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# Effect of Media Composition on Biohydrogen Production from Fig (*Ficus carica*) by Dark Fermentation

**[1,2,*]Abibu, W., [3] Kaya, M., [3] Karadas, Y., [2,3] Karapınar, İ.**

[1] Federal University of Agriculture, College of Biosciences, Department of Microbiology, 2240, Abeokuta, Nigeria

[2] Dokuz Eylul University, Natural and Applied Sciences, Biotechnology Department, 35390, İzmir, Turkey

[3] Dokuz Eylul University, Engineering Faculty, Environmental Engineering Department, 35390, İzmir, Turkey

## Abstract

In this study, mesophilic batch fermentation experiments were conducted to investigate the effects of varying C/N ratios (25 -100) on biohydrogen production. The fermentable sugar was obtained from microwave hydrolyzed waste fig (*Ficus carica*). A control experiment without external nitrogen addition was also set-up to evaluate if the nitrogen content of hydrolyzed fig could sustain biohydrogen production. The highest cumulative hydrogen volume (861± 0.5 ml) and volumetric production (4.3 ± 0.3 $L/L_{reactor}$) were obtained at C/N=50. The yields varied between 0.9 ± 0.21 and 1.1 ± 0.23 mol $H_2$ $mol^{-1}$ $TS_{consumed}$ for C/N= 25-100. The highest production rates achieved were 11.5 ± 0.12 ml $h^{-1}$ and 57.5 ± 0.14 ml $L^{-1}$ $h^{-1}$ at C/N=50. Thus, the best ratio in terms of substrate conversion to hydrogen is C/N=50 which can be evaluated as the nitrogen-sufficient condition. The nitrogen content of the hydrolyzed waste fig (control) was not sufficient for efficient biohydrogen production and external nitrogen addition is required to obtain a higher production rate. There were significant differences between the C/N ratio for cumulative hydrogen volumes, hydrogen production rate and volumetric hydrogen production rate.

***Keywords:*** Fig, biohydrogen, microwave, hydrolysis, total sugar production

*Corresponding author e-mail: abibu@funaab.edu.ng: *Orcid: https://orcid.org/0000-0002-1156-3779*

## Introduction

The environmental effect of fossil fuel shifted attention towards renewable energy which is environmentally friendly. Biohydrogen, a form of biofuel is a renewable energy and capable of contributing its quota to reducing dependency on fossil fuel. Fig (*Ficus carica*) is a Mediterranean fruit rich in sugar (Ercisli et al.,2012). Pretreated waste figs can be transformed into biofuels via anaerobic digestion. Carbon and Nitrogen are macronutrients needed by all life forms for existence (Li et al., 2014). Carbon to Nitogen ratio (C/N) measures nutrient balance availability needed for life processes (Argun et al., 2018).

Biohydrogen research stemmed out from the rising interest in renewable energy. It is a fermentation based, microbial mediated value-added product and it forms an integral part in the production, promotion, and sustainability of green energy technologies. Veeramalini et al. (2022) classified biohydrogen production technologies into photo-fermentation, bio-pyrolysis, microbial electrolysis cell, microbial fuel cell and dark fermentation. *Enterobacter, Escherichia,* and *Clostridium* are important microbial genera with high biohydrogen production potential (Goyal et al., 2013). Biohydrogen fermentation substrates pre-treatment is highly necessary. Pre-treatment makes fermentable sugar readily available for microbial utilization, thereby enhancing fermentation for biohydrogen production (Vivek et al., 2019). Pre-treatment may be physical, chemical, or biological. Microwaving is a physical pre-treatment type achieving lignocellulose substrate delignification within a short time via complete thermal penetration of biomass (Brasoveanu and Nemtanu, 2014)

Fig is widely grown in Turkiye with yearly export of approximately 200,000 tones (Ercisli et al., 2012). It is a rich source of sugar, organic acids, and protein (Slatnar et al., 2011). Aflatoxin contaminated fig is disposed by incineration. However, the high sugar content of fig can act as biohydrogen fermentation substrate.

