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Extractive Fed-Batch Bioacetone Fermentation with Free Cell and Immobilized *Clostridium Saccharoperbutylacetonicum* N1-4 in a Large Extractant Volume

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Abstract. This study compared free and immobilized cells with palm oil and hexanol as an extractant in a large volume ratio of extractant to the broth. Fermentation was conducted using TYA (Tryptone-Yeast-Acetate) media, glucose as the substrate and the palm oil, hexanol, and a mixture of hexanol and palm oil with a composition of 1:1 v/v as an extractant in the fermentation with a large volume ratio of extractant to broth of 5. The strain was inoculated as free-suspended cells of *C. saccharoperbutylacetonicum* N1-4 and immobilized cells in calcium alginate. The best extractant results for the acetone fermentation process in the ratio of extractive fermentation of free cells and immobilized cells were using palm oil as extractants. This extractant harvested acetone well, with the acetone concentration in the extractant at 18.41 g/L in free cell fermentation and 17.07 g/L in immobilized cell fermentation. The maximum total acetone concentration was up to 93.72 g/L broth. Using palm oil as an extractant in a large extractant volume combined with immobilization is an alternative to enhance bioacetone production by effectively reducing the toxicity effect of the products and minimizing nutrient requirements.

Keywords: Bioacetone; Cell immobilization; *Clostridium saccharoperbutylacetonicum* N1-4; Extractive fermentation; Free cell fermentation

1. Introduction

Most commodity chemicals and fuels are originated from non-renewable fossil reserves (Alimny *et al.,* 2019). As one of the products, acetone has a global market of about US$4 billion in 2021 (Amezquita-Ortiz *et al.,* 2022). In industry, acetone was utilized as a solvent and platform chemical to generate materials such as acrylic glass and polypropylene. Moreover, in the fuel sector, acetone was employed to boost the fuel's performance (Aguado-Deblas *et al.,* 2020). Acetone is generally manufactured via the cumene process as a by-product of phenol production (Kökdemir and Acaralı, 2021). The process involves propene cracking, which is an energy-intensive and hazardous process (Liew *et al.,* 2022). Acetone can also be obtained from renewable resources such as lignocellulosic biomass, algae, waste streams, industrial by-products, etc., through an

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acetone-butanol–ethanol (ABE) fermentation with a volumetric ratio of 3:6:1 (Etteh *et al.,* 2021; Veza, Said, and Latiff, 2021; Abo *et al.,* 2019; Liu *et al*., 2019). ABE fermentation using renewable raw materials consumes lower energy for production and releases no hazardous waste. This process is a promising alternative pathway for acetone production in the future for energy-efficient and environmentally friendly chemical manufacture.

C. saccharoperbutylacetonicum grows at the optimum temperature of 30 °C, relatively close to ambient temperature compared with other strains, which are mostly optimum at a higher temperature of 37 °C, reducing the energy requirement during fermentation. *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) was proven to be able to convert many kinds of substrates to ABE, such as glucose, lactic acid (Oshiro *et al.,* 2011), xylose (Zheng *et al.,* 2013), cellobiose (Noguchi *et al.,* 2013), xylan (Al-Shorgani, Kalil, and Yusoff, 2011), hydrolyzed agricultural waste (Wu *et al.,* 2021; Qureshi *et al.,* 2008), and wood (Zheng *et al.,* 2013).

