on the experience gleaned from Run 1.

The result showed that by reducing the column temperature from 80 to 37 °C, the concentration factor of acetoin reduced from 6 to 2 within the first 30 min contact time with a marginal decrease in percentage recovery (i.e., from 83 to 82% for Run 1 and Run 2 respectively as shown in Table 3). The acetoin concentration of the extract in separator 1 was about two times the concentration obtained during Run1 in the same separator that was tapped off at 90 min of operation, while the acetoin concentration in the extract from separator 2 was approximately half of the concentration obtained in separator 2 in Run 1 (Fig. 3b).

4.3 Effect of pressure and CO_2 flow rate

The effect of operating pressure on the extraction of acetoin was investigated at the fermentation temperature, 37 °C, and CO_2 feed rate of 10 kg/h at the same mode of operation. The data shows that increasing the operating pressure from 200 to 300 bar shows a modest decrease from 82 to 72% in the recovery of acetoin and a corresponding increase in a concentration factor of acetoin in the extract from 2 to 4 at 25 min contact time. The concentration of the extract in separators 1 and 2 increased by two and four-fold respectively to that of the feed after 50 min contact time (Fig. 3b). This study also showed that an increase in CO_2 flowrate to 15 kg/h at the operating pressure of 300 bar (Run 4) reduced the acetoin concentration

in the raffinate, thereby increasing the recovery of acetoin from 72 to 78% when compared to Run 3; this resulted to an increase in concentration factor from 4 to fivefold, i.e., there was a fivefold increase in the concentration of acetoin extract in separator 1 and 2 after 60 min (Fig. 3b). The reason could be that the mass transfer resistance of the solvent limits how much solute can be transferred to the bulk of the solvent, resulting in less solute transport into the bulk of the solvent and less saturating of sCO_2 after it exits the column. The mass transfer resistance decreases as the flow rate increases until the exiting solvent is saturated, and therefore equilibrium has been achieved and recovery may have increased [31].

4.4 Acetoin concentration and recovery from the fermentation broth

The fermentation broth was centrifuged and fed directly to the column of the pilot plant. The conditions for the acetoin recovery studies were selected based on the simulated experiments above and data from previous studies [21, 22]. The view was to show that the data derived from simulated broth can reasonably predict the acetoin recovery from fermentation broth.
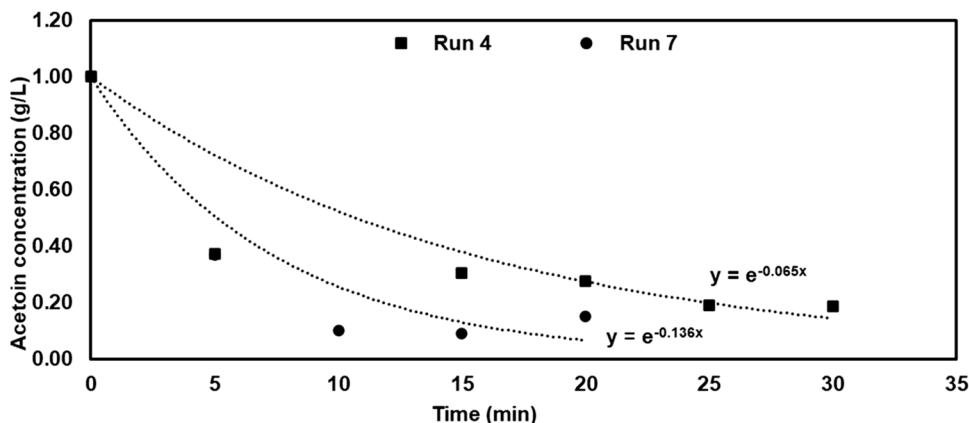
Figures 4 and 5 show the plots of the concentration of acetoin in the raffinate versus sampling time for simulated (Run 2 and 4) and fermentation broth (Run 6 and 7). It is clear from the figures that the two graphs have the same trend, and it can be shown that the concentration profile of acetoin in the fermentation broth can be reasonably predicted using the simulated data. It should be noted that the hydrodynamics in the column may not be the same at the same condition of operation; this may explain the scattered data around the line of best fit in the figures. Also, another factor is that the rheology of the simulated and fermentation broth may not be the same [32]. It has been earlier reported that factors such as the difference in feed composition would affect the rheological properties of the fermentation fluid in a system [33, 34].

