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Effect of Substrate Concentration on Simultaneous Acetone-Butanol-Ethanol Fermentation and Biohydrogen Production from Fig (*Ficus carica*)

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ABSTRACT

The growing demand for renewable and sustainable fuel alternatives has driven extensive research into biohydrogen production and acetone-butanol-ethanol (ABE) fermentation, with butanol as the primary product of interest. To address the fuel-versus-food dilemma, this study explores the biofuel potential of lignocellulosic biomass, specifically fig (*Ficus carica*), building upon our previous findings. Substrate concentration plays a pivotal role in fermentation outcomes, either enhancing or inhibiting product yields. This study investigates the effects of varying substrate concentrations (20–47 g L^{-1}) on ABE fermentation and biohydrogen production from fig hydrolysate. Results demonstrated that higher substrate concentrations significantly improved product yields: the maximum cumulative hydrogen production (1402 mL) and highest solvent concentrations (ethanol: 1.45 g L^{-1}; acetone: 8.08 g L^{-1}; butanol: 5.09 g L^{-1}) were achieved at 47 g L^{-1}. The biphasic ABE fermentation process yielded peak organic acid production (acetic acid: 10.49 g L^{-1}; butyric acid: 6.02 g L^{-1}) at 47 g L^{-1} and 39 g L^{-1}, respectively. These findings underscore the positive correlation between increased substrate concentration and enhanced ABE fermentation efficiency, highlighting the potential of fig as a sustainable feedstock for biofuel production.

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1. INTRODUCTION

Renewable energy offers a sustainable solution to growing energy demands while reducing environmental impact and dependence on finite fossil fuels (Sirajudeen & Annuar, 2021). Over the past three decades, biobutanol research has garnered substantial academic and industrial attention, increasing public recognition of its potential as an environmentally sustainable alternative to conventional fossil fuels and their detrimental ecological effects. Moreover, butanol's compatibility with existing fuel infrastructure—including pipeline transportability and favorable engine ignition properties—establishes it as a viable gasoline substitute with significant practical advantages (Dürre, 2007).

Initially, biobutanol research faced several challenges, such as low yield and productivity, high substrate costs, and difficulties in product separation and recovery. However, biotechnological advancements led to the discovery of butanol-producing Clostridial strains, which enhanced yield and productivity—an essential step for overcoming these limitations and advancing biobutanol research (Alam & Wang, 2019). Similarly, many researchers have focused on using second-generation substrates, namely lignocellulosic biomass, for acetone-butanol-ethanol (ABE) fermentation because they are readily available, inexpensive, and free from the food-versus-fuel debate associated with first-generation biomass.

Fig (*Ficus carica*), a Mediterranean lignocellulosic crop widely cultivated in Türkiye, is rich in C6 sugars. Our previous study confirmed the efficiency of thermal hydrolysis as a pretreatment option for fig biomass. Two different thermal pretreatment methods—microwaving and autoclaving—were compared. The process parameters tested for hydrolysis included particle diameter of dried fig (Dp = 200–375 µm), pH (3.0–5.0), and power (P = 250–670 W) for microwave pretreatment; and particle diameter (Dp = 200–375 µm), pH (3.0–5.0), temperature (T = 95–120°C), and time (t = 30–50 min) for autoclave pretreatment. Maximum sugar concentrations of 82.9 g L^{-1} and 70.0 g L^{-1} were obtained for microwave and autoclave pretreatments, respectively. These results demonstrate the effectiveness of these pretreatment methods for lignocellulosic biomass in biofuel research. The study also documented the elemental composition of figs (Abibu & Karapinar, 2023).

Microbial-mediated biofuel production is significantly influenced by critical parameters such as pH, temperature, and

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especially substrate concentration, which directly affect microbial activity and product yields in anaerobic fermentation systems. Microbial diversity plays a crucial role in substrate utilization and metabolic pathways, particularly when mixed cultures serve as inocula. The biphasic acetone-butanol-ethanol (ABE) fermentation process, consisting of acidogenesis—producing volatile fatty acids like acetic, lactic, butyric, and propionic acids—and solventogenesis—generating acetone, ethanol, and butanol—also yields gaseous byproducts such as methane (CH_4) and hydrogen (H_2) (Sołowski et al., 2020). This process exemplifies the microbial complexity involved in converting abundant lignocellulosic biomass—composed of lignin, hemicellulose, and cellulose—into renewable fuels. Lignocellulosic biomass requires pretreatment (physical, chemical, or microbial) to disrupt the lignin matrix and release fermentable sugars (Oke et al., 2016; Ranjithkumar et al., 2022).

