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Kinetic Study of Biohydrogen Production Improvement via Dark Fermentation of Sugarcane Molasses by *Escherichia marmotae*

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HYDROGEN (H_2) is expected to become the most sustainable and promising clean alternative fuel in the future. This study evaluated the potential of a new facultative anaerobic bacterium isolated from cow rumen to produce H_2 from sugarcane molasses. The bacterial isolate RM122 produced 451.67 ±12.14 and 387.67 ±19.23mL/L H_2 on 6% glucose and 6% molasses sugar, respectively. RM122 was characterized phenotypically, identified genotypically by 16S rRNA sequence analysis as *Escherichia marmotae,* and deposited in NCBI GenBank database with the accession number OP345936. H_2 production was improved to 1670.00 ±40.41mL/L by application of optimization experiments design and kinetic study. Sugarcane molasses was used as a fermentation substrate and the optimum sugar concentration was 4%, The recorded maximum hydrogen production (H_{max}) was 560.00±25.98mL/L with R_{max} (maximum hydrogen production rate) of 31.43 ±2.14mL/L/h, R_{max} (MGM) of 30.73 mL/L/h and λ (lag phase) of 15.19h for 52.43 fermentation time. H_{max} of 646.67 ±23.33mL/L with R_{max} (Exp) of 35.28 ±2.65mL/L/h, R_{max}(MGM) of 31.90mL/L/h, λ of 13.92h and R^2 of 0.9999 were obtained at the optimum pH 8. At the optimum fermentation temperature (35°C), λ of 4.12h was achieved to maximize hydrogen production to 828.33 ±21.67mL/L with R_{max}(MGM) of 27.98mL/L/h, and R^2 of 0.9594. At the optimum inoculum size (10%, v/v), the recorded H_{max} was 1670.00 ±40.41mL/L with R_{max} (Exp) of 60.00 ±2.04mL/L/h and λ of 13.50h. These findings suggest using *E. marmotae* RM122 as a prospective biohydrogen producer from cheap agro-industrial wastes.

Keywords: Agro-industrial wastes, Biohydrogen generation, Dark fermentation, Modified Gompertz model, Ruminant bacteria.

Introduction

Developing of sustainable and promising technologies to produce renewable clean fuels is a global concern today, this because the increased energy demand, gas emissions and climate changes (Rasmey et al., 2020). Hydrogen (H_2) is considered the most attractive alternative energy carrier with important features, for instance; its highest energy density and complete cleanness after burning (Navlani-García et al., 2018; Neuwirth et al., 2022). There are numerous exciting generation routes for H_2 such as natural gas thermal cracking & steam reforming, coal gasification, and water electrolysis, however,

all these routes are highly cost, time-consumed, energy-intensive, and less environmentally friendly (Benemann, 1996; Shown et al., 2014). Conversely, H_2 generation by biological routes has numerous important advantages like a simple operation system, availability of sustainable feedstocks, carbon neutrality, and cost-effectiveness (Han et al., 2016; Sun et al., 2019).

H_2 production by fermentation can be achieved through three main routes; Photo fermentation (PF), dark fermentation (DF) and photo-dark fermentation. DF is considered the ideal cost-effective way for H_2 production because of its higher formation rate and the

possibility of its production on different organic wastes (Kumar et al., 2018; Lee et al., 2021). DF of H_2 was conducted by various bacterial species for instance; anaerobes (*Clostridium* sp.) and facultative anaerobes (*Escherichia*, *Citrobacter*, *Enterobacter*, and *Bacillus* spp). Through dark fermentation, sugars are metabolized into pyruvates which oxidized into acetyl coenzyme A (acetyl-CoA), H_2, and CO_2 (Elsharnouby et al., 2013; Rao & Basak, 2021). Also, formic acid fermentation can be occurred by different members of Enterobacteriaceae in which formic acid is produced from pyruvic acid and is further metabolized into H_2 and CO_2 (Clion et al., 2015). Carbohydrates are the most effective feedstock for hydrogen production in DF through improving acetic acid and butyric acid production as intermediates (Barca et al., 2015). H_2 generation from carbohydrate-based substrates is twenty times higher than that from protein-based substrates (Cheng et al., 2015). Since low H_2 yields are reported using different sugars, it is crucial to find cheap feedstocks of high commercial value rather than using synthetic sugars. The use of sugar molasses (a by-product of sugar industry) as a feedstock led to higher H_2 production rate and yield (Tsioptsias et al., 2016; Mohammadi et al., 2019).

Different factors particularly substrate type, substrate concentration, bioreactor configuration, fermentation temperature, initial pH, inoculum size, and organic loading rate can affect hydrogen production through DF processes (Guellout et al., 2018). Some kinetic models have also been proposed to explain the effects of these factors on H_2 yield and its production rate. In addition, these models provide kinetic constants which give critical information about the analysis, design, and operation system of H_2 fermentation process (Wang & Wan, 2009). The two kinetic models; modified Gompertz model (MGM) and modified Logistic model (MLM) have been widely used to describe dark fermentative H_2 production (Pasupuleti & Venkata Mohan, 2015; Basak et al., 2018).

The current study aimed to improve biohydrogen production from sugarcane molasses by *Escherichia marmotae* as a ruminant facultative anaerobic bacterium. As well as, kinetic analysis of optimization experiments using modified Gompertz model (MGM) was applied.