The major limitation of the ABE fermentation process is the complex composition of products with low concentrations, which resulted in the high energy demand of downstream separation of the ABE fermentation from broth (Cai *et al.,* 2022). In addition, the inhibition of ABE products (i.e., butanol and acetone) limits product titer because of the toxicity to the producer strain. This condition challenges the economic viability of the whole ABE process (Sarangi and Nanda, 2018). The proposed solution is the acetone recovery as fast as it is generated, thus preventing the acetone concentration under the inhibition threshold. Some investigated options are adsorption, gas stripping, pervaporation, liquid-liquid extraction (LLE), etc. (Rathour *et al.,* 2018). The integration of ABE fermentation with LLE is an alternative method that is under great challenge to be developed (Al-Shorgani *et al.,* 2019; Jang *et al.,* 2012). Process with simpler configuration and lower energy consumption is more preferable (Muharja *et al.,* 2023a; 2022; 2020a). The LLE method has several astonishing advantages: it uses a simple setup and equipment, easy to recover the products from the extractant, and it has low energy use (Zhao *et al.,* 2019). Previous studies found that a large extractant volume of 5 – 10 to broth ratio significantly improved total ABE fermentation by diminishing butanol toxicity, led to high product yield and high cell density (Darmayanti *et al.,* 2019, 2021; Zhao *et al.,* 2019). Enhanced acetone to butanol product ratio in ABE fermentation up to 5:7 could be obtained by increasing the fermentation temperature to 43.5 °C (Wu *et al.,* 2021).

Another common method to enhance ABE fermentation product yield by protecting the cells from the product's harmfulness is the cell immobilization technique (Abo *et al.,* 2019). Various cell immobilization techniques have also been found to improve yield through the use of high cell density in continuous fermentation mode (Menchavez and Ha, 2019; Chang *et al.,* 2016). Al-Shorgani *et al.* (2019) reported an improvement in fermentation productivity and stability by using an immobilized cell of *Clostridium sp.* in continuous fermentation systems. Cell immobilization prevents bleeding during the process. There are many methods for cell immobilization, namely self-aggregation, entrapment, adsorption, or biofilm (Liu *et al.,* 2019). Cell immobilization via the gel entrapment method is extensively employed due to its high mechanical properties and flexible support structure (Gao *et al.,* 2021). Although research on the improvement of ABE productivity via LLE and cell immobilization has been progressively conducted, from the literature studies, there has been no publication focusing on the application of acetone production.

From the aforementioned background, this study aims to develop acetone production via the ABE fermentation process with a large volume ratio of extractant to the broth. As a result, a high level of acetone production was attained by utilizing immobilized *C. saccharoperbutylacetonicum* N1-4 in a fed-batch system.

2. Methods

2.1. Materials

The chemicals used for this experiment are hexanol (Merck, Darmstadt, Germany), palm oil, glucose, $CaCO_3$, $(NH_4)_2SO_4$, CH_3COONH_4, $MgSO_4.7H_2O$, $FeSO_4$ -$7H_2O$, KH_2PO_4, NaCl, $CaCl_2$, HCl and KOH (Pudak Scientific, Bandung, Indonesia), yeast extract and tryptone (Himedia, Mumbai, India).

2.2. Microorganism Refresh

The first process is mixing distilled water with 15 g/L of grated potato, 10 g/L of glucose, 0.5 g/L of ammonium sulfate, and 3 g/L of $CaCO_3$. The mixture was then heated in boiled water for 1 hour, then cooled to room temperature. The solid was filtered, and the suspension was sterilized and used for Potato-Glucose media. Five spoons of *C. saccharoperbutylacetonicum* N1-4 ATCC 13564 sand stock bacteria were added to 10 ml media, then heat shocked in boiling water for 1 minute. Then, it was cooled and set at room temperature for 24 hours anaerobically.

2.3. Preculture

Media was made of tryptone (Himedia, Mumbai, India) 6 g/L, yeast extract (Himedia, Mumbai, India) 2 g/L, CH_3COONH_4 3 g/L, $MgSO_4.7H_2O$ 0.3 g/L, $FeSO_4$ -$7H_2O$ 0.01 g/L, KH_2PO_4 0.5 g/L, and glucose 20 g/L. The pH of the solution was adjusted to 6.5 (HCl and KOH were used as pH-adjusting agents). Refreshed media was inoculated at 10% v/v and then placed at room temperature anaerobically.