Carbon and Nitrogen are vital elements needed by all life forms for energy, growth and genetic build up. Urea is a low cost, readily available and rich nitrogen source acting as protein source for microorganisms, enhancing microbial performance and improving fermentation yield (Mcguire et al., 2013; Voltolini et al., 2010). Also, urea can act as a fermentation media nitrogen source substitute for yeast extract and peptone in biohydrogen fermentation. Tareen et al. (2021) successfully investigated ethanol production using urea as nitrogen source. As reported by Li et al. (2014), ☐Carbon to Nitrogen ratio (C/N) translates into nutrient balance availability for microbial utilization in fermentation medium. Fig is naturally a rich source of carbon. Incorporating urea into fermentation media components improves the C/N ratio, thus, enhancing microbial activity. Considering this fact, this study explored hydrogen production potential under C/N ratios of 0, 25, 50 and 100. *Clostridium pasteuranium* DSM 525, a prolific biofuel producing microorganism was used as fermentation microbial source under mesophilic condition. Our previous research has shown that fig is rich in fermentable sugar and other important macro and micronutrients needed for microbial performance in the fermentation process for hydrogen and butanol production (Abibu and Karapinar, 2023). Anaerobic fermentation via co-digestion of Sugar cane molasses, distillery effluent and starchy wastewater for hydrogen and n-butanol production was done by Singh et al. (2020). Highest hydrogen and butanol productivities of 96 mL $L^{-1}$ $h^{-1}$ and 46 mL $L^{-1}$ $h^{-1}$ respectively were achieved in the pretreated water hyacinth and starchy wastewater.

Similarly, Mahato et al. (2020) pretreated fruit wastes via microwaving. Hydrogen production from hydrolysate via dark fermentation was varied with/without inoculum (*Clostridium* strain $BOH_3$). $H_2$ production recorded in the absence and presence of inoculum were 2526-3118 mL $L^{-1}$ and 10720 mL $L^{-1.}$ Also, in the study conducted by Rambabu et al. (2020), hydrolysate from acid pretreated date-palm fruit wastes was fermented for $H_2$ production with *Enterobacter aerogenes* as inoculum with $Fe_3O_4$/ Date seed-derived activated carbon (DSAC) nanocomposites addition to fermentation medium. Hydrogen productivity of 238.37 mL $H_2$ $L^{-1}h^{-1}$ was recorded from the study. Thus, this clearly indicates the potential of biomass pretreatment to achieve increased biohydrogen production.

Saidi et al. (2020) co-fermented fruit and fish wastes for hydrogen production using *Thermotoga maritima* as inoculum within a C/N ratio of 12 and 47. Total hydrogen production of 285 mmol $L^{-1}$ and $H_2$ yield of 3.86 mol $H_2$ $mol^{-1}$ were obtained at C/N ratio of 22. In the same vein, Gomez-Romero et al. (2014) studied $H_2$ from the co-fermentation of cheese whey with fruit vegetable waste at C/N ratios of 7- 46. Highest $H_2$ production yield and rate of 449.84 mL $H_2$/g COD and 10.68 mmol $H_2$ $L^{-1}h^{-1}$, respectively were recorded at C/N of 21. In another study by Argun et al. (2008) at C/N of 20 - 200 and C/P of 50 – 1000, maximum $H_2$ yield and rate of 281 ml $H_2$ $g^{-1}$ starch and 98 ml $H_2$ $g^{-1}$ biomass $h^{-1}$, respectively were recorded at C/N and C/P ratios of 200 and 1000, respectively.

This current study investigated effect of C/N ratio on $H_2$ production with *Clostridium pasteuranium* DSM 525 as inoculum and fig (*Ficus carica*) In addition, this is the only study that considered fig as substrate and *C.pasteuranium* DSM 525 as inoculum in biohydrogen research. Furthermore, this is the only study that considers the effect of C/N ratio on $H_2$ production in recent years.

## Materials and Methods

### *Substrate preparation*

Waste figs were obtained from the Aegean Exporter Association, Izmir, Turkiye. The figs were cut into small sizes (≤ 5cm) and dried at 72°C for 70 hours. Upon drying, figs were blended and sieved with the aid of a mechanized sieving machine to different sizes in the range of 150 - 300 µm. The pre-treatment conditions described by Abibu and Karapinar (2023) were employed in this study with total sugar concentration of 82g/L obtained.