Materials and Methods

Microorganism source and inocula preparation

The used H_2 producer bacterium was isolated from a cow rumen sample on thioglycolate agar medium (tryptone 15.0, L-cystine 0.5, glucose 5.5, yeast extract 0.5, NaCl 2.5, sodium thioglycolate 0.5, resazurin 0.001 and agar 18.0g/L). The anaerobic condition was adjusted by flashing the fermentation bottles with nitrogen gas and then sealed with rubber septa. The bacterium was preserved under anaerobic conditions at 4°C. Bacterial inoculum (3 x 10^6cells/mL) was prepared by inoculation of one mL of 48h old culture into 99mL sterilized fluid thioglycolate broth then incubated for 48h at 37°C (Deabes et al., 2020) under anaerobic conditions.

Pretreatment of sugarcane molasses

The used sugarcane molasses was pretreated and prepared as a feedstock for H_2 production by the tested bacterium according to Rasmey et al. (2018). Molasses was diluted to the required sugar concentration using distilled water. pH 7 of the diluted molasses was adjusted using concentrated H_2SO_4 and 0.1N NaOH. Then, molasses was heated at 95°C for 15min in a water bath and was left for 2h to cool and precipitate the impurities. The cleared molasses was transferred to the sterilized fermentation bottles for H_2 production.

Fermentation process

Modified 6% glucose T6 broth medium (tryptone 6.0, yeast extract 2.0, $MgSO_4$-$7H_2O$ 0.3, $FeSO_4$-$7H_2O$ 0.01, KH_2PO_4 0.5, ammonium acetate 3.0, sodium thioglycolate 2.0, L-cystine HCl 0.5, and glucose 60.0g/L) and 6% sugar concentration of pretreated molasses were used as fermentation media to demonstrates the ability of the selected bacterial isolate to produce H_2. In 600mL glass bottles, 5mL bacterial inoculum (1%, v/v) was inoculated to 495 mL of the studied fermentation medium. Bottles were sealed with rubber plugs and put on a shaking water bath with 60rpm for 72h at 37°C.

Hydrogen assay

The produced gas was passed through bottles containing 2M NaOH solution to absorb and precipitate carbon dioxide gas and the cumulative hydrogen gas was collected in an inverted cylinder by water displacement method (Mishra & Das, 2014). Confirmation of produced H_2 was performed using gas chromatograph (TRACE

GC Ultra Thermo Scientific) supplied with a thermal conductivity detector and a stainless-steel column covered with a molecular sieve. The column temperature was initially 50°C and increased at a rate of 30°C/min to 200°C. All gas volumes were reported at 1.0 atm and 25°C (Abd-Alla et al., 2019).

Phenotypic and genotypic identification of RM122

Morphological characters (color, shape, consistency, margin, and elevation) of colonies were observed on thioglycolate agar plates incubated at 37°C for 48h. According to Beveridge (2001), Gram staining of the bacterial culture was used to examine cell shape and other microscopic characteristics. Different biochemical tests (catalase, indole, glucose fermentation, methyl red, citrate utilization, nitrate reduction, H_2S production, oxidase, urease, and Vogas-Proskauer as described in Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005) were used for phenotypic characterization of the bacterial isolate RM122. The results were recorded for each test as positive or negative.

Bacterial DNA was extracted by QIAamp DNA Mini Kit (Qiagen). Polymerase chain reaction (PCR) of the extracted DNA was performed using the bacterial universal primers (forward and reverse), 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR was performed in 50µL (25-µL GoTaq DNA polymerase master mix (Promega, USA), 2-µL primer F, 2-µL primer R, template DNA, and nuclease-free water). PCR amplification reaction was achieved at 95°C for 5 minutes, then 40 cycles at 95°C for 1min, 50°C for 1min, and 72°C for 1.5min followed by 72°C for 10min in Veriti Thermal cycler (Applied Biosystems). Purified PCR product (1000bp) was analyzed by DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems). The resulting nucleotide sequence was compared to the recorded sequences at NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) and deposited in GenBank database under the accession number OP345936. The phylogenetic tree was constructed using MEGA 7 software (Kumar et al., 2016).

Improvement of hydrogen production by E. marmotae RM122

Distinct parameters were studied to enhance cumulative hydrogen production from sugarcane molasses. Effect of different molasses sugar concentrations (1-8%, 1% interval), initial fermentation pH (3-9), fermentation temperature (20-45°C, 5°C interval), and initial inoculum size (10-35%, 5% interval) were studied on the produced cumulative hydrogen.

Kinetics study

Modified Gompertz model (MGM) (Eq. 1) was applied to the obtained H_2 yields to determine hydrogen production potential (H), hydrogen production rate (r), and lag phase (λ). Hydrogen production rate can be calculated from Equation no. 2. Equation no. 3. is used to calculate t_{95}.

$$H(t) = H_{max}. exp\left\{-exp\left[\frac{R_{max}.e}{H_{max}}(\lambda - t) + 1\right]\right\} (1)$$

$$r_{H_2} = R_{max}. exp\left\{-exp\left[\frac{R_{max}.e}{H_{max}}(\lambda - t) + 1\right] + \left[\frac{R_{max}.e}{H_{max}}(\lambda - t) + 1\right] + 1\right\} (2)$$

$$t_{95} = \frac{H_{max}}{R_{max}.e}[1 - \ln(-\ln 0.95)] + \lambda \quad (3)$$

where, H(t) is the produced cumulative hydrogen (mL/L) at time t (h), r_{H2} is the rate of hydrogen production (mL/L/h) at time t (h), H_{max} is the maximum hydrogen production (mL/L), R_{max} is the maximum hydrogen production rate (mL/L/h), λ represents lag phase time (h) and e is exp. (1) = 2.71828. t_{95} is the time required to achieve 95% of the maximum hydrogen yield (h).

Statistical analysis

The statistical analysis of data was performed using SPSS (v.25; IBM Corp, Armonk, NY, USA) software. All experiments were done in three replicates. Means and standard errors were calculated by Duncan's multiple range tests. Determination Coefficient factor (R^2) was used to demonstrate the fitness degree of the obtained data with the studied kinetic model. Statistical significance was determined at 5% level (Aung & Eun, 2022).