2.4. Free Cell Extractive Fermentation

The TYA media contained tryptone 6 g/L, yeast extract 2 g/L, CH_3COONH_4 3 g/L, $MgSO_4$ – $7H_2O$ 0.3 g/L, $FeSO_4$ -$7H_2O$ 0.01 g/L, KH_2PO_4 0.5 g/L, and glucose 50 g/L (Darmayanti *et al.,* 2020). The pH of the solution was adjusted to 6.5. Palm oil, hexanol, a mixture of hexanol, and palm oil with a ratio of 1:1 was used as extractants, with an extractant-to-broth volume ratio of 5. The working volume of fermentation was 30 mL of broth and 150 mL of extractant (Figure 1A). It was in an incubator shaker at 160 rpm at 50°C. At the 12, 24, 48, and 72 hours, each sample was fed with 2 mL of 120 g/L glucose solution.

2.5. Extractive Fermentation with Immobilized Cells

The working volume of the fermentation was 30 mL of broth, 30 mL of immobilized cells beads, and 150 mL of extractant (Figure 1B). Immobilized cell beads were prepared by making sodium alginate gel (3% w/v) containing NaCl (0.85% w/v) and preculture media of 10% v/v. The gel mixture was then dropped in $CaCl_2$ solution (3% w/v) to form calcium alginate beads. The beads were filtered and used for fermentation.

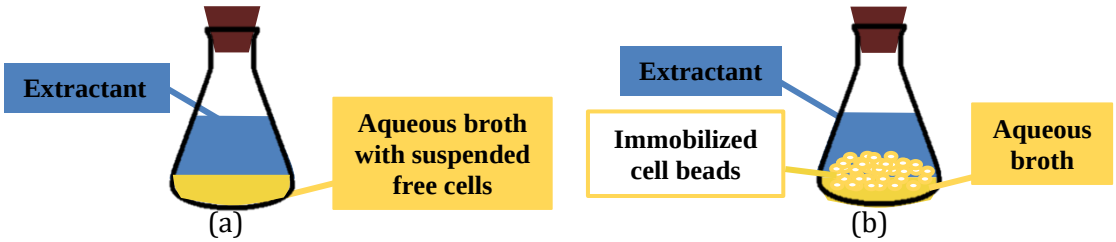

Figure 1 Extractive fermentation with (a) free and (b) immobilized cells

2.6. Analysis

The reducing sugar in the broth was measured using DNS (dinitrosalicylic acid) method (Muharja *et al.,* 2020b; 2019). The cell density of *C. saccharoperbutylacetonicum* was analyzed by measuring absorbance at 562 nm using a UV-vis spectrophotometer; one unit

of absorbance is equal to 0.246 g/L dry cell weight. Acetone, ethanol, acetic acid, and butanol were analyzed using gas chromatography (Darmayanti *et al.,* 2021).

3. Results and Discussion

3.1. Free Cell Extractive Fermentation

This study used palm oil and hexanol as representative natural and synthetic extractants because they have not been reported in previous studies for bioacetone fermentation. Acetone dissolves well in both of these organic solvents, so they were used as the extractant for acetone in this experiment. The initial pH was adjusted at 6.5 as this is the optimum acidity for the strain to form organic acid. The pH for free cell extractive fermentation with palm oil extractant decreased to around 5-6 (Figure 2A), while the pH in hexanol extractants and hexanol-palm oil mixtures was at pH around 6. During the acidogenesis phase, the pH tends to decrease due to the formation of acids, namely acetic acid, in the fermentation with palm oil extractant. The influence of external pH is one of the key factors involved in triggering the transition from the acidogenesis phase to the solventogenesis phase (Capilla *et al.,* 2024).

