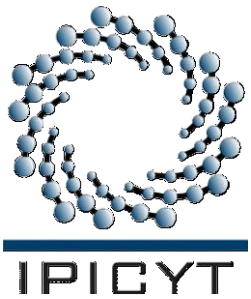

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

Optimization of hydrogen production by the psychrophilic strain G088

Tesis que presenta

Cecilia Lizeth Alvarez Guzmán

Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Codirectores de la Tesis:

Dr. Antonio De León Rodríguez

Dr. Victor Emmanuel Balderas Hernández

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Constancia de aprobación de la tesis

La tesis ***"Optimization of hydrogen production by the psychrophilic strain G088"*** presentada para obtener el Grado de Maestra en Ciencias en Biología Molecular fue elaborada por **Cecilia Lizeth Alvarez Guzmán** y aprobada el once de julio del dos mil dieciséis por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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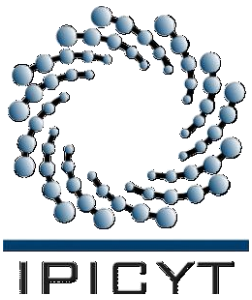

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MAESTRA EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Cecilia Lizeth Alvarez Guzmán

sobre la Tesis intitulada:

Optimization of hydrogen production by the psychrophilic strain G088

que se desarrolló bajo la dirección de

Dr. Antonio De León Rodríguez
Dr. Victor Emmanuel Balderas Hernández

El Jurado, después de deliberar, determinó

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Dedicatorias

A Dios, por permitirme hacer lo que más me gusta y darme la fuerza necesaria para llegar al final.

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Resumen

“Optimización de la producción de hidrógeno por la cepa psicrófila G088”

El hidrógeno (H_2) es considerado como uno de los vectores energéticos más prometedores para el futuro, posee características únicas como un alto rendimiento energético, es renovable, limpio y su combustión es sustentable y amigable con el medio ambiente debido a que no genera subproductos tóxicos. De los métodos de producción de H_2, la fermentación oscura es el método biológico más usado debido a las ventajas que presenta. Sin embargo, la mayoría de los estudios referentes a la producción fermentativa operan a temperaturas mesófilas, termófilas e incluso hipertermófilas, y poco se conoce de los procesos psicrófilos que tienen como ventaja requerir menor gasto de energía para calentar los reactores durante el proceso. En esta tesis se estudió la producción de biohidrógeno utilizando la cepa psicrófila G088 estrechamente relacionada a *Polaromonas rhizosphaerae* [EF127651] utilizando xilosa, glucosa, fructosa, galactosa, lactosa o sacarosa como fuente de carbono a una temperatura de 20ºC. Los resultados mostraron que la cepa G088 produjo biohidrógeno usando todos los sustratos evaluados, produciendo desde 91.7 hasta 439.8 ml para lactosa y glucosa respectivamente. Sin embargo, la glucosa fue el sustrato con el que se obtuvo la mayor velocidad de producción y rendimiento de 50.1 ml/l/h y 1.7 mol H_2/mol glucosa, respectivamente. Adicionalmente, la fermentación oscura es un proceso complejo el cual puede ser influenciado por diferentes factores, por lo tanto, también se evaluó el efecto de la temperatura, el pH y la concentración de sustrato sobre la producción de biohidrógeno, la velocidad de producción y el rendimiento, empleando glucosa. Se utilizó la metodología de superficie de respuesta con un diseño experimental Box-Behnken con el fin de determinar las condiciones óptimas de producción de biohidrógeno. De acuerdo al modelo matemático, las condiciones óptimas fueron temperatura de 26.3ºC, pH de 6.2 y concentración de glucosa de 25.31 g/l para una producción de biohidrógeno predicha de 503.34 ml de H_2, una velocidad de producción de 37.91 ml/l/h y un rendimiento de 1.93 mol H_2/mol glucosa. Se encontró que el efecto lineal del pH y el efecto cuadrático de la temperatura, pH y concentración de glucosa fueron los términos que afectaron a la producción de biohidrógeno. Por otra parte, la velocidad de producción fue afectada únicamente por el efecto cuadrático de la temperatura y concentración de glucosa, mientras que el efecto lineal y cuadrático de los tres factores afectó a la variable de rendimiento. Las condiciones óptimas fueron probadas experimentalmente por triplicado corroborando la correspondencia con el valor predicho por el modelo matemático. Los valores experimentales de la producción de biohidrógeno, velocidad y rendimiento bajo estas condiciones fueron 513±12.48 ml, 36.5±4.10 ml/l/h y 1.81±0.04 mol H_2, respectivamente. Estos resultados demuestran que la cepa G088 tiene potencial para ser usada para el desarrollo de nuevos procesos biotecnológicos para la producción de biohidrógeno.