Results and Discussion

Identification of the bacterial isolate RM122

The bacterial isolate RM122 was isolated on thioglycolate agar medium from a cow rumen

sample and was selected as a highly H$_2$ producer. This isolate produced 451.67 ±12.14mL/L and 387.67 ±19.23mL/L on 6% glucose T6 medium and pretreated sugarcane molasses (6% sugar concentration), respectively. RM122 appeared as light pink colonies on thioglycolate agar plates after 48h of anaerobic incubation. The colonies were slimy surface, circular shape, slightly convex elevation, entire margin, and semi-transparent. The isolate was Gram negative with non-spore forming short rod cells (Fig. 1). RM122 was negative for citrate utilization, lactose fermentation, H$_2$S production, indole production, oxidase production, urease production, and Vogas-Proskauer tests. Whereas, the isolate was positive for catalase production, glycerol fermentation, methyl red, and nitrate reduction tests (Table 1). Based on the obtained morphological and physiological characteristics, the isolate RM122 belongs to *Escherichia* sp. (Julian et al., 2015). The bacterial isolate RM122 was identified as *Escherichia marmotae* based on 16S rRNA gene sequence analysis. *E. marmotae* was first reported by Liu et al. (2015) where isolated from feces of *Marmota himalayana*. A phylogenetic tree of *E. marmotae* RM122 was constructed by MEGA 7 software using the Minimum Evolution method (Rzhetsky & Nei, 1992). The evolutionary relationship were presented in a phylogenetic tree (Fig. 2) between *E. marmotae* RM122 (OP345936) and the other related species from NCBI GenBank database. The sum of branch length of tree was 0.0377 and the scale of branch lengths was in the same units as those of the evolutionary distances used to assume the phylogenetic tree. Initial tree was generated using Neighbor-joining algorithm (Saitou & Nei, 1987). Maximum Composite Likelihood method was used to compute the evolutionary distances (Tamura et al., 2004).

Improvement of hydrogen production by E.

Fig. 1. Cells shape of *E. marmotae* RM122

TABLE 1. Phenotypic characteristics of bacterial isolate RM122

Characters	Results
Colony morphology	Slimy surface, circular, slightly convex, entire margin, semi-transparent, light pink
Cell shape	Non-spore forming short rod cells
Gram Staining	Negative
Catalase	Positive
Citrate utilization	Negative
Lactose fermentation	Negative
Glycerol fermentation	Positive
H$_2$S	Negative
Indole	Negative
Methyl Red	Positive
Nitrate Reduction	Positive
Oxidase	Negative
Urease	Negative
Voges-Proskauer	Negative

marmotae RM122

Sugarcane molasses is considered as one of the most critical renewable agro-industarial wastes for biohydrogen production which may result in low-cost viability. Different studies demonstrated the use of sugar molasses as a substrate for high cumulative hydrogen production levels compared to other different substrates (Mohammadi et al., 2019). Sugarcane molasses is characterized by containing high sugars (mainly sucrose, glucose, and fructose) concentration of 40-60% (w/w), vitamins (biotin, choline, pantothenic acid, riboflavin, and thiamine), amino acids, and inorganic salts (Ca^{+2}, Na$^+$, SO$_4^{-2}$) which works as essential supplements for microbial fermentation media (Lee et al., 2021; Zhang et al., 2021; Gao et al., 2022). Culture conditions such as sugar concentration, initial fermentation pH, fermentation temperature, and inoculum size had significant effects on hydrogen production. The calculated kinetic parameters (H$_{max}$, R$_{max}$, and λ) of modified Gompertz model and t$_{95}$ were studied to maximize the produced hydrogen yield by *E. marmotae* RM122 using sugarcane molasses.

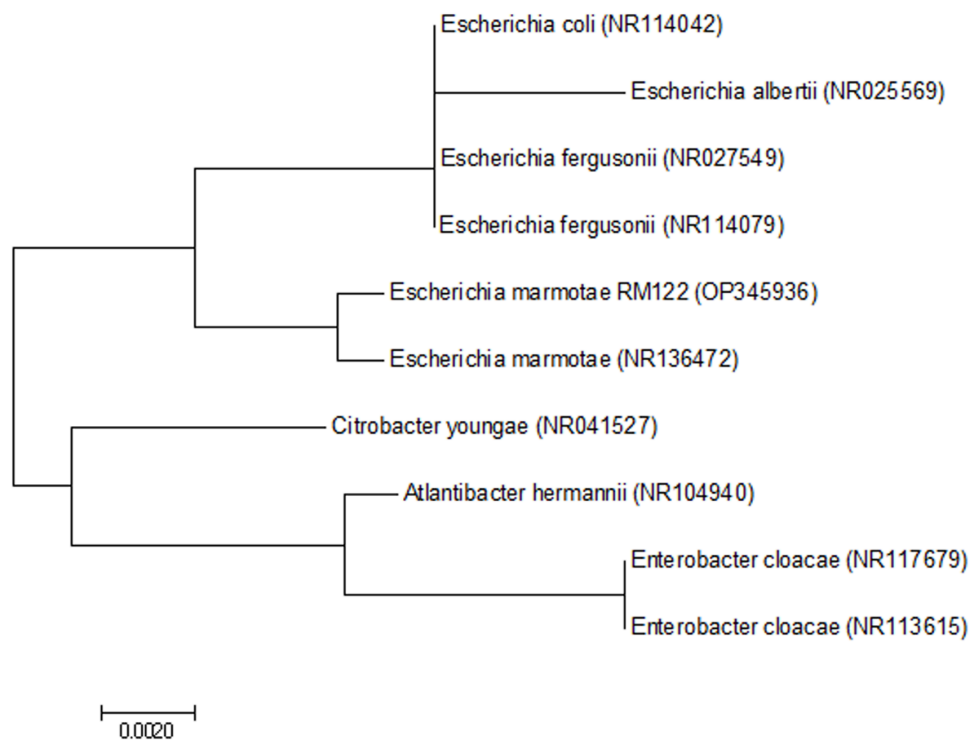*Effect of subatrate concentration*

Fig. 2. Evolutionary relationship of taxa deposited in GenBank with *E. marmotae* RM122