The addition of $CaCO_3$ (calcium carbonate), which is a reagent that serves to buffer the degree of acidity that can maintain the pH in the fermentation process. To achieve optimum conditions for the growth and metabolism of acetic acid bacteria, the environment and the state of the fermentation medium are well maintained. In this study, the effect of the use of $CaCO_3$ is very influential because it maintains the pH value of each extractant. The pH in this fermentation should not be below 5 to prevent the acid crash, which may cause death to the strain (Capilla *et al.,* 2022). Its low solubility in water can cause it to neutralize acids such as acetic acid and maintain pH at a certain level automatically (Valles *et al.,* 2020). For good ABE production, the pH should be buffered between 5-6 (Han *et al*., 2013), as occurred in the extractive fermentation with palm oil as an extractant. However, using hexanol as an extractant caused low cell growth, which consequently formed a lower acid product and no significant pH change during fermentation.

The fermentation with palm oil extractant has increased the cell density due to the effect of the extractant on free cell growth (Figure 2B). This was because fermentation in free cells was unimpeded, and these bacteria directly consumed the substrate (Aisyah *et al.,* 2023). The longer the fermentation time, the higher the cell density value, which makes bacteria grow and multiply well (Su *et al.,* 2020). Even after 96 hours, the cell kept growing in the fermentation using the palm oil as an extractant. Palm oil favored cell growth by reducing the toxicity effect of the fermentation products (Aisyah *et al.,* 2023; Hossain *et al.,* 2022). The results of research using hexanol extractants with mixed extractants of hexanol and palm oil tend to have similar results in each test, which indicates that cell density does not increase because hexanol is toxic during growth.

The curve in Figure 2C shows that the sugar concentration of the free cell extractive fermentation in the three extractant variations increased and decreased in a similar value. In the first 12 hours, a delay in glucose consumption was observed in the fermentation with each extractant. This is related to the lag phase during cell growth (Figure 2B) which is caused by the contact of the cells and extractant in a large volume hindering the glucose mass transfer from the media to the cell membrane, as also observed in the previous study (Darmayanti *et al.,* 2018). As a result of increasing fermentation time, the glucose consumption activity decreased according to the reduction in available substrates and nutrients (Hawashi *et al*., 2019). The glucose feeding maintained the available carbon source for the strain to grow and produce solvents (Niglio, Marzocchella, and Rehmann,

2019). The highest glucose consumption in the fermentation with free cells was obtained at 43.85 g/L using palm oil as an extractant.