While increased substrate concentration can enhance biohydrogen production performance (Kim et al., 2024), excessive concentrations often inhibit fermentation due to organic acid accumulation and toxicity (Basak et al., 2020). Maintaining optimal substrate levels is crucial for hydrogenase enzyme activity, as demonstrated by studies reporting 10 g/L substrate yielding 45.6 mL/g-VS hydrogen in sewage sludge/grass residue co-fermentation (Hastuti et al., 2016; Wang & Yin, 2018). Similarly, up-flow packed bed reactors achieved optimal hydrogen production at 25 g L^{-1} substrate concentration, yielding 4275 mL/day (Karaosmanoglu & Karapinar, 2019), and cacao pod husk fermentations optimized at 35 g VS L^{-1} produced 257.05 ± 7.97 mL L^{-1} hydrogen (Kriswantoro & Chu, 2024).

Substrate concentration also critically influences microbial consortia dynamics. For instance, *Clostridium butyricum and Lactobacillus casei* cocultures exhibit carbon source preference variations depending on substrate concentration (Park et al., 2018). In the ABE fermentation, Capilla et al. (2021) showed that combining an optimal pH of 5.1 with glucose concentrations exceeding 60 g L^{-1} increased biobutanol production by 1.5 to 1.7 times using *Clostridium acetobutylicum* DSM 792. This highlights the delicate balance between substrate availability and microbial performance central to biofuel production systems, which are often dominated by *Clostridia*, *Bacillus*, and *Thermoanaerobacterium* species (Hassan et al., 2015; Wang & Yin, 2019).

Engliman et al. (2022) emphasized the need for kinetic studies to determine the maximum substrate concentration and micronutrient levels that inhibit biohydrogen production. Accordingly, their study employed both the Monod and Andrews models. They concluded that substrate concentrations exceeding 10 g L^{-1} and iron nanoparticle concentrations above 200 mg L^{-1} negatively affected bacterial performance and biohydrogen yields. At these levels, lactic acid and propionic acid—known inhibitors of biohydrogen production—were also observed. Although numerous studies have reported different optimal substrate concentrations for maximizing biohydrogen production, such variation often stems from differences in substrate type and inoculum used across studies. While the effects of substrate concentration have been investigated in various lignocellulosic biomasses such as cacao pod husk (Kriswantoro & Chu, 2024), sugarcane bagasse, and wheat straw, this study is, to the best of our knowledge, the first to examine this parameter using fig (*Ficus carica*) hydrolysate as the sole substrate. The focus on fig hydrolysate constitutes the principal novelty of the present work. Therefore, this study explored the simultaneous production of biohydrogen, organic acids (acetic and butyric), and solvents (acetone, ethanol, and butanol) across a range of fig fruit substrate concentrations.

2. EXPERIMENTAL

2.1 Substrate and inoculum preparation

Waste figs were collected from local farmers in Izmir, Türkiye, and oven-dried at 72°C for 72 hours. Microwave pretreatment was conducted under optimized conditions—100 g L^{-1} substrate loading, 370.72 µm particle size, pH 4.96, 253.67 W power, and a 50-minute duration—resulting in a sugar concentration of 80.5 ± 1 g L^{-1}. These results are consistent with our previous findings (Abibu & Karapinar, 2023), where total sugar concentrations of ≥ 80 g L^{-1} were achieved under the same pretreatment conditions. A pure culture of *Clostridium pasteurianum* DSM 525, obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), was used as the inoculum in all experiments. The fig hydrolysate was autoclaved prior to inoculation to sterilize it and eliminate potential microbial contamination. This aseptic treatment was deemed sufficient to obviate the need for microbial community analysis. For microbial fermentation, *Clostridium pasteurianum* DSM 525 was cultivated in a growth medium containing 40 g L^{-1} glucose, 20 g L^{-1} $CaCO_3$, 10 g L^{-1} yeast extract, and 0.1 g L^{-1} L-cysteine. The culture (10% v/v inoculum) was incubated at 37°C with an initial pH of 7.0 for 48 hours.

2.2 Experimental setup

Batch fermentation was conducted in 310 mL airtight serum bottles, each with a working volume of 200 mL, leaving 110 mL of headspace for gas accumulation. The fermentation medium comprised the following components (g L^{-1}): K_2HPO_4 (2.8), KH_2PO_4 (3.9), $MgSO_4$ (0.25), yeast extract (0.6), and L-cysteine (0.1). The pH was adjusted to 7.0 before autoclaving (121°C for 15 minutes). To establish anaerobic conditions, the headspace of each bottle was purged with nitrogen gas prior to incubation under mesophilic conditions at 37 °C. During fermentation, the pH was monitored daily and maintained between 6.5 and 7.0 by the addition of 5 N NaOH when it dropped below the lower threshold. Experiments were conducted using fig hydrolysate with initial total sugar concentrations ranging from 20 to 47 g L^{-1}. A control experiment containing 50 g L^{-1} glucose and identical nutrients was run in parallel. The fermentation medium was inoculated with 10% (*v/v*) of actively growing bacterial culture derived from the stock under aseptic conditions. Fermentation was terminated upon achieving steady-state bioproduct formation.