PALABRAS CLAVE: Biohidrógeno, bacteria psicrófila, metodología de superficie de respuesta

Abstract

“Optimization of hydrogen production by the psychrophilic strain G088”

Hydrogen (H_2) is considered as one of the most promising energy carriers for the future, it has several unique characteristics, such as high energy yield, it is renewable, clean and its combustion is sustainable and environmentally friendly since it does not generate toxic byproducts. Among hydrogen production methods, dark fermentation is the biological method most widely used. However, most of the studies addressing fermentative hydrogen production operate at mesophilic, thermophilic and even hyperthermophilic temperatures and little information is available using psychrophilic microorganisms. These processes consume less energy for heating the reactors during the process. In this thesis, the biohydrogen production was studied using the psychrophilic strain G088 closely related to *Polaromonas rhizosphaerae* [EF127651] using xylose, glucose, fructose, galactose, lactose or sucrose as a carbon source at temperature of 20ºC. The results showed that G088 strain produced biohydrogen using all the evaluated substrates, ranging from 91.7 to 439.8 ml for lactose and glucose respectively. Nevertheless, glucose was the best substrate with maximum values of biohydrogen production rate and yield of 50.1 ml/l/h and 1.7 mol H_2/ mol glucose respectively. In addition, dark fermentation is a complex process, and can be influenced by different factors; thus, the effect of temperature, pH and substrate concentration on biohydrogen production, biohydrogen production rate and yield was also studied using glucose. Response surface methodology with a Box-Behnken design was used to determine the optimal biohydrogen production conditions. According to the mathematical model, temperature 26.30ºC, pH 6.2 and glucose 25.31 g/l were the optimum conditions for a predicted biohydrogen production of 503.34 ml of H_2, and a biohydrogen production rate and yield of 37.91 ml/l/h and 1.93 mol H_2/ mol glucose, respectively. The linear effect of pH and the quadratic effect of temperature, pH and glucose concentration were the most significant terms affecting biohydrogen production. Otherwise, biohydrogen production rate was affected only by the quadratic effect of temperature and glucose concentration, while biohydrogen yield was affected by the lineal and quadratic effect of the three factors. The optimal production conditions were experimentally tested by triplicate corroborating the correspondence with the predicted value by the mathematical model. The experimental values for biohydrogen production, yield and rate under these conditions were 513±12.48 ml, 1.81 mol H_2 / mol glucose and 36.5±4.10 ml/l/h, respectively. These studies demonstrate that the G088 strain has potential to be used for developing new biotechnological processes for biohydrogen production.

KEY WORDS: Biohydrogen, Psychrophilic bacteria, response surface methodology

Chapter 1

Biohydrogen production by the psychrophilic G088 strain using single carbohydrates as substrate

Abstract

The production of biohydrogen by psychrophilic G088 strain ([EU636029]), closely related to *Polaromonas rhizosphaerae* ([EF127651]) was evaluated using xylose, glucose, fructose, galactose, lactose or sucrose as a carbon source. Biohydrogen production was performed in 120 ml serological bottles with a production medium containing 2.75 g/l tryptone, 0.25 g/l yeast extract, and 20 g/l of each carbohydrate. Results showed that the G088 strain produced biohydrogen using all the evaluated substrates, ranging from 91.7 to 439.8 ml for lactose and glucose, respectively. However, glucose was the substrate with the highest consumption rate, accompanied by the maximum values of biohydrogen production rate and a biohydrogen yield of 50.1 ml/l/h and 1.7 mol H_2/ mol glucose, respectively. Analysis of the secreted metabolites showed that ethanol and organic acids were the main by-products. Our results demonstrate that the G088 strain has potential to be used for developing new biotechnological processes for biohydrogen production.