Biohydrogen production using different molasses sugar concentrations (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0%) of the pre-treated sugarcane molasses and the assessed kinetic parameters were investigated (Table 2). Highest levels of cumlative hydrogen (H_{max} 560.00 ±25.98mL/L and R_{max} (Exp) 31.43 ±2.14 mL/L/h) were produced at 4% molasses sugar concentration. These results were in agreement with Wang & Jin (2009) who stated that 4% molasses sugar concentration was the optimal for hydrogen production by *Clostridium butyricum* W5 from molasses. kinetic studies by MGM revealed that λ and R_{max} (MGM) were 15.19h and 30.73mL/L/h respectively at 4% for 52.43h fermentation time to achieve 95% production. Decreasing in the obtained hydrogen yield by increasing molasses concentration may result from the decrease of free electrons collected from NADH by high lactic acid concentrations (Lee et al., 2021).

Effect of initial fermentation pH

Cumulative H_2 production and H_2 production rate was studied at different initial fermentation pH from 3 to 9. Figure 3 shows the effect of initial fermentation pH on the produced H_2 yields and the calculated kinetic parameters. At pH 8, maximum

hydrogen yield was 646.67±23.33mL/L when R_{max}(Exp) was 35.28±2.65mL/L/h. Resulted data demnostrated a significant decrease in cumulative hydrogen production when the fermentation pH was below 7.0. Decreasing of produced hydrogen yield from pH 7.0 to 3.0 could be attributed to the effect of the lower pH values on biomass growth and the activity of hydrogenase enzymes which are involved in anaerobic hydrogen production pathways of the hydrogen producing bacteria (Hawary et al., 2019; Sivagurunathan & Lin, 2020). Also, these data were in agreement with Stavropoulos et al. (2016) who stated that efficient pH ranges for biohydrogen production were from 4.5 to 9.0. Kinetics analysis of the obtained data at the optimum pH revealed that R_{max} (MGM) was 31.90mL/L/h when λ was 13.92h and t_{95} was 53.90h. R^2 was 0.9999 indicating the produced hydrogen yield at pH 8 was well fitted to MGM. Valdez-Vazquez et al. (2006) stated that the time of lag phase for hydrogen production depends on the value of the initial fermentation pH value.

Effect of fermentation temperature

Effect of various fermentation temperatures (20, 25, 30, 35, 40, and 45°C) on hydrogen production was studied and the resulted data was shown in Fig. 4. Maximum hydrogen level

(H_{max} 828.33 ±21.67mL/L) was obtained at 35°C. The produced hydrogen yield was reduced when fermentation temperatures were below or above 35°C. The resulted data were harmony with Usman et al. (2019) who revealed that mesophilic temperature range from 30°C to 49°C was the optimal for hydrogen production. Similar findings were achieved by Sivaramakrishnan (2021) who revealed that 35°C and 37°C were the optimal fermentation temperatures for hydrogen production by *Clostridium* sp. and *Enterobacter* sp., respectively. kinetic studies of the obtained data revealed that the lowest λ (4.12h) was achieved at the optimal temperature (35 °C) with R_{max} (MGM) 27.98mL/L/h, t_{95} 53.62h and R^2 of 0.9594. In contrast, lag phase time was increased by increasing or decreasing of fermentation temperature than the optimum levels resulting in decreasing of produced hydrogen yield, this is due to the denaturation of hydrogen production enzymes (Hernandez-Mendoza et al., 2014).

Effect of inoculum size

Hydrogen production was significantly affected by different concentrations of bacterial inoculum size. Table 3 represents the obtained H_2 yield and the resulted kinetic parameters by inoculating the fermentation medium with different inoculum concentrations (10, 15, 20, 25, 30, and 35%, v/v) of *E. marmotae* RM122. Maximum cumulative hydrogen (1670.00 ±40.41mL/L) with

R_{max} (Exp) (60.00 ±2.04mL/L/h) was achieved by inoculating the fermentation medium with 10% (v/v). Kinetic studies of hydrogen production indicated that R_{max} (MGM) (59.98mL/L/h) was achieved at the optimum inoculum size when λ and t_{95} were 13.50h and 64.56h, respectively, with R^2 of 0.9720. These results were in agreement with Wang & Jin (2009) who stated that initial cell concentration of the inoculum directly affects the growth lag phase and hydrogen productivity. Biohydrogen production was negatively affected by increasing the fermentation inoculum concentration than the optimum inoculum size. Zhao et al. (2011) mentioned that the overgrowth of bacterial cells and overconsumption of used substrate was increased by increasing the inoculum size more than the optimum levels resulting in a rapid decrease in hydrogen production.

Data in Table 4 represent the effect of the studied hydrogen production optimization experiments on the produced hydrogen yield. Improvement of hydrogen production by *E. marmotae* RM122 from sugarcane molasses reached to 1282.33mL/L (330.78%) compared to the initially produced hydrogen yield (387.67 ±17.33mL/L) at the optimal levels of molasses sugar concentration, fermentation pH, temperature, and inoculum size. Alavi-Borazjani (2021) stated that optimization of nutritional and environmental factors is highly important for improvement of hydrogen production by bacteria.