2.3 Analytical Methods

Following centrifugation at 8,000 rpm, the supernatants were analyzed for total sugar content, organic acids, and solvents. Total sugar consumption was quantified spectrophotometrically using the Dubois method (DuBois et al., 1956). Daily monitoring included total gas production, measured by the water displacement method (Tosuner et al., 2019), and the percentage of hydrogen in the produced gas. Hydrogen production volumes were calculated according to the method described by Eker and Erkul (2018). Hydrogen content was determined using gas chromatography (Agilent 6890 series) equipped with ChemStation software (Abibu & Karapinar, 2024). An Alltech HayeSep D column (80/100

mesh, 6″ × 1/8″ × 0.085″) was used for the analysis, with nitrogen as the carrier gas at a flow rate of 30 mL/min. Operational temperatures were set at 35 °C for the oven, 120 °C for both the injector and detector, and 140 °C for the filament. Gas chromatography with the Agilent system was calibrated exclusively for hydrogen detection and quantification, as hydrogen is the targeted gas in our fermentation laboratory. The concentrations of ABE solvents and organic acids (lactic, acetic, propionic, and butyric acids) were analyzed using high-performance liquid chromatography (HPLC) with an Agilent 1200 series system (Abibu & Karapinar, 2024). A 5 mM H_2SO_4 solution served as the mobile phase, delivered at a flow rate of 0.6 mL/min. An Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm), maintained at 50 °C and coupled with a refractive index detector, was used for the detection of ABE solvents.

For the analysis of organic acids, the same column was utilized at a lower temperature of 40 °C, and detection was done at 280 nm with a diode array detector. The complete bioproduct fermentation scheme is presented in Figure 1.

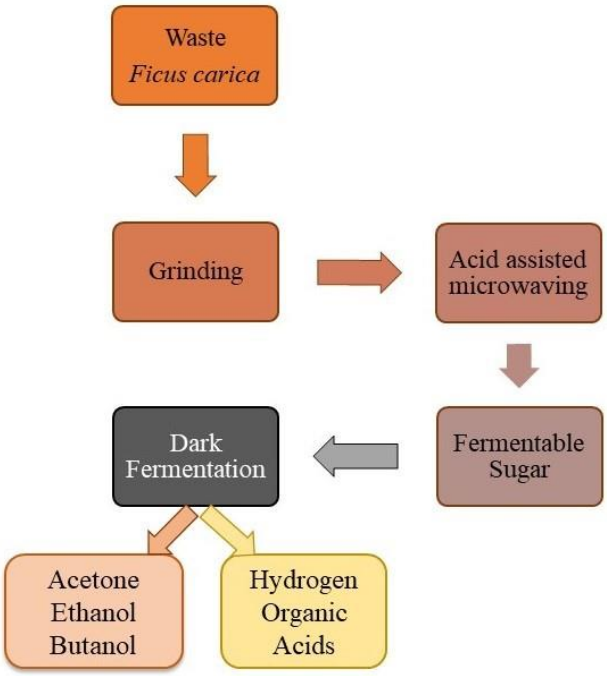

Figure 1. Process flow-scheme for ABE fermentation

3. RESULTS AND DISCUSSION

3.1 Hydrogen production

Figures 2(A–D) present hydrogen production results at various substrate concentrations. Specifically, cumulative hydrogen production, hydrogen yield, and hydrogen production rates (in mL L^{-1} and mL h^{-1}) are shown in Figures 2A, 2B, 2C, and 2D, respectively. The temporal variation of hydrogen production revealed a rapid increase within the first 24 hours of fermentation, indicating a short microbial lag phase and swift metabolic activation.

As illustrated in Figure 2A, a cumulative hydrogen production volume of 316 mL was recorded at a substrate concentration of 47 g L^{-1} after 24 hours. In contrast, the lowest cumulative hydrogen production, 64 mL, was observed at 20 g L^{-1} under the same time frame. By 48 hours, cumulative hydrogen production had increased significantly, reaching 851 mL and 943 mL at 39 g L^{-1} and 47 g L^{-1}, respectively. This enhanced production can be attributed to the availability of sufficient substrate to sustain microbial activity during ABE fermentation. The highest cumulative hydrogen production observed during the entire fermentation period (120 hours) was 1402 mL at a substrate concentration of 47 g L^{-1}, demonstrating a strong positive correlation between substrate availability and hydrogen output. Typically, low substrate concentrations result in reduced hydrogen production due to limited carbon availability required for microbial growth. According to Tao et al. (2007), the low hydrogen yield at low substrate concentrations is attributable to the consumption of simple sugars by microorganisms for biomass formation rather than for fermentation into hydrogen. This explains the observed decline in cumulative hydrogen production under substrate-limiting conditions. Conversely, while high substrate concentrations can enhance microbial activity, they may also negatively affect inoculum metabolism by lowering the pH, leading to the accumulation of organic acids. This acid build-up can inhibit the transition into the solventogenic phase of ABE fermentation. Wang and Wan (2009) concluded that elevated substrate concentrations support increased hydrogen production only within an optimal range; beyond this range, substrate inhibition reduces hydrogen yield.

