Isolation and Identification of Ruminant Bacteria for Biohydrogen Production from Sugarcane Molasses

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ABSTRACT

Biohydrogen production by fermentative bacteria is one of the most prospective alternative ways for fossil fuels. Therefore, this study aimed to isolate new facultative anaerobic bacteria from cow rumen as highly hydrogen producers from sugarcane molasses. Among the isolated ruminant anaerobic bacteria, the isolate RM92 was the highest hydrogen producer with maximum cumulative hydrogen (H_{max}) of 426.67±14.56 mL/L. This bacterial isolate (RM92) was identified phenotypically and genotypically as *Escherichia fergusonii*. The 16S rRNA gene sequence was deposited in NCBI GenBank database under the accession number OP185369. Optimization experiments design for maximization of hydrogen production by *E. fergusonii* RM92 increased the H_{max} to 1280.00 ±34.64 ml/L with maximum hydrogen production rate (R_{max}) of 36.92±0.89 ml/L/h on 4% molasses sugar concentration, pH 8, incubation temperature 40 °C and initial inoculum size 30% (v/v). These findings suggest that *E. fergusonii* RM92 could be used as a potential hydrogen producer from cheap agro-industrial wastes.

1. Introduction

The global demand for energy increases daily by increasing the industrialization and motorization of the world, this will lead to the depletion of fossil fuels. Moreover, the total depending on the petroleum-based fuels to fulfil the energy demand will increase the greenhouse gas (GHG) emissions and climate changes [1]. Therefore, the search for discovering new eco-friendly renewable energy sources is required. The renewable energy should be satisfied to overcome the current problems resulted from fossil fuels using like the price rising, air pollution and global warming. Hydrogen is one of the clean renewable energy and recognized as a common potential fuel for many petro-chemical industries during the last 20 years [2]. Hydrogen demand is increasing as a potential renewable energy source for its clean combustion and its high specific energy (~123 MJ/kg) comparing to fossil fuels (~46 MJ/kg) [3].

Hydrogen can be produced by various physico-chemical routes, however the biological route using the anaerobic fermentative bacteria is considered as the cheapest sustainable way. Bio-hydrogen could soon be entering the fuel market [4,5]. Different mechanisms for biohydrogen production are available depending on the used substrate and microorganism such as biophotolysis, indirect photolysis, dark fermentation, photofermentation, and microbial electrolysis.

Dark fermentation (DF) was recommended as the highly effective way for biohydrogen production. Bacteria such as *Clostridium* spp. [6,7], *Enterobacter* spp. [8], *Escherichia* spp, and *Bacillus* spp. [9,10] are commonly used to obtain high hydrogen yield.

The efficiency of the microbial fermentation process for biohydrogen production is depending on the used substrate and its properties. Nowadays, various challenges appeared which slow down scaling up of H_2 production via microorganisms when applying different substrates: particularly, the pretreatment of organic wastes and its nutritional composition [11]. Therefore, the search for an agro-industrial waste that possess high carbon and nutrients content applicable for hydrogen production by anaerobic fermentative bacteria has been ongoing for the last years. The use of molasses (waste of sugar industry) in hydrogen production was one of suggested substrates due to its variable physicochemical characteristics, environmental impacts, and the accessible wide range of hydrogen-producing microorganisms [12, 13].

Molasses is produced as a by-product of the sugar cane and sugar beet refining industry in large amounts sufficient for feeding other industries. The molasses contains high amounts of organic nitrogen sources and high amounts of essential vitamins and salts which are biochemically accessible to the fermentation bacteria [14]. So, the use of molasses as a substrate in continuous fermentation processes leads to much higher hydrogen yield and production rate compared to synthetic glucose [15,16]. Also, using molasses as a substrate can be considered as an available and inexpensive material for the process [17,18,19].

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The current research aimed to isolate ruminant bacteria capable of producing hydrogen under dark fermentation from sugarcane molasses as a substrate. Optimization of hydrogen production by *E. fergusonii* RM92 from sugaracane molasses was conducted by studying the effect of substrate concentration, incubation temperature, initaial pH value and inoculum size.