TABLE 2. Kinetic parameters of hydrogen production at different levels of molasses sugar concentration using the modified Gompertz model (MGM)

| Sugar concentration (%)	Experimental		MGM		t_{95} (h)	R^2
	H_{max} (mL/L)	R_{max} (mL/L/h)	λ (h)	R_{max} (mL/L/h)		
1	513.33 ±20.28	24.29 ±3.57	5.34	24.18	47.89	0.9118
2	520.00 ±23.09	35.71 ±3.71	15.55	35.66	56.19	0.9954
3	516.67 ±18.56	32.86 ±3.09	15.48	32.46	51.91	0.9145
4	560.00 ±25.98	31.43 ±2.14	15.19	30.73	52.43	0.9872
5	460.00 ±23.09	31.43 ±1.81	24.84	31.41	66.62	0.9376
6	398.33 ±21.67	31.43 ±1.39	20.37	31.08	59.03	0.9832
7	375.00 ±18.93	31.43 ±1.81	21.99	30.48	56.98	0.9355
8	385.00 ±25.74	31.43 ±2.50	22.51	30.79	60.57	0.9723
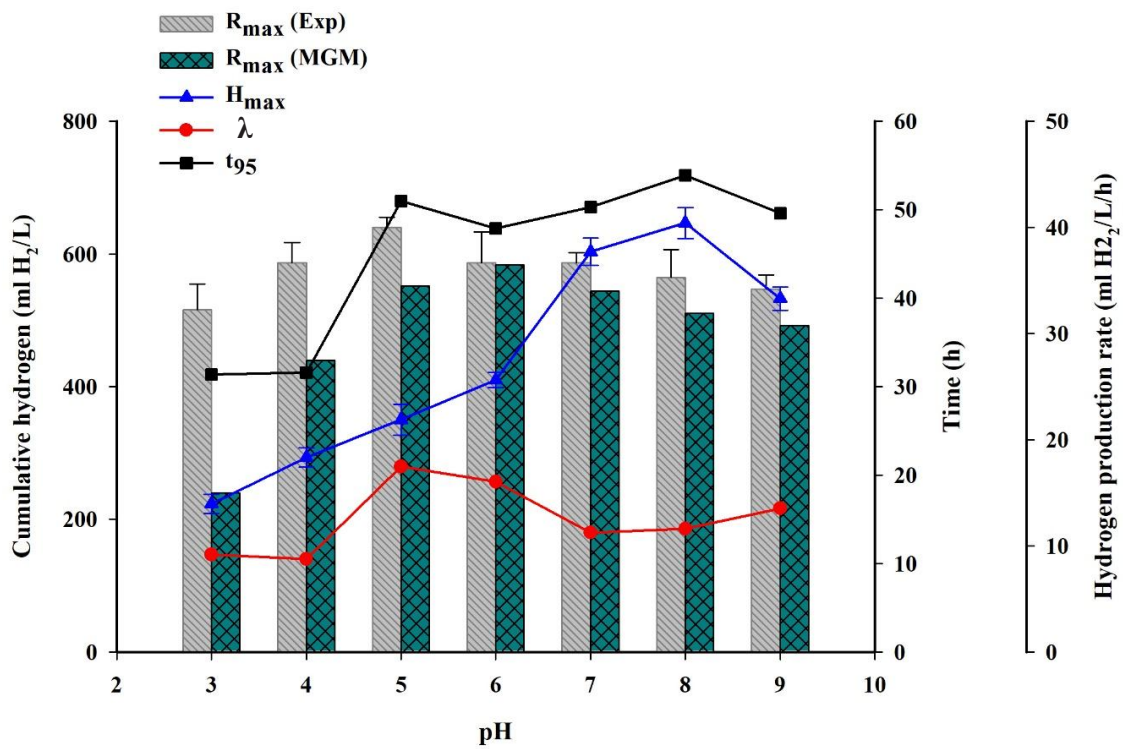
Fig. 3. Effects of different pH levels on H$_{max}$, R$_{max}$ (Exp), R$_{max}$ (MGM), λ, and t$_{95}$
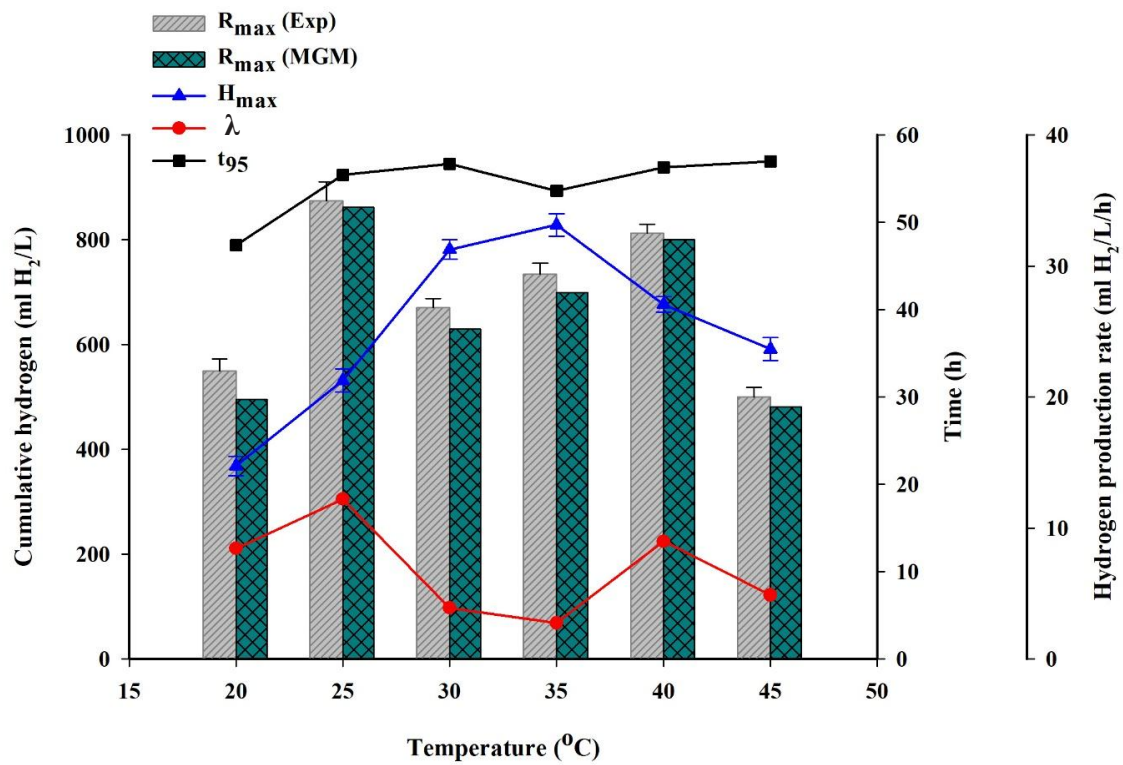