Similarly, Karaosmanoglu and Karapinar (2019) examined the effect of varying influent substrate concentrations (10–35 g L^{-1}) on biohydrogen production from waste wheat. Their findings showed that hydrogen production increased with substrate concentration, peaking at 25 g L^{-1} (4274 mL day^{-1}), but declined at concentrations above this level. This supports the inference that both substrate limitation and inhibition negatively impact cumulative hydrogen production at low and high concentrations, respectively. Our findings align with this pattern. The hydrogen production rate (in mL h^{-1}) was lowest at 20 g L^{-1} due to substrate limitation. In contrast, significantly higher production rates were observed at 39 and 47 g L^{-1} total sugar, as well as at 50 g L^{-1} glucose, indicating an absence of substrate inhibition. These results suggest that ABE fermentation with simultaneous hydrogen production can tolerate higher sugar concentrations. As shown in Figure 2B, a maximum hydrogen yield of 1.4 mol H_2/mol glucose was achieved at 47 g L^{-1} total sugar concentration, compared to 0.65 mol H_2/mol glucose in the control experiment. Volumetric hydrogen production rates also reached satisfactory levels, with a peak value of 7000 mL L^{-1} at 47 g L^{-1} hydrolysate concentration (Figure 2C). The superior volumetric rate and yield observed in fig hydrolysate compared to glucose may be attributed to the presence of essential trace metals in figs, which can act as cofactors enhancing microbial metabolism and bioproduct formation.

3.2 Utilization of total sugar

Most agricultural residues contain fermentable sugars, though their concentrations vary depending on the biomass type. Figure 3 illustrates total sugar consumption by microorganisms for growth, metabolic activity, and metabolite production. As shown, the initial sugar concentrations in the fermentation media—comprising fig hydrolysate at varying substrate levels (20, 39, and 47 g L^{-1}) and glucose at 50 g L^{-1} as the control—were rapidly consumed following inoculation. After 120 hours of anaerobic fermentation, the percentage of sugar utilization was 98.22%, 86.5%, 75.89%, and 75.68% for the 20, 39, 47 g L^{-1} and control experiments, respectively. These results show that sugar consumption was highest at the lowest substrate concentration (20 g L^{-1}).

This aligns with the findings of Yang and Wang (2019), who reported maximum sugar utilization efficiency at low substrate concentrations (5–10 g L^{-1}), with a noticeable decline in utilization as initial substrate concentrations exceeded 10 g L^{-1}. Steady-state sugar consumption was reached by the 96th hour of fermentation. However, the substrate concentration with the highest sugar utilization does not necessarily correspond to the most favorable condition for targeted product yields.

This is because low substrate concentrations often coincide with insufficient nutrient availability relative to microbial demands, leading to faster nutrient depletion and potentially reduced fermentation performance. Interestingly, the sugar content in fig hydrolysate was consumed more rapidly than that of glucose, which is traditionally considered the most preferred carbon source for microorganisms. This finding suggests that fig hydrolysate, rich in fermentable sugars and potentially beneficial macro- and micronutrients, can serve as an effective alternative to glucose for bioproduct generation through fermentation.

3.3 Soluble metabolites formation

As mentioned earlier, the ABE fermentation process is a biphasic process comprising acidogenesis and solventogenesis stages. Hydrogen production is typically observed during ABE fermentation, especially when inocula with hydrogen-producing potential are employed. The key metabolites of the ABE process include organic acids (acetate, propionate, butyrate, and lactate), organic solvents (acetone, butanol, and ethanol), and hydrogen. The timely initiation of the acidogenesis stage is marked by a decrease in pH, indicating efficient microbial activity. Furthermore, early progression into the solventogenesis stage is always desirable. However, product inhibition caused by ABE solvents (above certain concentrations) remains a major challenge in the process. Although researchers such as Yang and Wang (2019) and Wang et al. (2008) have reported that increasing total organic acid production negatively affects cumulative hydrogen production—primarily due to blockage of microbial cell membranes by non-dissociated metabolites.