2. Materials and Methods

2.1. Isolation of ruminnat bacteria

The used bacteria were isolated from two rumen samples of cows collected from Cattle Butchery at Suez, Egypt, using anaerobic enrichment method. One ml rumen solution was inoculated in 50 ml sterilized fluid thioglycolate broth medium (tryptone 15.0, L-cystine 0.5, glucose 5.5, yeast extract 0.5, NaCl 2.5, sodium thioglycolate 0.5 and resazurin 0.001 g/l) covered with paraffin oil. The bottles were incubated for 48 hrs at 37 °C under anaerobic conditions. The enrichment cultures were straked on fluid thioglycolate agar plates and incubated under the previous conditions for 48 hrs, the grown separated colonies were picked up. The purified cultures were conserved under anaerobic condition at 4°C [20].

2.2. Preparation of bacterial inoculum

Bacterial inoculum (3×10^6 cells/ml) was prepared by inoculating one ml of 48 hrs old culture into 100 ml sterilized fluid thioglycolate broth and was incubated at 37°C under static conditions for 48 hrs under anaerobic conditions.

2.3. Substrate pretreatment

Sugarcane molasses was used as a substrate for production of hydrogen by the isolated bacteria. Molasses was pretreated before using according to Rasmey et al. [21]. Molasses was diluted by distilled water to the desired sugar concentration and the pH was adjusted to 6 by concentrated sulfuric acid. The diluted molasses was heated for 15 minutes at 95 °C in water bath, then was left for 2 hrs to cool and precipitate the impurities. The cleared molasses was transferred to the sterilized fermentation bottles.

2.4. Screening of hydrogen production from sugarcane molasses

Five ml bacterial inoculum (at the ratio of 1 %, v/v) were inoculated to 495 mL molasses fermentation medium in 600 ml glass bottles. Bottles were sealed with rubber plugs and incubated at 37°C for 72 hrs. The produced gas was passed on bottles containing 2M NaOH solution to absorb carbon dioxide and the produced hydrogen gas was collected in a cylinder by water displacement method as shown in Fig.1 [22]. The produced hydrogen was confirmed by gas chromatograph (Thermo Scientific TRACE GC Ultra) according to Abd-Alla et al. [23].
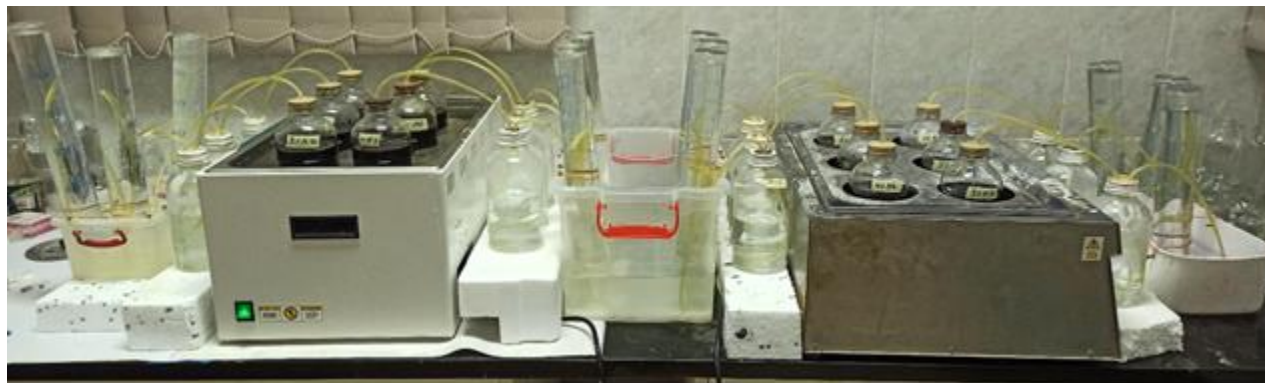

Fig. 1: Designed hydrogen production system using water displacement method.

2.5. Identification of the bacterial isolate RM92

2.5.1. Morphological characterization

Morphological characters (color, size and edge) of colonies were observed on thioglycolate agar plates incubated at 37°C for 48 hrs. Cells shape and microscopic characters were observed by Gram staining of the bacterial culture according to Beveridge [24].

2.5.2. Biochemical characterization

The bacterial isolate RM92 was subjected to different biochemical tests (catalase, glucose fermentation, indole, methyl red, nitrate reduction, oxidase, citrate utilization, H_2S production, urease and Vogas-Proskauer) as described in Bergey's Manual of Systematic Bacteriology [25]. The results were recorded as positive or negative for each test.