### *Experimental set up*

Mesophilic batch fermentation involving 310 ml capacity serum bottles were used in this study. The fermentation total working volume was 200 ml. Urea was used as the nitrogen source, and it was externally added to the fermentation medium. A control experiment without nitrogen addition was conducted and named C/N=Control. The Total Kjeldahl Nitrogen (TKN) and $NH_4$-N concentration in the 100 g/L microwave fig hydrolysate were 98 mg/L and 0.24 mg/L.

The fermentation medium contained 3.9 $KH_2PO_4$, 0.05 $FeSO_4.7H_2O$, 0.1 L-cysteine and 0.25 $MgSO_4$ in g $L^{-1}$ at pH 7.0. Medium sterilization was done with autoclave at 121°C for 15 min. Anaerobic condition was ensured by passing all experimental set-ups under nitrogen gas from head space of the bottles.

### *2.3 Organism*

The organism (*C. pasteuranium* DSM 525) was inoculated (10% v/v) in an inoculation 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 organism was incubated at 37°C with pH 7.0 for 48 hrs before being used as inoculum for fermentation.

### *2.4 Analytical Methods*

Daily hydrogen production from the experimental set-ups were carefully monitored via taking gas samples through the head space of the bottles and also determining the total gas volume by water displacement method (Tosuner et al., 2019). Agilent Gas Chromatograph (1100 series) with TCD and connected to ChemStation (a computer software) was used for percentage hydrogen determination (Eker and Erkul, 2018). The $H_2$ production rate in this study represented the overall cumulative $H_2$ production per reactor volume in 1 h (mL $H_2$ $L_{reactor\ volume}^{-1}$ $h^{-1}$).

pH range 6.5-7 was ensured throughout the study. Daily collection of samples was done for seven (7) days. The Dubois method (Dubois et al., 1956) was used for the total sugar analysis while the high-performance liquid chromatography (Agilent 1200 series, USA) was used for organic acid analysis.

### *Data analysis*

Data obtained were analysed using Analysis of Variance (ANOVA) and means were separated using SNK (Student-Newman-Keuls) statistics at p- value of 0.05

## Results and Discussion

The effect of the C/N ratio (25, 50 and 100) on biohydrogen production was studied via batch fermentation under mesophilic conditions. The time course of total hydrogen production at varying C/N ratios is depicted in Figure 1. Hydrogen production started immediately after inoculation of hydrogen-producingspore-forming *C. pasterianum*. The substrate is the waste fig fruit with high sugar content which was made more available by microwave pretreatment. Therefore, there was no lag phase or delay in the fermentation or hydrogen production, which is mostly observed in lignocellulosic wastes. Our previous study showed that some toxic substances were formed during microwaving. Hydroxy-methyl furfural (HMF) is one of the common toxic substances generated during acidic or heat treatment of sugar containing wastes (Rosatella et al., 2011). The concentration of HMF formed from fig hydrolysis was HMF=2.29 g $L^{-1}$ under optimized conditions with the sugar content of around 80 g/L (Abibu and Karapinar, 2023). Hydroxy-methyl furfural concentration in this study corresponded to 1.145 g $L^{-1}$ with initial sugar concentration of $TS_0$=40 g $L^{-1}$. The lack of lag phases for C/N= 0-100 in this study showed that HMF concentration in the fig hydrolysate did not substantially affect the hydrogen production. There was a slight delay in hydrogen production for the control experiment without external nitrogen source addition. This result indicated that fig hydrolysate is nitrogen deficient for effective hydrogen production.

The cumulative hydrogen volumes and hydrogen yield obtained at the end of fermentation for different C/N ratios were summarized in Figure 2 (a and b). The production volume increased with C/N ratio up to C/N=50 with maximum volume of CHV= 861± 0.5 ml and then decreased for the C/N>50. However, the yield was almost constant at around 1.1 ± 0.23 mol mol$^{-1}$ TS$_{consumed}$ for the C/N ratios between 25 and 100. The yield of hydrogen formation in the control experiment without nitrogen source addition was around 0.9 ± 0.21 mol mol$^{-1}$ TS$_{consumed}$ which was not substantially different from the ones with nitrogen added. The sugar content of the hydrolyzed waste fig was effectively (80%) consumed for all C/N ratios studied.