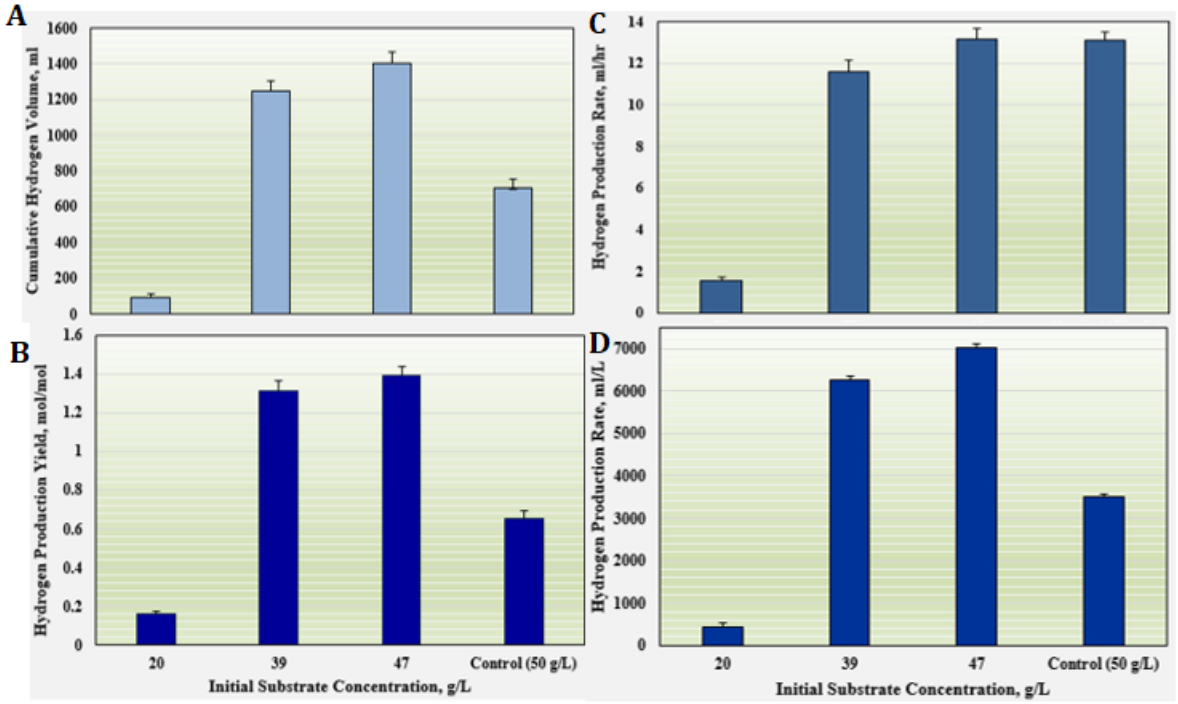

Figure 2. Effect of initial substrate concentration on (A): Cumulative hydrogen volume (ml), (B): Hydrogen production yield (mol mol^{-1}), (C): Hydrogen production rate (ml hr^{-1}), and (D): Volumetric hydrogen production (ml L^{-1})

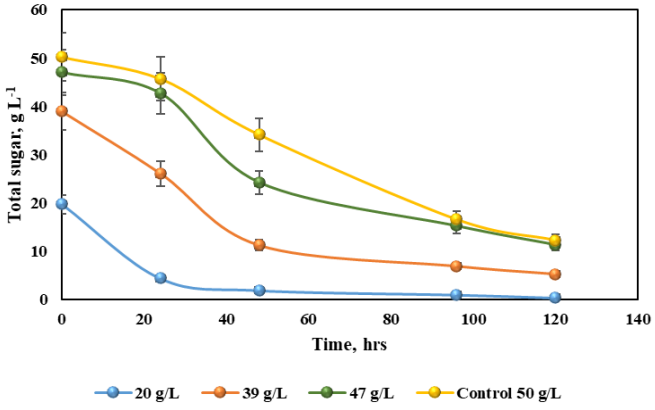

Figure 3. Variation of total sugar consumption with time at different substrate concentrations

Gorgec and Karapinar (2019) concluded that a direct relationship exists among substrate concentration, total organic acid production, hydrogen production, and sugar utilization. Operating the fermentation system in a continuously operated packed-bed system at low hydraulic retention times (HRTs) has been suggested to prevent the accumulation of organic acids that inhibit hydrogen production.

Figure 4A shows the variation of acetic acid production over time at different substrate concentrations. It is not surprising that high acetate production was observed in this research. The figure indicates that the highest and lowest acetate productions were recorded at 47 g L^{-1} (10.49 g L^{-1}) and in the control experiment (4.7 g L^{-1}), respectively. Thus, acetic acid production increases with substrate concentration.

Figure 4B presents the variation of butyric acid production over time at different substrate concentrations. The highest and lowest butyrate productions were obtained at 39 g L^{-1} (6.02 g L^{-1}) and 20 g L^{-1} (4.24 g L^{-1}), respectively. Like acetic acid, butyric acid production also increased with substrate concentration. Lu et al. (2019) reported a similar trend in acetic and butyric acid production with increasing substrate concentration. Within a concentration range of 10 to 40 g L^{-1}, acetate and butyrate production increased from 22.17 to 35.17 mM and from 5.91 to 23.30 mM, respectively. Kriswantoro and Chu (2024) observed acetic acid levels between 712.3 and 1,251 mg L^{-1} and butyric acid levels between 581.5 and 707.1 mg L^{-1} for substrate concentrations of 50 and 100 g L^{-1}.

Lactate and propionate were not observed throughout this experiment. These two organic acids are often produced during ABE fermentation when inocula with the appropriate metabolic potential are used. Their presence typically leads to competition with the targeted organic products. Therefore, their absence in this study may indicate a metabolic diversion favoring the production of butyrate and acetate only.