2.5.3. Genotypic characterization

The genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen) according to Knüpfer et al. [26]. The extracted DNA was subjected to polymerase chain reaction using the universal primers, 27F (5′ AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR reaction was performed in a total volume of 50 µL (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 done at 95°C for 5 minutes, then 40 cycles at 95°C for 1 minute, 50°C for 1 minute, and

72°C for 1.5 minutes followed by 72°C 10 minutes in Veriti Thermal cycler (Applied Biosystems). The PCR bands were visualized on 1% agarose gel electrophoresis stained with ethidium bromide using UV light. Purified PCR product (approximately 1100 bp) was loaded and analyzed by DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems). The resulted nucleotide sequence was compared to bacterial sequences at NCBI database (http:// www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was constructed using MEGA 7 software.

2.6. Optimization of hydrogen production

Distinct parameters were studied to optimize hydrogen production from sugarcane molasses. Effect of different substrate concentrations from 1 to 8 % molasses sugar concentration with 1 % interval, initial fermentation pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0), incubation temperature (20 - 45 °C) with 5 °C interval and inoculum size from 10 to 35 % with 5 % interval were tested on hydrogen production by selected bacterial isolate.

2.7. Statistical analysis

The data were statistically analysed using the SPSS 25.0 software program. All experiments were achieved with three independent replicates. Means and standard errors were calculated for three replicates and compared by Duncan's multiple range tests. Statistical significance was determined at 5% level.

3. Results and Discussion

3.1. Isolation and screening of hydrogen producing bacterial isolates

Biohydrogen can be produced by hydrogen fermentative bacteria using different substrates rather than glucose. A total of seven ruminant bacterial isolates were isolated from the cow rumen samples and were screened for their hydrogen productivity on the pretreated sugarcane molasses. Interestingly, the bacterial isolate RM92 was the highest producer for hydrogen with H_{max} of 426.67±14.56 mL/L. Sugarcane molasses is considered as one of the most important sustainable agro-idustarial wastes for production of different valuable products by microorganisms. According to Lay et al. [27] and Lee et al. [28], molasses may regarded as an appropriate feedstock for hydrogen production because it contains different nuterients (sugars, amino acids, microbial proteins and vitamins) which are essential supplements for microbial fermentation.

3.2. Identification of bacterial isolate RM92

3.2.1. Phenotypic characterization

Bacterial isolate RM92 was appeared as pink colonies after 48 hrs incubation on thioglycolate agar plates (Fig. 2a). The isolate RM92 was non-spore forming, Gram negative and rod-shaped cells (Fig. 2b). The isolate was positive for catalase production, glucose fermentation, indole production, nitrate reduction and methyl red test. While the isolate was negative for oxidase production, citrate utilization, H_2S production, urease production and Vogas-Proskauer test (Table 1). According to Julian et al.

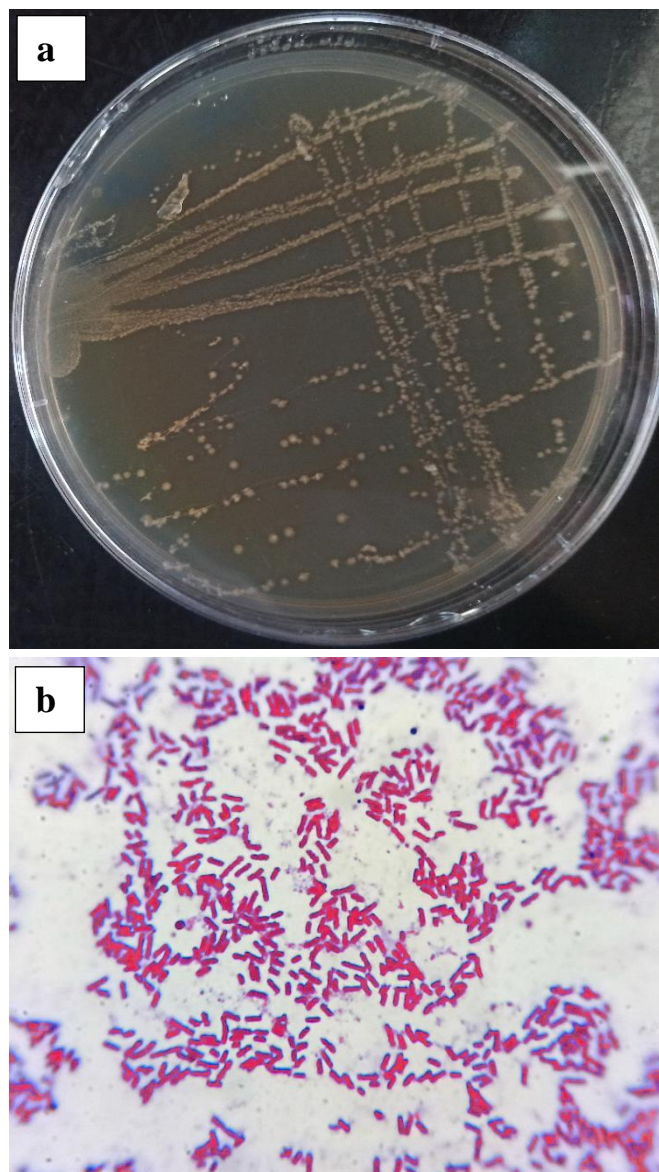[29], the selected isolate belongs to *Escherichia* sp. based on its phenotypic characteristics.