Fig. 4. Effects of different fermentation temperatures on H$_{max}$, R$_{max}$ (Exp), R$_{max}$ (MGM), λ, and t$_{95}$

TABLE 3. Kinetic parameters of hydrogen production using the modified Gompertz model (MGM) at different concentrations of initial inoculum size

Inoculum size (%)	Experimental		MGM		t_{95} (h)	R^2
	H_{max} (mL/L)	R_{max} (mL/L/h)	λ (h)	R_{max} (mL/L/h)		
10	1670.00 ±40.41	60.00 ±2.04	13.50	59.98	64.65	0.9720
15	950.00 ±23.09	27.14 ±0.82	2.44	27.14	53.87	0.9815
20	910.00 ±28.87	34.00 ±0.72	12.28	33.90	53.10	0.9424
25	860.00 ±11.55	36.92 ±0.89	4.50	36.42	44.79	0.9428
30	800.00 ±23.09	36.15 ±1.33	8.28	35.05	50.99	0.9766
35	771.67 ±21.67	20.00 ±0.89	5.06	19.97	67.73	0.9999

TABLE 4. Hydrogen production improvement at optimum levels of molasses sugar concentration, fermentation pH, fermentation temperature, and inoculum size from the initially produced hydrogen yield (387.67 ±17.33mL/L)

Variable	Optimum	Optimized H_{max} (mL/L)	Hydrogen improvement	
			(mL/L)	(%)
Molasses sugar conc. (%)	4	560.00 ±25.98	172.33	44.45
pH	8	646.67 ±23.33	248.34	66.81
Temperature (°C)	35	828.33 ±21.67	440.66	113.67
Inoculum size (%)	10	1670.00 ±40.41	1282.33	330.78

Conclusion

Biohydrogen is an eco-friendly renewable energy carrier that will help in the reduction of the global demand for petroleum-derived fuels, greenhouse gas emissions, and climate changes. Hydrogen production by anaerobic digestion of agro-industrial wastes using fermentative bacteria is considered the favorable cheapest solution. Kinetic study of biohydrogen production improvement through dark fermentation of sugarcane molasses using *Escherichia marmotae* RM122 was investigated here. The maximum hydrogen production (1670.00 ±40.41 mL/L) was achieved by optimization of the fermentation conditions. A future research can be conducted on hydrogen production from molasses on semi-industrial scale.

Conflict of interest: The authors declare that they have no conflict of interest.

Authors' contributions: Research idea: A.A.A., A.M.R. and M.A.T., experimental data: M.A.T. and A.M.R., data analysis and discussion: M.A.T. and A.M.R., writing and preparation of paper draft: M.A.T. and A.M.R., revision and editing: A.A.A., A.M.R., M.A.T. and S.H. All authors have revised the proof and agreed to publish this version of the manuscript.

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References

Abd-Alla, M.H., Gabra, F.A., Danial, A.W., Abdel-Wahab, A.M. (2019) Enhancement of biohydrogen production from sustainable orange peel wastes using *Enterobacter* species isolated from domestic wastewater. *International Journal of Energy Research*, **43**(1), 391-404.

Alavi-Borazjani, S. A., da Cruz Tarelho, L. A., Capela, M.I. (2021) Parametric optimization of the dark fermentation process for enhanced biohydrogen production from the organic fraction of municipal solid waste using Taguchi method. *International Journal of Hydrogen Energy*, **46**(41), 21372-21382.

Aung, T., Eun, J.B. (2022) Impact of time and temperature on the physicochemical, microbiological, and nutraceutical properties of laver kombucha (*Porphyra dentata*) during fermentation. *Lebensmittel-Wissenschaft + Technologie (LWT),* **154**, 112643.

Barca, C., Soric, A., Ranava, D., Giudici-Orticoni, M.T.,

Ferrasse, J.H. (2015) Anaerobic biofilm reactors for dark fermentative hydrogen production from wastewater: a review. *Bioresource Technology*, **185**, 386-398.

Basak, B., Fatima, A., Jeon, B.H., Ganguly, A., Chatterjee, P.K., Dey, A. (2018) Process kinetic studies of biohydrogen production by co-fermentation of fruit-vegetable wastes and cottage cheese whey. *Energy for Sustainable Development*, **47**, 39-52.

Benemann, J. (1996) Hydrogen biotechnology: progress and prospects. *Nature Biotechnology*, **14**(9), 1101-1103.