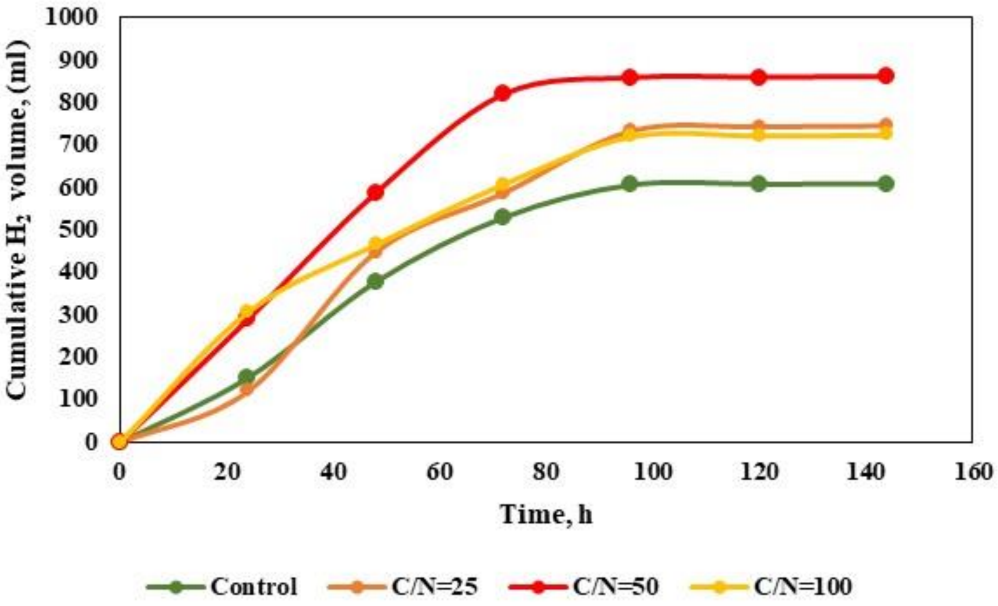

Fig 1: Time course of hydrogen production at different C/N in the fermentation media

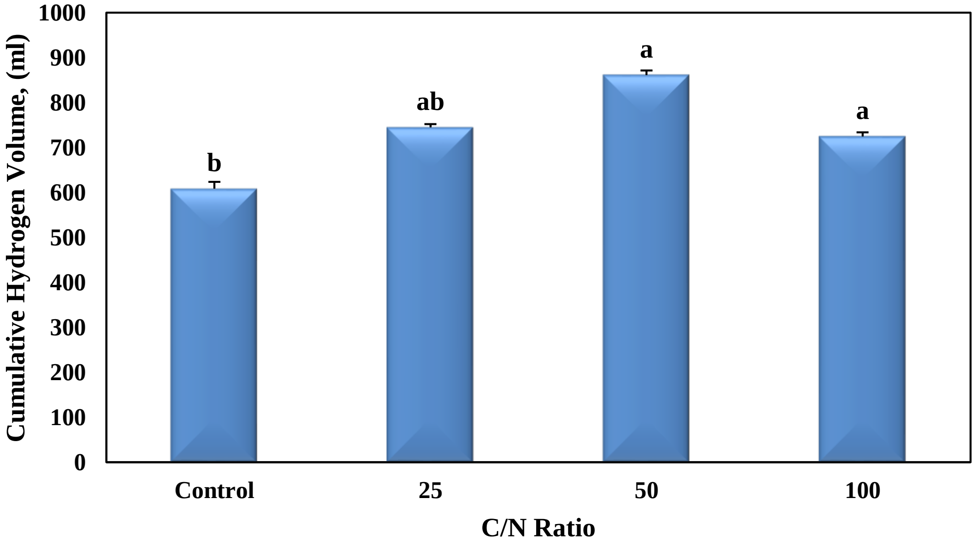

Fig 2a: Cumulative hydrogen volumes at different C/N ratio in fermentation media (Means with different letters along the bars are significantly different (P< 0.05)