Fig. 2: Morphological characters of the isolate RM92; a) colonies on thioglycolate agar medium b) cells shape.

3.2.2. Genotypic identification

The bacterial isolate RM92 was identified based on the analysis of 16S rRNA gene sequence as *Escherichia fergusonii*. The sequence was deposited in GenBank database under accession number OP185369. A phylogenetic tree was constructed using Jukes-Cantor model with neighbour-joining algorithm of MEGA 7 [30,31]. The phylogenetic tree (Fig.3) shows the evolutionary relationship between the identified bacterial isolate with other related species from NCBI GenBank database.
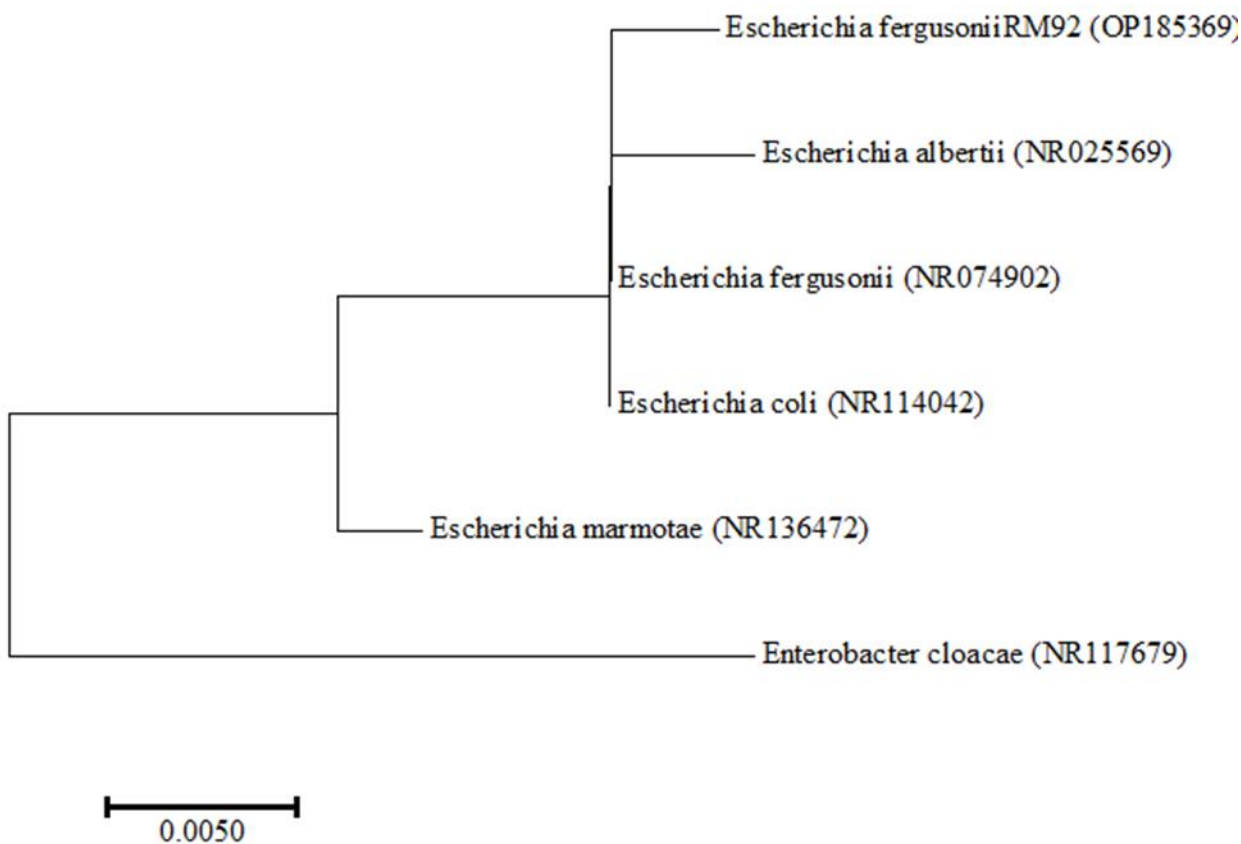
Fig. 3: Evolutionary relationship of taxa deposited in GenBank with *E. fergusonii* RM92.