Alvarez-Guzmán CL, Oceguera-Contreras E, Ornelas-Salas JT, Balderas-Hernández VE, De León Rodríguez A. Biohydrogen production by the psychrophilic G088 strain using single carbohydrates as substrate. International Journal of Hydrogen Energy. 2016;41:8092-100.

1.1 Introduction

Environmentally friendly energy carriers and sources are the most outstanding topics in the energy and environmental sector. The current global energy demand is mostly dependent on reserves of fossil fuel uses [1]. In recent years, various studies have been conducted to obtain a sustainable source of energy that can replace fossil fuels and its negative impact on the environment. In this regard, hydrogen was found as a promising clean and environmental friendly energy carrier [2], furthermore its energy value is 122 kJ/g, which is 2.75 times higher than hydrocarbon fuels [3] and upon oxidation hydrogen produces water [4]. In addition, handling of hydrogen gas is safer in comparison to other known natural gases. These features make hydrogen an ideal candidate to replace fossil fuels [5].

Hydrogen is a valuable energy carrier, an important feedstock to the chemical industry, and useful in detoxifying a wide range of water pollutants. As an energy carrier, it is especially attractive due to its potential to be used to power chemical fuels. In industry, hydrogen is used for the hydrogenation of many products, including heavy oils in gasoline production, foods, and ammonia for fertilizer. Nowadays, hydrogen is mainly produced by reforming fossil fuels, 40% hydrogen is produced from natural gases, 30% from heavy oil and naphtha, 18% from coal, 4% from electrolysis and about 1% from biomass. Therefore, hydrogen is currently neither renewable nor carbon-neutral. Instead, hydrogen manufacturing has a large greenhouse-gas footprint.

Among various hydrogen production processes, the biological method is known to be less energy intensive; compared with the chemical processes for hydrogen

production, biological hydrogen production by fermentative process can be operated at ambient temperatures and normal pressures [6, 7]. There are different biological methods of hydrogen production, such as photosynthetic and fermentative processes. Dark fermentation is a process in which microorganisms utilize carbohydrates to produce biohydrogen in anaerobic fermentation conditions. However, low yields and production rates have been the main barriers for practical applications [8]. Most of the studies addressing fermentative hydrogen production operate on anaerobic digesters at mesophilic (24-40°C), thermophilic (40-65°C) or hyperthermophilic (>80°C) [5] temperatures. Whereas, to our knowledge, only two studies have been reported on biohydrogen production using psychrophilic bacteria [9, 10].

Most microorganisms isolated from cold environments are either psychrotolerant or psychrophilic. Psychrotolerant organisms grow well at temperatures close to the freezing point of water, but have the fastest growth rates above 20°C, whereas psychrophilic organisms grow fastest at temperatures of 15°C or lower, but are unable to grow above 20°C. Irrespective of how they may be defined, ‘psychro’ microorganisms are cold-adapted and exhibit properties which are distinctly different from other thermal classes [11]. These microorganisms have slower metabolism rates and higher catalytic efficiencies than mesophiles [12], the high activity of psychrophilic enzymes at low and moderate temperatures offers potential economic benefits due to the substantial energy savings in large-scale processes that would not require the expensive heating of reactors [13]. In addition, the temperature range prevents the risk of microbial contamination [12]. These advantages make the psychrophilic bacteria a good candidate for

biohydrogen production. Currently, these microorganisms are being exploited as cell factories for the production of unstable compounds as well as for bioremediation of polluted cold soils and wastewaters. Furthermore, their enzymes have already found useful applications in various domains such as molecular biology, medical research, industrial food or feed technologies, detergents or cosmetics [14] However, in the biofuels field their application has not been widely explored due to only a few studies addressing hydrogen, methane and biodiesel production having been reported [9, 10, 15-18].

In this study, the effectiveness of biohydrogen production from single carbohydrates using a psychrotolerant G088 strain closely related to Polaromonas rhizosphaerae was assessed. This microorganism was isolated from samples of glacier sediment from Antarctica [19]. The carbohydrates evaluated were glucose, xylose, fructose, galactose, lactose and sucrose. Currently there is only one study reporting biohydrogen production from psychrophilic bacteria isolated from Antarctica, which was reported by our research group [9].