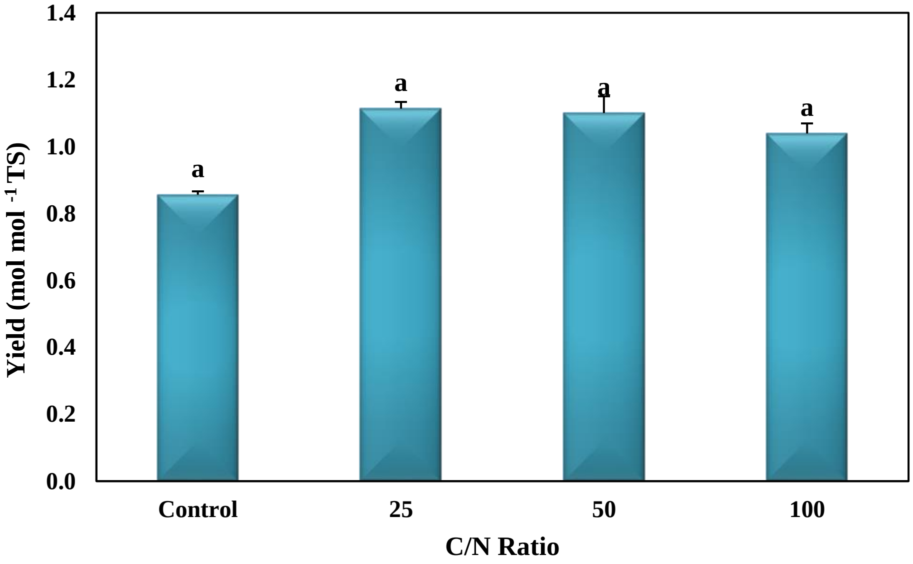

Fig 2b. Variation of yield of hydrogen formation with C/N ratio in fermentation media (Means with different letters along the bars are significantly different (P< 0.05)

The main organic acids produced were acetic acid (HAc) and butyric acid (HBt) as given in **Figure 3 (a and b)**, respectively. No lactic acid and propionic acids formed. The concentrations of acetate in these experiments were more than that of butyrate. The final acetic and butyric acid concentrations were in the range of 6.74 -7.39 g L$^{-1}$ and 4.99 - 7.39 g L$^{-1}$, respectively. The nitrogen concentration did not affect the organic acid types and their concentrations. The cumulative hydrogen production trend was almost like acetic acid production. Hydrogen production terminated around 80th h of fermentation period at which acetic acid concentration reached its maximum value. In the case of butyric acid generation, a fast production was observed for the first 20 h of fermentation and then, production continued at a slow rate. This result indicated that acetic acid accumulation in the media slowed down the butyric acid generation. In addition, nitrogen rich conditions such as C/N=25 slightly favored the butyric acid production. According to Gonzales et al. (2016), high butyrate concentrations translated to higher conversion of metabolites into butyrate production pathway during hydrogen production. In addition, Bundhoo et al. (2016) concluded that low propionate concentrations is desirable as it implies reduced potential of $H_2$ as electron donor by propionic acid producing microorganisms. This is supported by this study as no propionate was observed throughout the experiment.