Beveridge, T.J. (2001) Use of the Gram stain in microbiology. *Biotechnic & Histochemistry*, **76**(3), 111-118.

Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M. (2005) "*Bergey's Manual of Systematic Bacteriology*". New York: Springer.

Cheng, J., Ding, L., Xia, A., Lin, R., Li, Y., Zhou, J., Cen, K. (2015) Hydrogen production using amino acids obtained by protein degradation in waste biomass by combined dark-and photo-fermentation. *Bioresource Technology*, **179**, 13-19.

Clion, V., Dumas, C., Collin, S., Ernst, B. (2015) Key factors for biohydrogen production by dark fermentation. *The Canadian Journal of Chemical Engineering*, **93**(2), 309-316.

Deabes, M.M, Allayeh, A.K., Seif, M.M., Rasmey, A.H.M., Naguib, K.M. (2020) Antiviral, Antifungal, and Antibacterial Potential Activities of *Ephedra Sinica* in Vitro. *Jordan Journal of Biological Sciences*, **13**(3), 313-320.

Elsharnouby, O., Hafez, H., Nakhla, G., El Naggar, M.H. (2013) A critical literature review on biohydrogen production by pure cultures. *International Journal of Hydrogen Energy*, **38**(12), 4945-4966.

Gao, R., Xiong, L., Wang, M., Peng, F., Zhang, H., Chen, X. (2022) Production of acetone-butanol-ethanol and lipids from sugarcane molasses via coupled fermentation by *Clostridium acetobutylicum* and oleaginous yeasts. *Industrial Crops and Products*, **185**, 115131.

Guellout, Z., Clion, V., Benguerba, Y., Dumas, C., Ernst, B. (2018) Study of the dark fermentative hydrogen production using modified ADM1 models. *Biochemical Engineering Journal*, **132**, 9-19.

Han, W., Yan, Y., Shi, Y., Gu, J., Tang, J., Zhao, H. (2016) Biohydrogen production from enzymatic hydrolysis of food waste in batch and continuous systems. *Scientific Reports*, **6**(1), 1-9.

Hawary, H., Rasmey, A.H.M., Aboseidah, A.A., El-Morsi, E.S., Hafez, M. (2019) Enhancement of glycerol production by UV-mutagenesis of the marine yeast *Wickerhamomyces anomalus* HH16: kinetics and optimization of the fermentation process. *3 Biotech*, **9**(12), 1-14.

Hernandez-Mendoza, C.E., Moreno-Andrade, I., Buitron, G. (2014) Comparison of hydrogen-producing bacterial communities adapted in continuous and discontinuous reactors. *International Journal of Hydrogen Energy,* **39**(26), 14234-14239.

Julian, T.R., Islam, M.A., Pickering, A.J., Roy, S., Fuhrmeister, E.R., Ercumen, A., et al. (2015) Genotypic and phenotypic characterization of *Escherichia coli* isolates from feces, hands, and soils in rural Bangladesh via the Colilert Quanti-Tray System. *Applied and Environmental Microbiology*, **81**(5), 1735-1743.

Kumar, G., Shobana, S., Nagarajan, D., Lee, D. J., Lee, K. S., Lin, C. Y., et al. (2018) Biomass based hydrogen production by dark fermentation—recent trends and opportunities for greener processes. *Current Opinion in Biotechnology*, **50**, 136-145.

Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, **33**(7), 1870-1874.

Lee, K.S., Chen, S.L., Lin, C.Y., Chang, J.S. (2021) Converting waste molasses liquor into biohydrogen via dark fermentation using a continuous bioreactor. *International Journal of Hydrogen Energy*, **46**(31), 16546-16554.

Liu, S., Jin, D., Lan, R., Wang, Y., Meng, Q., Dai, H., et al. (2015) *Escherichia marmotae* sp. nov., isolated from faeces of Marmota himalayana. *International Journal of Systematic and Evolutionary Microbiology*, **65**(Pt_7), 2130-2134.

Mishra, P., Das, D. (2014) Biohydrogen production from

Enterobacter cloacae IIT-BT 08 using distillery effluent. *International Journal of Hydrogen Energy,* **39**(14), 7496-7507.

Mohammadi, M., Mohammadi, P., Karami, N., Barzegar, A., Annuar, M.S.M. (2019) Efficient hydrogen gas production from molasses in hybrid anaerobic-activated sludge-rotating biological contactor. *International Journal of Hydrogen Energy*, **44**(5), 2592-2602.

Navlani-García, M., Mori, K., Kuwahara, Y., Yamashita, H. (2018) Recent strategies targeting efficient hydrogen production from chemical hydrogen storage materials over carbon-supported catalysts. *NPG Asia Materials*, **10**(4), 277-292.

Neuwirth, M., Fleiter, T., Manz, P., Hofmann, R. (2022) The future potential hydrogen demand in energy-intensive industries-a site-specific approach applied to Germany. *Energy Conversion and Management*, **252**, 115052.

Pasupuleti, S.B., Venkata Mohan, S. (2015) Acidogenic hydrogen production from wastewater: Process analysis with the function of influencing parameters. *International Journal of Energy Research,* **39**(8), 1131–1141.

Rao, R., Basak, N. (2021) Optimization and modelling of dark fermentative hydrogen production from cheese whey by *Enterobacter aerogenes* 2822. *International Journal of Hydrogen Energy*, **46**(2), 1777-1800.

Rasmey, A.H.M., Hassan, H.H., Abdulwahid, O.A., Aboseidah, A.A. (2018) Enhancing bioethanol production from sugarcane molasses by *Saccharomyces cerevisiae* Y17. *Egyptian Journal of Botany*, **58**(3), 547-561.