Table 1: Phenotypic characteristics of the isolate RM92.

Test	Result
Cells shape	Non-spore forming rod cells
Gram staining	-ve
Catalase production	+ve
Citrate utilization	-ve
Glucose fermentation	+ve
H_2S production	-ve
Indole production	+ve
Methyl Red	+ve
Nitrate Reduction	+ve
Oxidase production	-ve
Urease production	-ve
Voges-Proskauer	-ve

3.3. Optimization of hydrogen production by *Escherichia fergusonii* RM92

3.3.1. Substrate concentration

The effect of different molasses sugar concentrations (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 %) on hydrogen production by *Escherichia fergusonii* RM92 was studied (Fig.4). Resulted data revealed that 4 % molasses sugar concentration was the optimum for hydrogen production (676.67±14.53 mL/L) with maximium hydrogen production rate (31.43±2.77 mL/L/h). Similar results were obtained by Wang and Jin [32] who reported the optimum molasses sugar concentration for hydrogen production was 4.4 %. Decreasing of hydrogen production occur by increasing molasses concentration more than 4% might due to the increasing of the pre-existing lactic acid concentration which acts as a sink collecting free electrons from NADH [28]. Molasses contains glutamate which considered as a good substrate for hydrogen producing bacteria that have the ability to convert it into hydrogen [29].

3.3.2. Effect of fermentation pH

Productivity of hydrogen by *E. fergusonii* RM92 was tested at different fermentation initial pH values (3, 4, 5, 6, 7 and 8). The maximum hydrogen production was plotted in Fig. 5. There are significant changes in hydrogen

production by changing the fermentation initial pH. Highest maximum hydrogen production (H_{max} 713.33±20.28 ml/L and R_{max} 46.11±3.38 ml/L/h) was obtained at initial fermentation pH 8 followed by (H_{max} 690.00±25.98 ml/L and R_{max} 43.89±3.38 ml/L/h) at pH 7 and then the hydrogen productivity decreased by decreasing of pH values. Similar results were obtained by Baek et al. [33] who reported that the optimum pH for hydrogen production was 7. Stavropoulos et al. [34] stated that the suitable pH range for biohydrogen production from 4.5 to 9. Hydrogen production was significantly reduced below pH 6 due the lower pH values inhibit biomass growth and affects hydrogenase enzymes activity [35,36].

3.3.3. Effect of fermentation temperature

Effect of different fermentation temperatures (20, 25, 30, 35, 40 and 45 °C) on hydrogen production were studied and the resulted data demonstrated in Fig.6. Optimum temperature for maximum biohydrogen production was at 40 °C (741.67 ±24.55 ml/L and R_{max} 25.83±0.62 ml/L/h). while, the fermentation temperature above or less than 40 °C was negatively affected hydrogen production yeild. Similar finding were obtained by Mishra and Das [37] who revealed that 40°C was the optimum fermentation

temperature for hydrogen production by *Enterobacter* sp. CN1. Also, Usman et al. [38] revealed that mesophilic temperature range of 30-49 °C was the optimum for hydrogen production. Increment of temperature from the optimium level leads to lowering hydrogen production yield as a result of degradation of hydrogen production enzymes [39].