As previously mentioned, organic acid production is accompanied by a decrease in pH in the fermentation medium. Daily pH monitoring was conducted, and a general trend of pH reduction (to around 5.0–5.3) was observed, signaling organic acid production (Figure 4 C). However, after sample collection, the pH was typically adjusted back to 7.0. A drop in pH negatively affects hydrogen-producing microorganisms, thereby reducing hydrogen production. Lay (2001) reported that members of the *Clostridium* genus are hydrogen-producing bacteria that become inactivated at low pH, thus halting hydrogen production.

The total organic acid production (including lactate, propionate, acetate, and butyrate) observed in this study was 9.59, 16.11, 16.21, and 9.28 g L^{-1} for substrate concentrations of 20, 39, 47 g L^{-1}, and the control, respectively. This clearly shows that total organic acid production increases with increasing substrate concentration.

The variation in acetone, butanol, and ethanol concentrations after 120 hours of ABE fermentation at different initial substrate concentrations is presented in Figure 5 (A–C). Production of these solvents was enhanced with fig hydrolysate, particularly at high initial total sugar concentrations of 39 and 47 g L^{-1}. Thus, increasing substrate concentrations favored improved ABE solvent production. The fermentation followed a specific sequence in terms of product formation and termination. In Figure 5A, acetone formation exhibited a continuous increasing trend at all substrate concentrations, including glucose. In Figure 5B, ethanol production concluded within 20 hours for all substrate concentrations. In Figure 5C, butanol production ceased at 20 hours for both the 20 g L^{-1} substrate concentration and glucose, while it steadily increased over the fermentation period at 39 and 47 g L^{-1} sugar concentrations in the hydrolysate. Butanol is the desired product in ABE fermentation due to its superior biofuel properties. The maximum butanol concentration in our experiments was 5.09 g L^{-1} at the highest initial sugar concentration, while only 1.03 g L^{-1} was achieved with glucose. A similar trend was observed for acetone and ethanol production.

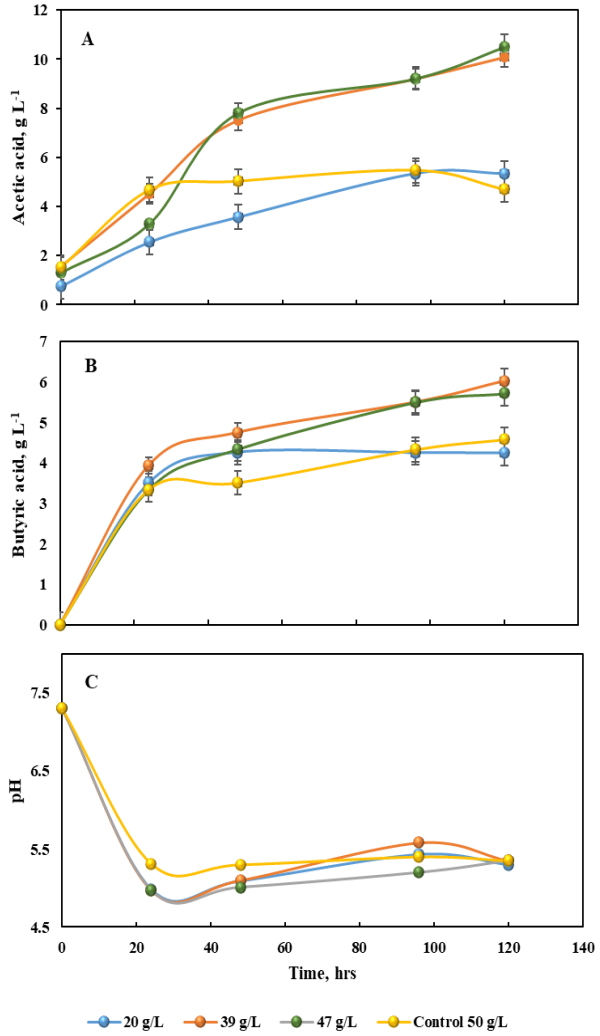

Figure 4. Variation of acetic acid (A), butyric acid production (B), and pH (C) with time at different initial substrate concentrations

In general, substrate type, inoculum, and reactor configuration also influence the concentration of bioproducts during ABE fermentation. This aligns with the findings of Ghimire et al. (2015), who studied methods to improve biohydrogen production. Early hydrogen generation increases the partial pressure inside the reactor, leading to product accumulation in the headspace. This results in reduced ferredoxin availability, thereby lowering hydrogen production. The inoculum concentration in the fermentation medium also affects volatile fatty acid production. Kriswantoro and Chu (2024) compared 5% and 10% inoculum additions and observed increased organic acid production with 10% inoculum. As noted earlier, a positive relationship exists between organic acid production and hydrogen generation up to a certain substrate concentration—this threshold varies

depending on the inoculum and substrate type used in fermentation.