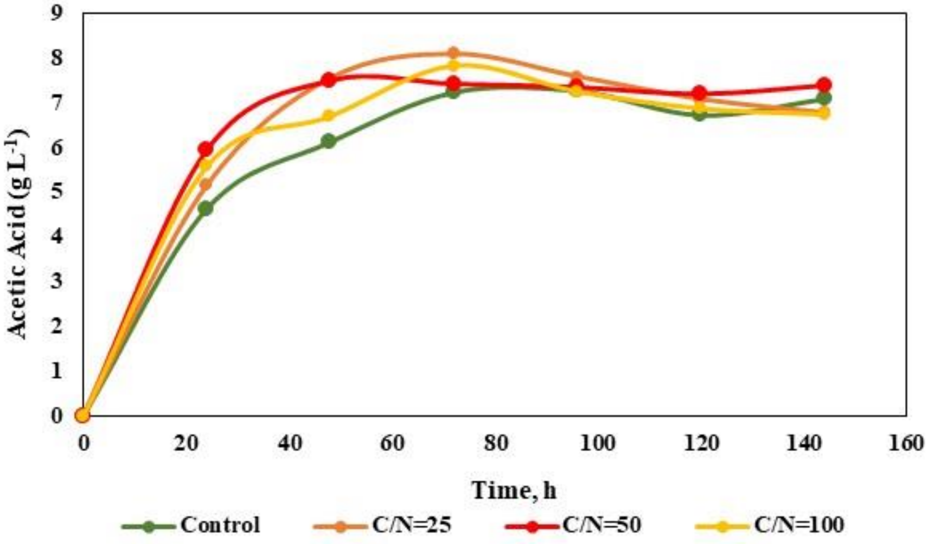

Figure 3a. Acetic acid production at different C/N ratio

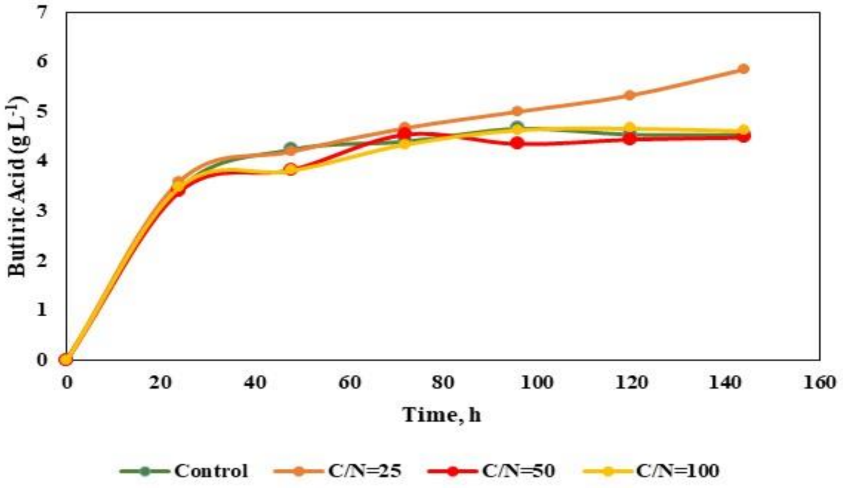

Fig 3b. Butyric acid production at different C/N ratio

The maximum rate of production was 11.5 ± 0.12 ml $h^{-1}$ (Figure 4a) and 57 ± 0.14 mL $L^{-1}$ $h^{-1}$ (Figure 4b) attained at C/N=50. The hydrogen purity in the total gas reached up to 54% which indicated a good purity of hydrogen for a biological process. Saidi et al. (2020) studied $H_2$ production from co-digestion of wastes with the bacterium *Thermotoga maritima* as microbial source. C/N ratios used in the study to determine hydrogen production potentials were achieved from the co-digestion ratios. In addition, the effect of nitrogenous sources ($NH_4Cl$ and cysteine HCl) on the experimental setup at different C/N ratios was studied. It was discovered in the study that co-fermentation in the presence of $NH_4Cl$ and cysteine HCl at the lowest C/N ratio of 12 (used in the study) improved cumulative $H_2$ production 176 mmol $L^{-1}$ and $H_2$ yield of 3.87 mol $H_2$ $mol^{-1}$ glucose. Similarly, the co-fermentation in the absence of $NH_4Cl$ and cysteine HCl at the lowest C/N ratio of 12 gave highest cumulative $H_2$ production of 132 mmol $L^{-1}$. However, a further increase in substrate concentration to C/N ratio= 22 yielded $H_2$ production rate of 28 mmol $H_2$ $L^{-1}h^{-1}$.

The variation of total sugar concentration at different C/N ratio with time in this study is shown in Figure 5.