3.3.4. Effect of inoculum size

The maximium hydrogen yield (H_{max} 1280.00 ±34.64 ml/L with R_{max} 36.92±0.89 ml/L/h) was achieved at 30% (v/v) inoculum size of the fermentation medium (Fig.7). Hydrogen production was negatively affected by deviating the inoculum percent from the optimal. This might be attributed to the growth lag phase which was notably affected by the initial cell concentration that consequently affect hydrogen productivity [32]. Also, Zhao et al. [40] reported that increasing of inoculum volume than the optimum levels led to overconsumption of used substrate and overgrowth of bacterial cells in a short time, which resulted in a rapid decrease in hydrogen production.
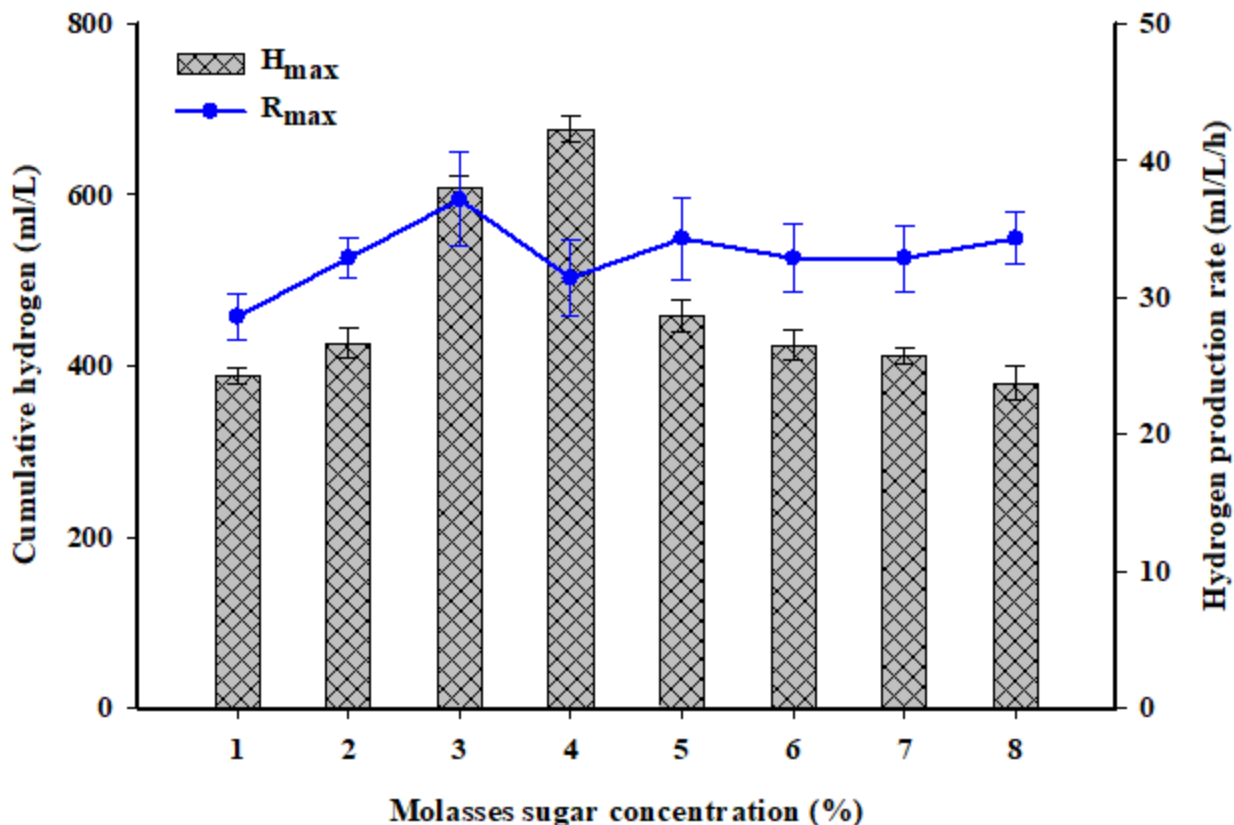