Figure 6 represents the concentration of acetoin in the extract tapped from separators for simulated (Run 2 and Run 4) and fermentation broth (Run 5, Run 6, and Run 7). Although the concentrations of the acetoin extract in the separators were less than 2 g/L for all the studies involving fermentation broth, it can be shown that the acetoin recovery from the simulated and actual fermentation broth were 77 and 78% respectively (Table 3). The result shows for the first time that pilot plant data of simulated broth could be used as a basis to predict operating conditions for the recovery of acetoin from the actual fermentation broth.

Fig. 4 Acetoin concentration in the raffinate versus sampling time at 37 °C, 200 bar, 10 kg/h for simulated broth (Run 2), and fermentation broth (Run 6)

Run 2   Run 6

$y = 1.001e^{-0.033x}$

$y = 1.001e^{-0.082x}$
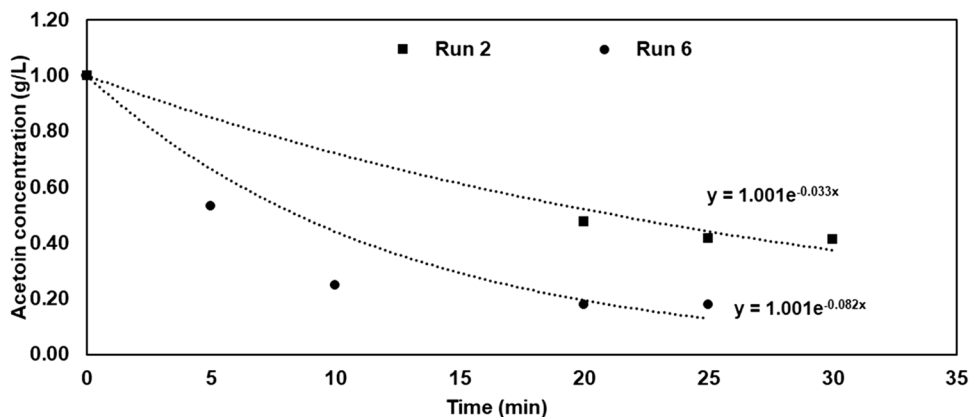
Fig. 5 Acetoin concentration in the raffinate against sampling time at 37 °C, 300 bar, 15 kg/h for simulated broth (Run 4), and fermentation broth (Run 7)

Run 4   Run 7

$y = e^{-0.065x}$

$y = e^{-0.136x}$

Fig. 6 The concentration of acetoin in the extract tapped from the extractor

Simulated broth (g/L)   Fermentation broth (g/L)

3.832

1.71

0.91

1.41

1.06

Run 2 Run 6   Run 5   Run 4 Run 7
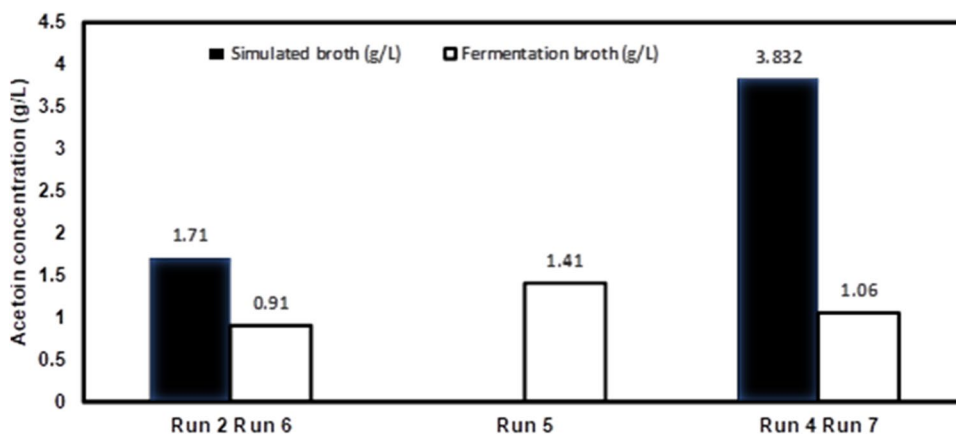
5 Conclusions

Although several separation techniques have been used for acetoin extraction with improved recovery yield, the subject of harmful residues and environmental unfriendliness associated with the solvent utilized in previous extraction research remains unsolved. The results of this study reveal that using supercritical CO_2, acetoin can be recovered at 77 and 78% in simulated and fermented broth respectively (Table 1), and the discrepancy may be explained by the inconsistency of the hydrodynamics in the pilot plant as well as the difference in the rheological properties of both simulated and fermentation broth. This finding could serve as a basis for the development of a database for the downstream processing of fermentation broth of bioproducts which could find use in the fermentation industry.

Data availability All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication All the authors approved the consent for publishing the manuscript to Biomass Conversion and Biorefinery.

Competing interests The authors declare no competing interests.

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