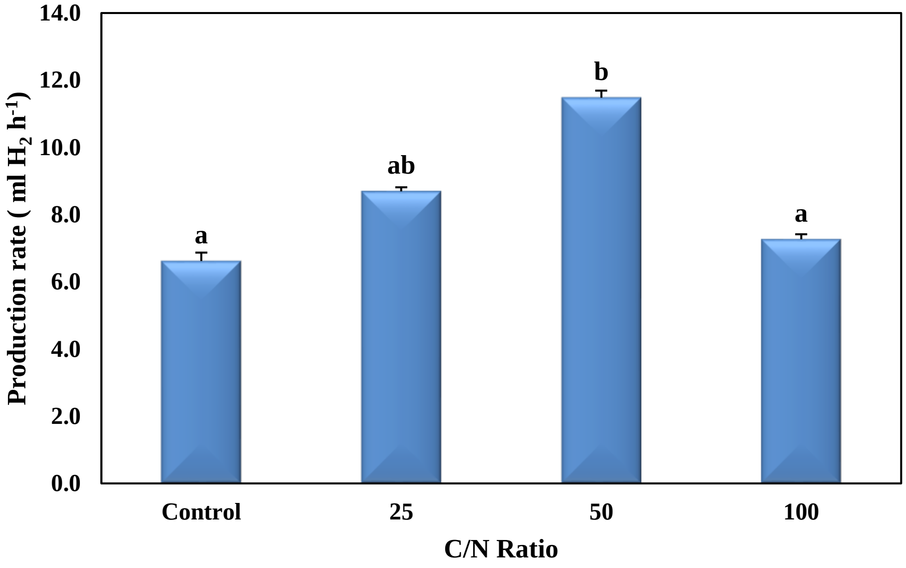

Fig 4a. Variation of hydrogen production rate with C/N ratio (Means with different letters along the bars are significantly different (P< 0.05)

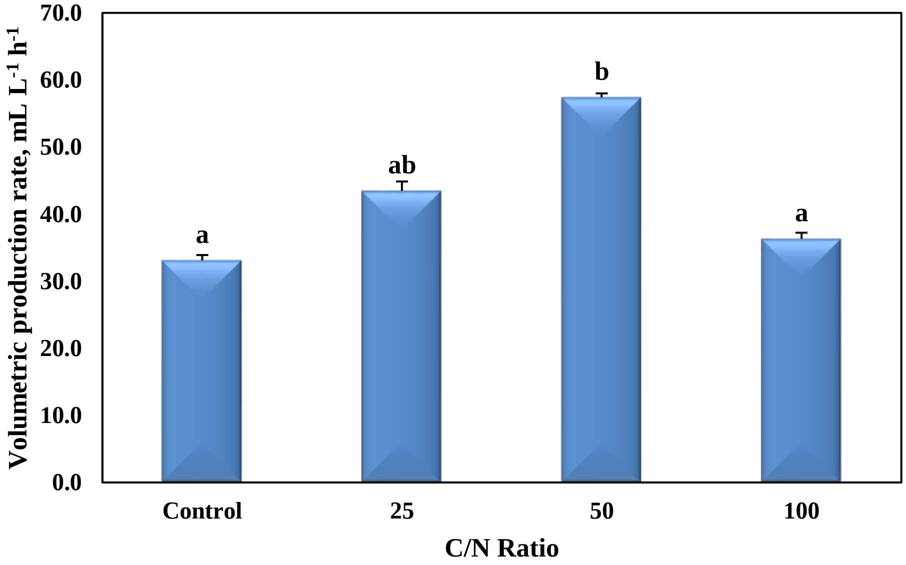

Fig 4b. Variation of volumetric hydrgen production rate with C/N ratio (means with different letters along the bars are significantly different (p< 0.05)