Fig. 4: Effect of molasses sugar concentration on hydrogen production by *E. fergusonii* RM92.
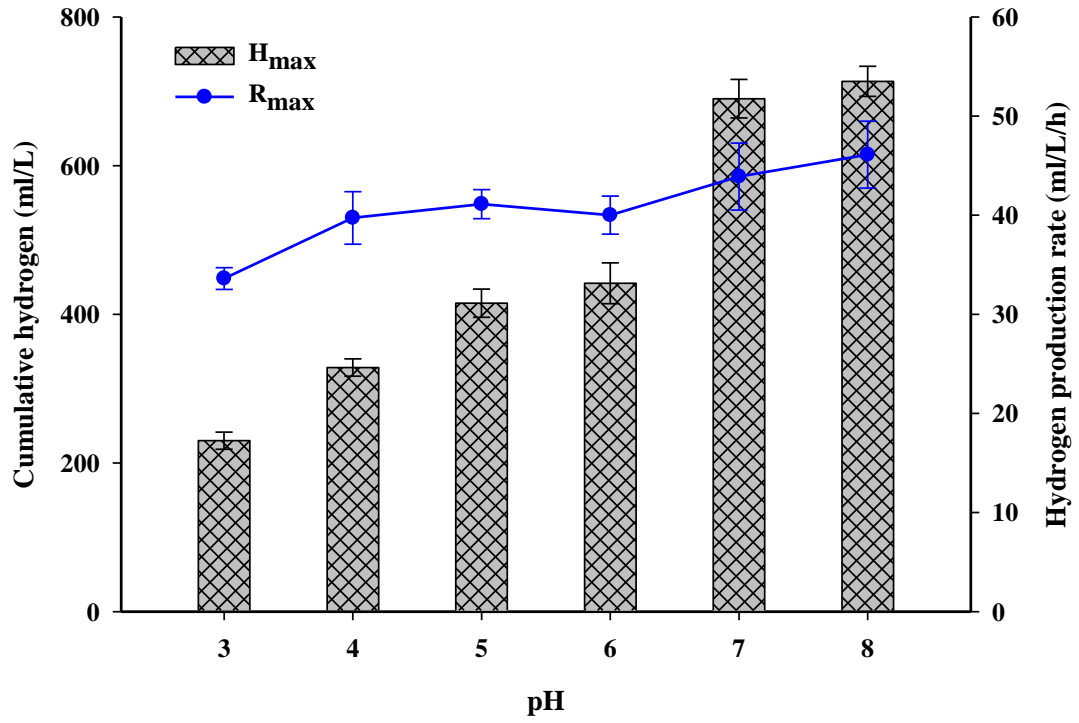
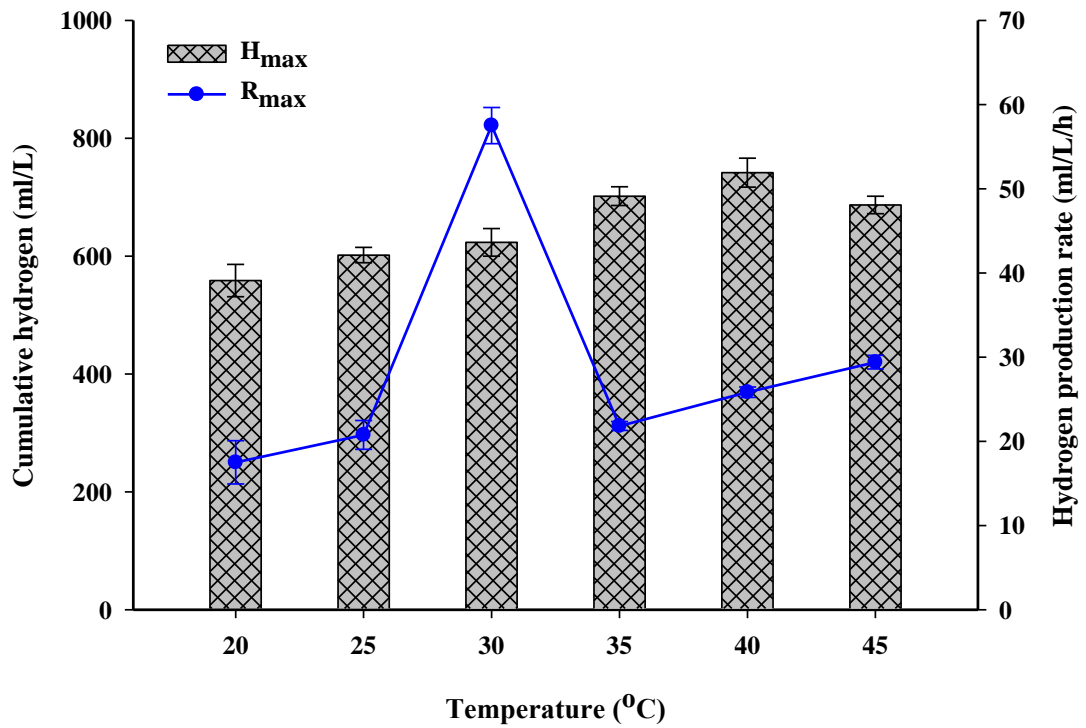**Fig. 5:** Effect of initial pH on hydrogen production by *E. fergusonii* RM92.