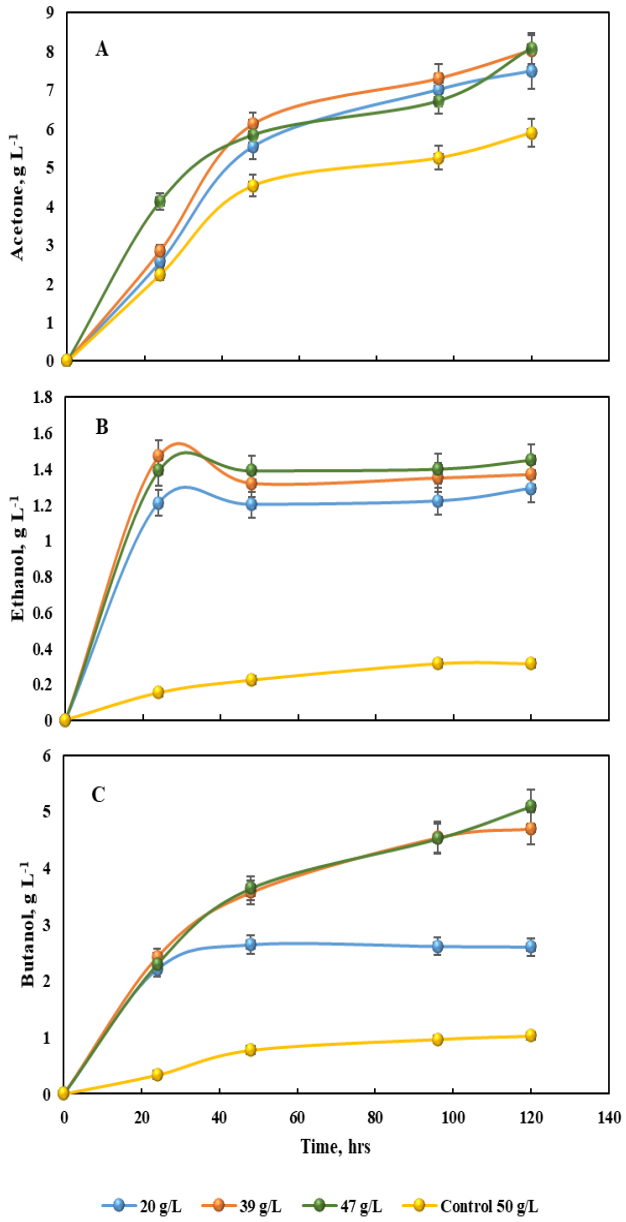

Figure 5. Variation of acetone (A), ethanol (B), and butanol (C) productions with time at different substrate concentrations

Capilla et al. (2021) investigated the effect of initial glucose concentrations (33, 66, and 100 g L^{-1}) on ABE fermentation. Final acetic and butyric acid concentrations were around 5 and 6 g L^{-1}, respectively. They attributed increased organic acid production (acetate and butyrate) to calcium ion (Ca^{2+}) supplementation in the fermentation media. Interestingly, the final total organic acid concentrations reported were similar to those found in the present study. In our previous work on the effects of metals on ABE fermentation and biohydrogen production—using the Plackett-Burman experimental design—we also confirmed that Ca^{2+} has a significant positive impact on acetate and butyrate production (Abibu & Karapinar, 2024).

The study by Capilla et al. (2021) remains one of the few known investigations into the effect of glucose concentration on ABE fermentation. As expected, better results were achieved using pure glucose as the fermentation substrate. Their study reported maximum sugar consumption of 33 g L^{-1} and final butanol concentrations of 3.35, 7.47, and 8.01 g L^{-1} at initial glucose concentrations of 33, 66, and 100 g L^{-1}, respectively. A similar trend was observed in the current study, with the highest sugar utilization occurring at the lowest substrate concentration (20 g L^{-1}), resulting in a final butanol concentration of 2.6 g L^{-1}.

Daily pH control has been shown to reduce the accumulation of undissociated organic acids that could otherwise inhibit the transition from the acidogenic to the solventogenic phase of ABE fermentation. Furthermore, elevated initial glucose concentrations may induce substrate inhibition in the fermentation system.

Comparing Figure 3 with Figure 5, it is clear that product formation (organic acids and solvents) peaked when total sugar was nearly completely consumed by the microorganisms involved in the fermentation process (Figure 3). This is particularly evident around the 120th hour of fermentation. Furthermore, the 20th hour marked a critical point characterized by a sharp decrease in total sugar (Figure 3) alongside a corresponding sharp increase in product formation (Figures 4 and 5). Thus, an inverse relationship between total sugar utilization and product formation can be concluded from this study.

The results showed that hydrogen production (including hydrogen yield, cumulative hydrogen production, and production rate), organic acids, and ABE products were higher at relatively elevated total sugar concentrations (39–47 g L^{-1}) compared to glucose. Our previous study demonstrated that microwave pretreatment of fig often produces some inhibitory compounds, albeit in low amounts (Abibu & Karapinar, 2023). Hydroxymethylfurfural (HMF) is one such inhibitory byproduct identified in our earlier work. An HMF concentration of 2.29 g L^{-1} was detected following microwave pretreatment of waste fig, alongside fermentable sugar production of 80 g L^{-1}. At an initial total sugar concentration of 47 g L^{-1} in fig hydrolysate, the HMF concentration corresponds to approximately 1.35 g L^{-1}.