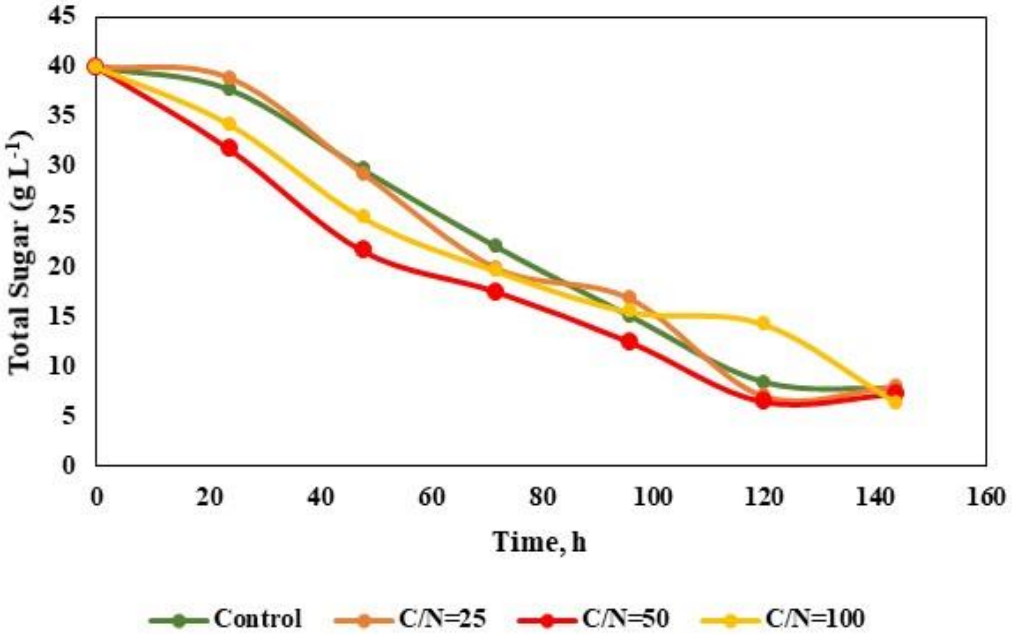

Fig 5. Time variation of total sugar concentration at different C/N ratio

In this study, urea was the only source of nitrogen used for fermentation (in the absence of yeast extract, $NH_4Cl$, cysteine HCl and peptone). From the results, cumulative hydrogen production (CHP) increased from the setup within C/N ratio 0 (600 ml) and 50 (900 ml). Above C/N ratio 50, CHP reduced to almost 1/3rd of that recorded in C/N ratio 50. Similarly, the highest $H_2$ yield of 1.1 ± 0.23 mol $mol^{-1}$ $TS_{consumed}$ was recorded around C/N= 25-50. These results are similar to that of Saidi et al. (2020) that lower C/N ratio achieved better $H_2$ production. The low $H_2$ production results from this study may be attributed to low availability of nitrogen source for microbial activity during fermentation when compared with other studies that considered numerous nitrogen sources as composition of fermentation medium. This is clearly indicated in the $H_2$ yield of 0.9 ± 0.21 mol $mol^{-1}$ $TS_{consumed}$ in the controlled experiment (C/N=0) and 1.1 ± 0.23 mol $mol^{-1}$ $TS_{consumed}$ for C/N= 50. Furthermore, Saidi et al. (2020) best result was at C/N ratio = 22 which was lower than C/N ratio=25-100 tested in our study. Thus, a lower C/N ratio is expected for improved $H_2$ production results. C/N ratio was within 20 and 200 in the study of Argun et al. (2008), the maximum production yield was obtained at C/N=200. Öztekin et al. (2008) used acid hydrolyzed waste wheat and the optimal C/N ratio with highest yield of formation was obtained at C/N=50. Similarly, Argun and Dao (2016) reported that C/N=45.7 is optimal for maximum hydrogen production from peach pulp.

## Conclusion

The ever-increasing human population translates into an increasing need for energy which is mostly derived from fossil fuels. The environmental effect of fossil fuels shifted attention toward environmentally friendly renewable energy. Biohydrogen, a form of biofuel is a renewable energy and capable of contributing its quota to reducing dependency on fossil fuels. Pretreated waste figs can be subjected to anaerobic fermentation to yield value-added products like biohydrogen. Batch dark fermentation of microwave hydrolyzed waste fig for biohydrogen production showed that waste fig has a high potential for hydrogen generation. Nitrogen content up to C/N=100 in the fermentation medium did not substantially affect the yield of hydrogen formation.. However, the hydrogen formation rate was affected by the nitrogen concentration and the maximum rate was obtained at C/N of 50. Thus, a C/N ratio of 50 is highly recommended in terms of hydrogen formation rate.

## Declaration for competing interest

The authors declare no competing interests could have influenced the work reported in this study.

## Acknowledgement

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