1.2 Material and methods

1.2.1 Strain and Culture media

In this study, the psychrotolerant G088 strain obtained of samples of glacier sediment from Antarctica was used. The accession number EU636050 and closest relativity of this strain according to NCBI is Polaromonas rhizosphaerae [EF127651] [16]. The strain was grown routinely in YPG agar plates [0.25 g/l Bacto-tryptone (Difco), 0.25 g/l yeast extract (Difco), 0.25 g/l glucose (Sigma) and

15 g/l Bacto-agar (Sigma)] and maintained at 4°C. Six carbohydrates were used as substrates (xylose, glucose, fructose, galactose, sucrose or lactose). Biohydrogen production experiments were done in a rich production medium containing 2.75 g/l Bacto-tryptone (Difco), 0.25 g/l yeast extract (Difco) and 20 g/l of the corresponding carbohydrate mentioned above (Sigma) [20].

1.2.2 Biohydrogen production experiments

To evaluate hydrogen production by the G088 strain, preinocula were grown in a rich production medium under anaerobic conditions at 20ºC. Cells were harvested, centrifuged, washed and inoculated into 120 ml anaerobic serological bottles (Prisma, DF, Mex) containing 110 ml of production medium with 20 g/l of the respective carbohydrate supplemented with 1 ml/l of trace elements solution (0.015 g/l FeCl3.4H2O, 0.00036 g/l Na2MoO4.2H2O, 0.00024 g/l NiCl2.6H2O, 0.0007 g/l CoCl2.6H2O, 0.0002 g/l CuCl2.2H2O, 0.0002 g/l Na2SeO3, 0.01 g/l MgSO4). The cultures were started at an optical density at 600 nm (OD600nm) of 1, pH was adjusted at 6.8 and were incubated at 20°C and 150 rpm [21]. All experiments were carried out in triplicate.

1.2.3 Analytical methods

Hydrogen produced was measured by water displacement with NaOH 1N in an inverted burette connected to serological bottles with rubber tubing and a needle and validated by Gas chromatography using a thermal conductivity detector as described elsewhere [21]. All the experiments were carried out in triplicate.

Samples of 1 ml were taken at different times during fermentation, they were then diluted and filtered through a 0.22 mm membrane (Millipore, Bedford, Massachusetts, USA) [9]. Concentrations of xylose, glucose, fructose and galactose and several metabolites such as succinic acid, lactic acid, acetic acid and butanol were analyzed by High Performance Liquid Chromatography (HPLC, Infinity LC 1220, Agilent Technologies, Santa Clara CA, USA) using a Refraction Index Detector, a column Phenomenex Rezex ROA (Phenomenex, Torrance, CA, USA) at 60°C, and 0.0025 M H2SO4 as mobile phase at 0.41 ml/min. Sucrose was analyzed by the colorimetric method for determination of sugars and related substances [22] and lactose was analyzed by the 3, 5-dinitrosalicylic acid (DNS) method [23]. Ethanol, butyric acid, propionic acid, and acetone were analyzed in a Gas Chromatograph (GC, 6890N Network GC System, Agilent Technologies Wilmington, DE, USA) using a flame ionization detector (Agilent Technologies Wilmington). The column used was a capillary column HP-Innowax with the following dimensions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness (Agilent, Wilmington, DE, USA). Temperatures of the injector and flame ionization detector (FID) were 220 and 250°C respectively. Helium was used as carrier gas at a flow rate of 25 ml/min. The analyses were performed with a split ratio of 5:1 and a temperature program of 25°C for 10 min to 280°C, and was maintained at this temperature for a final time of 10 min [9].

1.3 Results

1.3.1 Fermentation of pentoses

We conducted an experiment with xylose as a substrate to evaluate hydrogen production because of the importance of this sugar as the main pentose obtained from the hydrolysis of hemicellulosic materials. Figure 1.1 shows a typical batch culture of the G088 strain using xylose as a single substrate. As observed, xylose started to be consumed 16 h after initiating the culture and hydrogen production started at 34 h of incubation with a volume of 8.7 ml. Hydrogen production attained its maximum volume at 333 h with 349.9 ml; however at that point there was still 5 g/l of non-consumed substrate. The maximum hydrogen production rate reached using xylose was 23.1 ml/l/h at 145 h. Hydrogen yield achieved was 1.4 mol H_2/ mol xylose (Table 1.1).