Rasmey, A.H., Tawfik, M.A., Abdel-Kareem, M.M. (2020) Direct transesterification of fatty acids produced by *Fusarium solani* for biodiesel production: effect of carbon and nitrogen on lipid accumulation in the fungal biomass. *Journal of Applied Microbiology*, **128**(4), 1074-1085.

Rzhetsky, A., Nei, M. (1992) A simple method for estimating and testing minimum-evolution trees. *Molecular Biology and Evolution*, **9**(5), 945-967.

Saitou, N., Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**(4), 406-425.

Shown, I., Hsu, H.C., Chang, Y.C., Lin, C.H., Roy, P.K., Ganguly, A., et al. (2014) Highly efficient visible light photocatalytic reduction of CO_2 to hydrocarbon fuels by Cu-nanoparticle decorated graphene oxide. *Nano Letters*, **14**(11), 6097-6103.

Sivagurunathan, P., Lin, C.Y. (2020) Biohydrogen production from beverage wastewater using selectively enriched mixed culture. *Waste and Biomass Valorization,* **11**(3), 1049-1058.

Sivaramakrishnan, R., Shanmugam, S., Sekar, M., Mathimani, T., Incharoensakdi, A., Kim, S. H., et al. (2021) Insights on biological hydrogen production routes and potential microorganisms for high hydrogen yield. *Fuel*, **291**, 120136.

Stavropoulos, K.P., Kopsahelis, A., Zafiri, C., Kornaros, M. (2016) Effect of pH on continuous biohydrogen production from end-of-life dairy products (EoL-DPs) via dark fermentation. *Waste and Biomass Valorization,* **7**(4), 753-764.

Sun, Y., He, J., Yang, G., Sun, G., Sage, V. (2019) A review of the enhancement of bio-hydrogen generation by chemicals addition. *Catalysts*, **9**(4), 353.

Tamura, K., Nei, M., Kumar, S. (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, **101**(30), 11030-11035.

Tsioptsias, C., Lionta, G., Deligiannis, A., Samaras, P. (2016) Enhancement of the performance of a combined microalgae-activated sludge system for the treatment of high strength molasses wastewater. *Journal of Environmental Management*, **183**, 126-132.

Usman, T.M., Banu, J.R., Gunasekaran, M., Kumar, G. (2019) Biohydrogen production from industrial wastewater: an overview. *Bioresource Technology Reports,* **7**, 100287.

Valdez-Vazquez, I., Ríos-Leal, E., Carmona-Martínez, A., Muñoz-Páez, K.M., Poggi-Varaldo, H.M. (2006) Improvement of biohydrogen production from solid wastes by intermittent venting and gas flushing of batch reactors headspace. *Environmental Science*

& *Technology*, **40**(10), 3409-3415.

Wang, J., Wan, W. (2009) Kinetic models for fermentative hydrogen production: a review. *International Journal of Hydrogen Energy*, **34**(8), 3313-3323.

Wang, X., Jin, B. (2009) Process optimization of biological hydrogen production from molasses by a newly isolated *Clostridium butyricum* W5. *Journal of Bioscience and Bioengineering,* **107**(2), 138-144.

Zhang, S., Wang, J., Jiang, H. (2021) Microbial production of value-added bioproducts and enzymes from molasses, a by-product of sugar industry. *Food Chemistry*, **346**, 128860.

Zhao, X., Xing, D., Fu, N., Liu, B., Ren, N. (2011) Hydrogen production by the newly isolated *Clostridium beijerinckii* RZF-1108. *Bioresource Technology*, **102**(18), 8432-8436.

تحسين إنتاج الهيدروجين الحيوي بواسطة دراسة حركية لتخمر مولاس قصب السكر بواسطة *Escherichia marmotae*

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تعتبر ظاهرة التغيرات المناخية والاحتباس الحرارى الناتجة من الاستخدام المتزايد للوقود البترولى من اهم المشكلات التى نعانى منها فى وقتنا الحالى. ولذلك يعتبر الوقود الحيوى من اهم مصادر الطاقة البديلة التى تساهم فى حل هذة المشكلات والتى سوف تقلل الطلب المتزايد للوقود البترولى. ويعتبر الهيدروجين الحيوى هو أحد هذة المصادر الصديقة للبيئة التى يمكن استخدامها فى المستقبل على نطاق واسع. تهدف هذه الدراسة إلى تقييم مقدرة البكتيريا اللاهوائية الاختيارية المعزولة من معدة الابقار على انتاج الهيدروجين الحيوى من مولاس قصب السكر. تمت دراسة انتاج الهيدروجين بواسطة العزله البكتيرية RM122، وكانت كمية الهيدروجين الناتجة من استخدام وسط غذئى يحتوى على 6% جلوكوز هى 451.67 ± 12.12 مل / لتر واستخدام المولاس المعامل بتركيز سكر6% هى 387.67 ± 19.23 مل / لتر. تم التعرف على هذه العزلة من خلال الصفات المظهرية والوراثية على انها *Escherichia marmotae*. ومن خلال تطبيق تصميم تجارب تحسين انتاج الهيدروجين والدراسة الحركية لهذه التجارب، تم زيادة الهيدروجين الناتج الى 1670.00 ± 40.41 مل / لتر عند تخمر مولاس قصب السكر بتركيز 4% سكر عند درجة الاس الهيدروجينى 8 وتلقيح وسط التخمر بنسبة 10% (حجم/حجم) والتحضين عند درجة حرارة 35 ° مئوية. تشير هذه النتائج إلى امكانية استخدام *Escherichia marmotae* RM122 كمنتج جيد للهيدروجين من المخلفات الصناعية الزراعية الرخيصة.