This suggests that fig hydrolysate, despite potentially containing inhibitory compounds such as furfural and hydroxymethylfurfural, does not limit hydrogen production, organic acid formation, or ABE production when compared with the glucose control, which is free of HMF and furfural. Although some studies suggest that high HMF concentrations negatively affect microbial communities and performance, low HMF levels have been reported to favor hydrogen production in fermentation processes. According to Muñoz-Páez et al. (2018), HMF can also be biodegraded, contributing to hydrogen production. The inhibitory effects of HMF on biohydrogen production are not yet fully understood.

Nevertheless, the ability of *Clostridium pasteurianum* DSM 525 to produce organic acids, solvents, and hydrogen indicates a notable tolerance to HMF. It can also be inferred that reduced metabolic activity observed at substrate concentrations above 47 g L^{-1} may reflect pronounced inhibitory effects of HMF. This study did not include a quantitative assessment of HMF's specific impact on fermentation kinetics or microbial stress responses. The ABE formation, particularly ethanol and butanol production, was significantly enhanced using fig hydrolysate. Butanol concentrations recorded at 39 and 47 g L^{-1} total sugar showed a 500% improvement compared to those reported by Capilla et al. (2021) for glucose fermentation. The concentrations of butyric acid and butanol in this study are

consistent with previously published fermentation experiments involving *Clostridium* spp.

The highest butyric acid concentration observed here (6.02 g L^{-1}) aligns with Atasoy and Cetecioglu (2020), who reported 2.889 g L^{-1} from dairy industry wastewater. Similarly, the peak butanol concentration (5.09 g L^{-1} at 47 g L^{-1}) is comparable to the 15.8 g L^{-1} butanol reported by Capilla et al. (2021) at 100 g L^{-1} glucose using *Clostridium acetobutylicum* DSM 792 as inoculum. The relatively low acetone concentration observed in our study, compared to other ABE fermentation works, aligns with the natural metabolic profile of *C. pasteurianum*, which favors butanol production over acetone. Since butanol is the major solvent of interest in ABE fermentation, these comparative results suggest that fig hydrolysate is an excellent substrate for producing organic acids, solvents, and hydrogen.

The superior product yields with fig hydrolysate may be attributed to the naturally occurring metals in figs, which significantly enhance ABE solvent production, as highlighted in our recent publication (Abibu & Karapinar, 2024). Thus, this study confirms that waste fig is a promising substrate for ABE fermentation. It is worth noting that comparisons between fig hydrolysate and glucose-based fermentations are based on observed sugar utilization and product formation trends within this study.

The increased hydrogen yields observed at 47 g/L may be attributed to substrate abundance supporting enhanced metabolic activity by the inoculum used in this study. Key biochemical pathways, including glycolysis and the acetyl-CoA step, are likely actively engaged, directing increased flux toward organic acid and solvent production during the acidogenesis and solventogenesis stages, respectively. Higher sugar concentrations favor the availability of NADH (Mostafazadeh et al., 2016), which in turn promotes greater solvent and hydrogen formation. The best hydrogen and solvent yields were recorded at 47 g/L, suggesting an optimal metabolic shift toward solvent generation under moderate substrate conditions that maintain redox balance with minimal metabolic stress. However, substrate concentrations exceeding 47 g/L resulted in decreased product concentrations, likely due to substrate inhibition and osmotic stress. It is important to emphasize that these findings are preliminary and do not conclusively define the optimal conditions. Therefore, further optimization through fed-batch or continuous fermentation approaches, coupled with scale-up studies, is needed to confirm process robustness and industrial viability. Additionally, downstream product recovery and techno-economic assessments will be systematically addressed in future work.

4. CONCLUSIONS

Incineration, the primary disposal method for waste figs, contributes to greenhouse gas emissions. As a lignocellulosic biomass, fig remains underutilized in biofuel production. This study evaluates its potential for ABE fermentation and biohydrogen production using *Clostridium pasteurianum* DSM 525, highlighting the crucial influence of substrate concentration. Maximum cumulative hydrogen production (1402 mL) and peak organic acid concentrations (acetic acid: 10.49 g L^{-1}; butyric acid: 6.02 g L^{-1}) were observed at 47 g L^{-1} and 39 g L^{-1}, respectively. The highest solvent yields (ethanol: 1.45 g L^{-1}; acetone: 8.08 g L^{-1}; butanol: 5.09 g L^{-1}) occurred at 47 g L^{-1}, identifying this as the optimal substrate concentration for butanol, the primary ABE product. Elevated substrate levels (39–47 g L^{-1}) promoted organic acid accumulation, which enhanced hydrogen production, with butanol formation closely linked to butyric acid concentration. Acetone production depended on acetic acid conversion. Future research should focus on kinetic and metabolic studies to better balance hydrogen and solvent yields—particularly butanol—for sustainable valorization of fig waste.

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