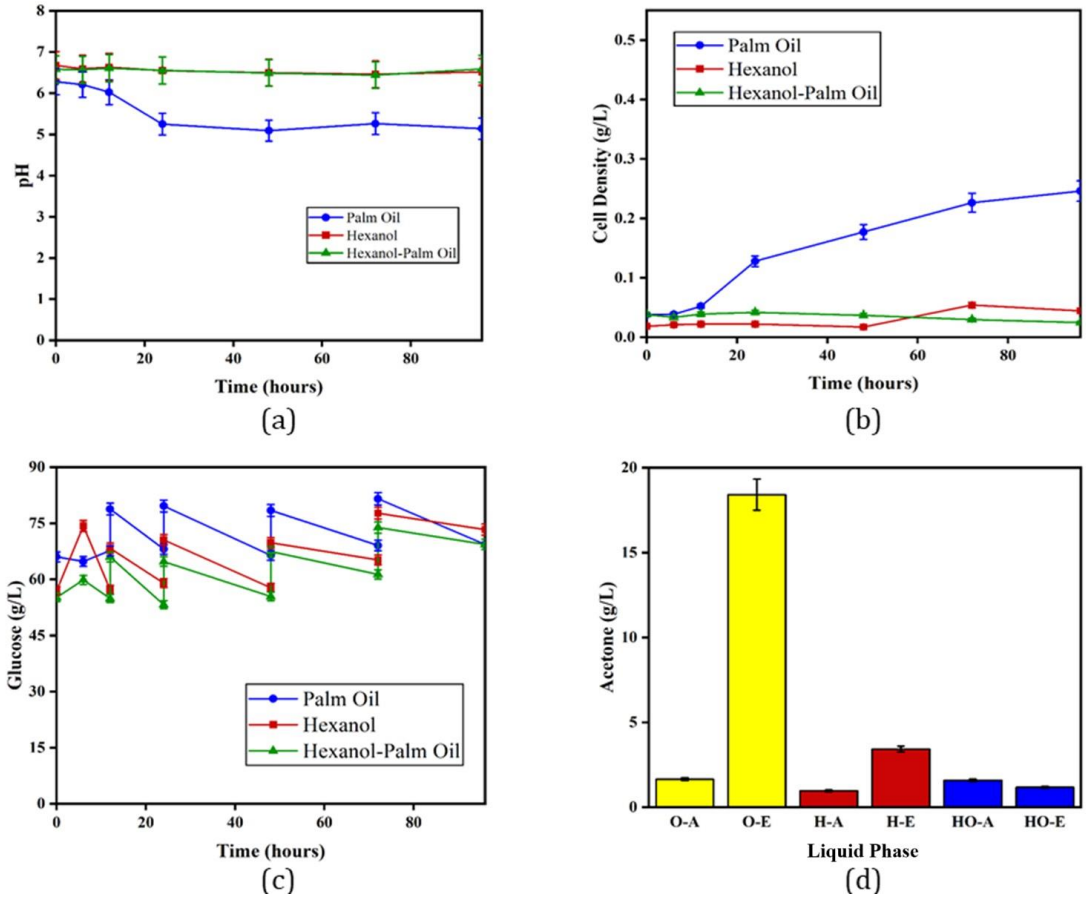

Figure 2 Free cell extractive fermentation pH test curve (a), cell density (b), glucose concentration (c), and acetone concentration (d) with ♦ palm oil, ■ hexanol, and ▲ hexanol-palm oil mixture as extractant. O is oil, H is hexanol, A is the aqueous phase, and E is the extractant phase

Figure 2D shows that the concentration of acetone in the free cell extractive fermentation was the highest using palm oil as extractant with a value of 18.414 g/L, with a high distribution coefficient of acetone in the palm oil–water mixture of 11.2. This study attempted a higher temperature of 50 °C to enhance the acetone product than butanol. As reported in the previous study with SSF of corn stover, a higher temperature of 43.5 °C could improve the acetone-to-butanol product ratio (Wu *et al.,* 2021). The substrate and nutrients in the free-cell extractive fermentation using palm oil were directly consumed by the free cells and no toxic barriers. Hence, the value obtained was relatively high. As the palm oil reduced the acetone concentration effectively from the media, the acetone was produced in a higher amount compared with the other extractant (Cai *et al.,* 2022).

3.2. Immobilized Cell Fermentation

The pH for fermentation with immobilized cells on palm oil extractants decreased by about 5-6 (Figure 3A), which was caused by acidogenesis, which produced acetic acid, followed by solventogenesis, which produced acetone. While the hexanol extractant and the mixture of hexanol with palm oil experienced a stable pH of around 6-7 because the fermentation product produced was not as much as the fermentation product using the palm oil extractant. This happened even though the bacteria were protected by the alginate matrix, and the hexanol was still toxic, so the production of solvents by the strain was

inhibited (Zhao, Tashiro, and Sonomoto, 2019). A similar phenomenon occurred in free-cell extractive fermentation.