Fig. 6: Effect of fermentation temperatures on hydrogen production by *E. fergusonii* RM92.
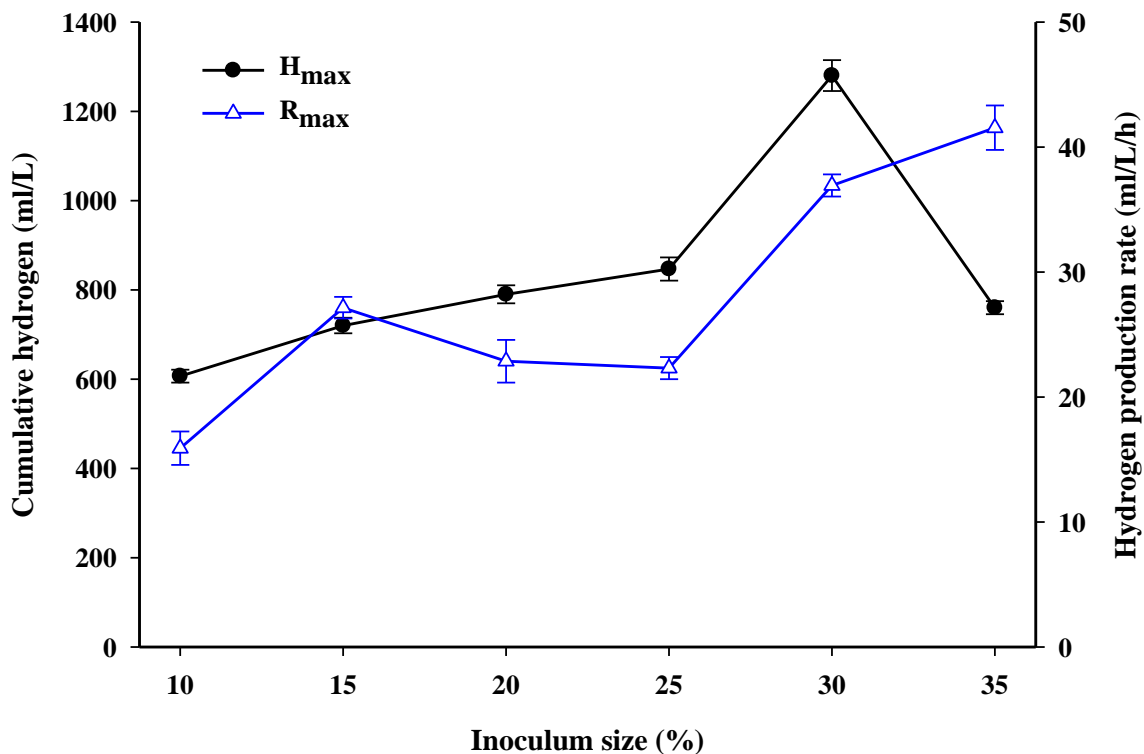
Fig. 7: Effect of inoculum size on hydrogen production by *E. fergusonii* RM92

4. Conclusion

Climatic changes and increasing of greenhouse gases are two serious problems face the world in the last century. These two prolems are resulted from using petrolium-derived fuels for energy. Thus, discovering new eco-friendly renewable energy sources like biohydrogen is required. Hydrogen production from agro-industrial wastes is considered the promising alternative and cheapest affordable solution. The current investigation investigated the biohydrogen production from anaerobic fermentation of sugarcane molasses using *Escherichia fergusonii* RM92. The highest hydrogen production (1280.00 ±34.64 ml/L) with maximum hydrogen production rate (36.92±0.89 ml/L/h) was achieved by optimization of the ferementation conditions. Future research can be conducted on biohydrogen production from sugaracne molasses on industrial scale.

Conflict of interest

The authors declare that they have no conflict of interest.

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