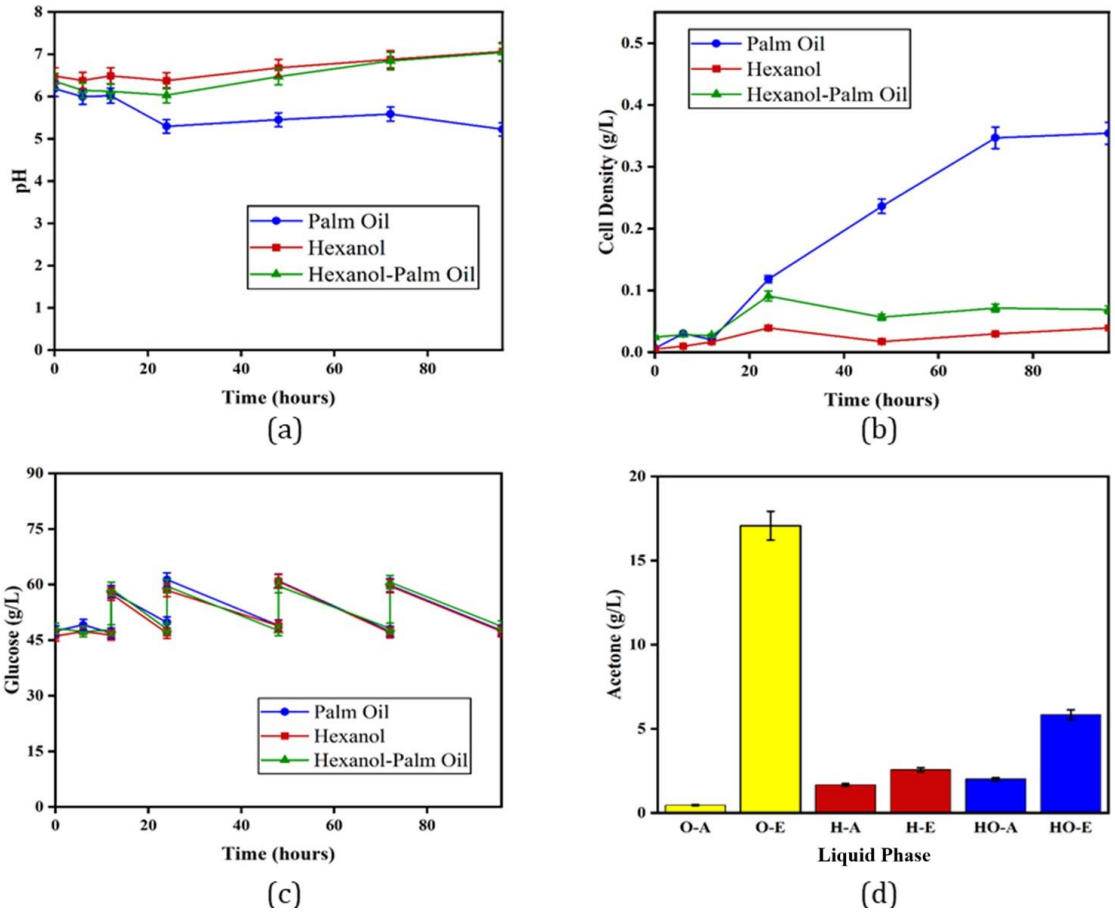

Figure 3 Immobilized cell fermentation pH (a), cell density (b), glucose concentration (c), and acetone concentration (d) with ♦ palm oil, ■ hexanol, and ▲ hexanol-palm oil mixture as extractant. Acetone concentration curve in immobilized cell extractive fermentation. O is oil, H is hexanol, A is the aqueous phase, and E is the extractant phase

Fermentation using palm oil extractant demonstrated a high cell concentration increase (Figure 3B); namely, the maximum cell density is obtained at the 96th hour of 0.354 g/L. Compared to the cell growth using free cell extractive fermentation (0.246 g/L), immobilization increased the growth. Immobilization protected the cells inside the alginate matrix, resulting in more accumulated cells entrapped in the beads, more than the density of free cells (Darmayanti *et al.,* 2018). In the previous study using a low ratio of toxic extractant to broth volume, immobilization could promote cell growth in the alginate beads (Ye *et al.,* 2018). In this study, a high-volume ratio of toxic extractant to broth exposed the cells to a more extreme condition, so they could not grow even entrapped in the calcium alginate.

Figure 3C shows that the glucose concentration curve for immobilized cell fermentation had a stable increase and decrease in all fermentation. The highest sugar consumption was 47.01 g/L, using palm oil as the extractant. The decrease in glucose concentration in the hexanol extractant and the mixture of hexanol with palm oil occurred because the bacteria were protected by the alginate and required a substrate for growth (Muharja *et al.,* 2023b). Although hexanol is a toxic substance, sugar consumption was more stable than fermentation with free cells. The role of immobilized cells in the fermentation of acetone products is to increase the resistance of microbial cells from the influence of

environmental conditions such as pH, temperature, organic solvents, and toxic substances, thus causing the viability of the cells to be maintained better (Ye *et al.,* 2018).

Figure 3D shows that the acetone concentration curve in the extractive fermentation of immobilized cells has increased in the palm oil extractant, which is 17.069 g/L, so the palm oil extractant is the best extractant to produce acetone products in the extractive fermentation using immobilized cells. Cell immobilization aims to make the cell immobile or reduce its space to move so that it inhibits its growth outside the beads, and the substrate is used only to produce products (Hastuti *et al.,* 2019).

3.3. Comparison of Free Cells and Immobilized Cells in Extractive Fermentation

Figure 4 shows the total acetone concentration to the volume of the medium based on the method of adding glucose to food nutrients in each fermentation obtained a high enough value for the palm oil extractant with free cell extractive fermentation, which was 93.72 g/L. This is because the free cells are in direct contact with the glucose substrate in the media cells. The second highest value was found in the palm oil extractant with extractive fermentation of immobilized cells, which was 85.81 g/L. This is because the immobilized cells were entrapped in the alginate matrix, so the substrate mass transfer to the immobilized cells was decelerated compared to free cells, which directly contacted the substrate with no hindrance. However, the difference was insignificant if immobilized cells were considered for the ease of recycling and eliminating the nutrient requirement for the preculture of the next fermentation batch (Zhao *et al.,* 2019). A previous study had proven the reusability of calcium alginate for Abe fermentation with stable results in three consecutive cycles (Kheyrandish *et al.,* 2015).

According to this study's result, the extractant's biocompatibility played a stronger role than the immobilization. In the previous study using octanol as the extractant with a low extractant-to-broth volume ratio of 0.33, entrapment in calcium alginate protected the cells from the toxic effect of the solvent (Ye *et al.,* 2018). In this study, the hexanol extractant in immobilized cells with a large volume-to-broth ratio of 5 contacted the beads in a high concentration of solvents, hampered the cell's growth, and consequently resulted in a low acetone concentration. The use of a biocompatible extractant (palm oil) in a large extractant-to-broth volume ratio favored the fed-batch fermentation by eliminating the toxic effect of the produced solvent in broth (Da-Costa-Nogueira *et al.,* 2021). The viability of the cells was maintained in a productive condition, giving a high total acetone concentration reaching 93.72 g/L and 85.81 g/L.

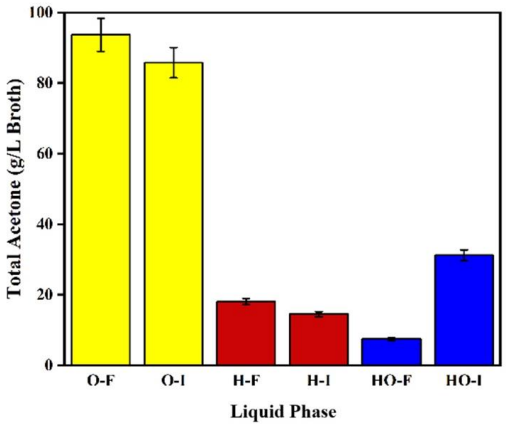

Figure 4 Total acetone concentration per media (broth) volume. O is oil, H is hexanol, A is the aqueous phase, and E is the extractant phase. Total acetone concentration is the total mass of the acetone produced in the aqueous and extractant phase divided by the total volume of broth (aqueous phase)

The data in Table 1 shows that in the extractant and aqueous phases, there are acetone, butanol, and ethanol which are the end products of the solventogenesis stage, and acetic acid, which is an intermediate product of the acidogenesis stage. The fermentation in this study using a fermentation temperature of 50 ^{o}C and acetone selective extractant succeeded in producing acetone selectively rather than the other products.

Table 1 Concentration of fermentation products in each phase

Sample Name	Acetone (g/L)	Ethanol (g/L)	Butanol (g/L)	Acetic Acid (g/L)
O-A (F)	1.652	-	-	-
O-A (I)	0.461	-	-	-
H-A (F)	0.965	0.706	-	0.559
H-A (I)	1.671	0.212	0.075	6.492
HO-A (F)	1.580	-	-	0.800
HO-A (I)	2.006	-	-	-
O-E (F)	18.414	-	-	1.262
O-E (I)	17.069	0.337	-	-
H-E (F)	3.424	-	-	-
H-E (I)	2.566	-	0.117	-
HO-E (F)	1.170	-	0.099	-
HO-E (I)	5.836	0.155	-	4.629

Information:
O = Fermentation with palm oil
H = Fermentation with hexanol
HO = Fermentation with palm oil-hexanol mixture
A = Aqueous phase
E = Extractant phase
F = Fermentation with free cells
I = Fermentation with immobilized cells

Table 2 Comparison of acetone-enhanced ABE fermentation

Fermen-tation	Process modification	Strain	Tempe-rature (^{o}C)	Total acetone concentration (g/L broth)	References
Batch	Extraction using octanol with extractant to broth ratio of 0.33	*C. acetobutylicum*	37	3.33	(Ye *et al.*, 2018)
Batch	Acetate addition	*C. acetobutylicum* and *S. cerevisiae*	37	7.0	(Luo *et al.*, 2016)
Batch	Acetate addition	*C. acetobutylicum* and *S. cerevisiae*	37	10.5	(Luo *et al.*, 2017)
Fed-batch	pH-stat with acetate and glucose co-feeding	*C. saccharoper-butylacetonicum*	30	8.74	(Gao *et al.*, 2016)
Batch	SSF of corn stover using thermotolerant strain	*C. acetobutylicum*	43.5	5.0	(Wu *et al.*, 2021)
Fed-batch	Extraction using oleyl alcohol and tributyrin mixture with extractant to broth ratio of 5	*C. saccharoper-butylacetonicum*	30	13.0	(Darmayanti et al., 2018)
Fed-batch	Extraction using palm oil with extractant to broth ratio of 5	*C. saccharoper-butylacetonicum*	50	93.72 with free cells and 85.81 with immobilized cells	This study.

Several studies enhancing acetone production in ABE fermentation were reported but in a limited number (Table 2). This study using high temperature and palm oil extractant in a large extractant-to-broth volume ratio reached the highest total acetone concentration

compared with the other reported research. The total concentration of produced acetone was calculated using the volume of broth as the basis because broth or media requires costly nutrient ingredients. Besides, in industrial applications, the extractant is recyclable, while the nutrient in the broth is consumable. A large extractant-to-broth ratio led to a higher capacity of the broth to produce six times more than the fermentation without extraction. High acetone concentration was produced by the strain because of the toxicity-eliminating effect from the use of biocompatible acetone selective extractant in a large volume. Using extractants in a large volume is a novel option to enhance the total concentration of product per broth so that the required nutrient during fermentation can be minimized (Darmayanti *et al.,* 2023). It was combined with the immobilization of cells to ease the reuse of the cells beads to remove the preculture step during long operations, so the nutrient required for the preculture step could also be eliminated. These advantages are solutions to overcome the issues of bringing ABE fermentation to a largescale production, improving the economic feasibility of bioacetone production.

4. Conclusions

The best extractant results for the acetone fermentation process were using free cells extractive fermentation with palm oil extractants with a total acetone concentration of 93.72 g/L broth. The large volume of biocompatible acetone selective extractant favored fermentation by reducing the toxicity effects of the products. Combining the use of a large volume of extractant and immobilization is a promising method for conducting fermentation with minimized nutrient requirements. The research could be further investigated for the purification of products and larger-scale applications.

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