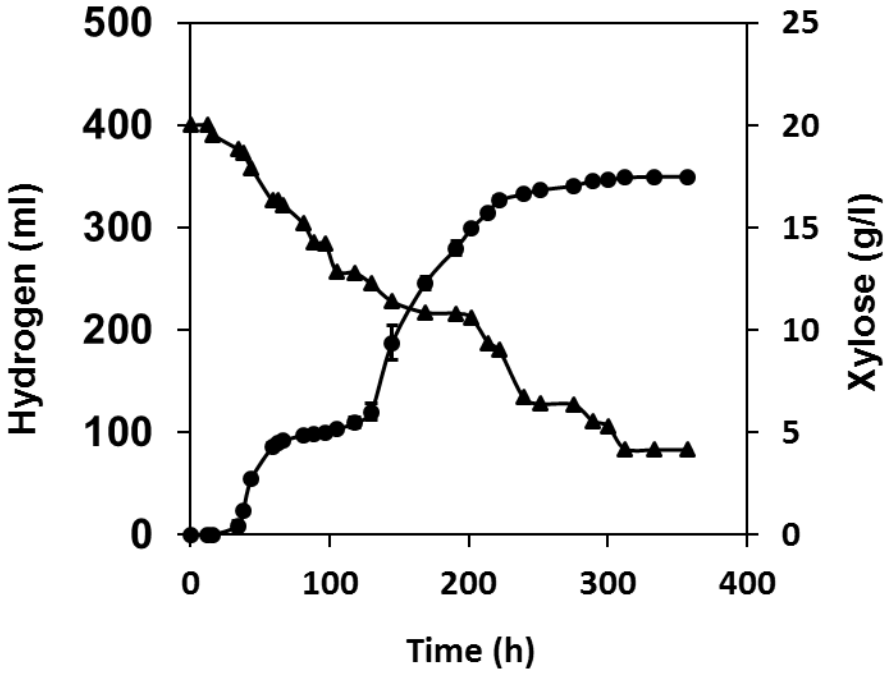

Figure 1.1 Batch culture of psychrotolerant G088 strain closely related to *Polaromonas rhizosphaerae* using xylose as substrate. Hydrogen production (●) and remaining amount of carbon source in the container (▲). Each data point represents the average of triplicate experiments ± SD.

Table 1.1 Hydrogen production parameters by the G088 strain using different substrates.

Substrate	Production (ml)	Hydrogen production rate (ml/l/h)	Yield (mol H_2/ mol hexose)
Glucose	439.8 ± 64.3	50.1 ± 6.7	1.7 ± 0.3
Fructose	388.1 ± 17.8	29 ± 6.9	1.3 ± 0
Xylose	349.9 ± 35	23.1 ± 1.3	1.4 ± 0.1*
Galactose	293.3 ± 8.0	7.9 ± 3.1	1.3 ± 0
Sucrose	201.7 ± 9.2	14.1 ± 1.4	1.7 ± 0.1
Lactose	91.7 ± 8.1	8.8 ± 1.6	1.5 ± 0.1

*mol H_2/mol pentose

1.3.2 Fermentation of hexoses

Lignocellulosic biomass contains 70-80% carbohydrates and could serve as the ideal feedstock for fermentative hydrogen production [24]. In this regard we evaluated the capability of the G088 strain to metabolize glucose, which is currently obtained by hydrolysis of starch, cellulose and hemicellulosic materials [25], as well as fructose that is mainly extracted from a fructan called inulin [26]. In addition galactose was tested too, which like glucose, is obtained from hemicellulosic material (lignocellulose). All of them constitute the major component of hemicellulosic biomass.

1.3.2.1 Glucose

In the case of the cultures using glucose as substrate, its consumption started at 20 h as seen in figure 1.2. The beginning of hydrogen production was 43 h after the culture started, with a volume of 12 ml. Afterward, the maximum hydrogen production was attained after exponential phase, generating a final volume of 439.8 ml in 306 h. In the middle point of fermentation, the glucose concentration was 6.9 g/l, and as expected the G088 strain completely utilized the available substrate since no glucose was detected at the end of the fermentation. Moreover, production rate and yield were 50.1 ml/l/h and 1.7 mol H_2/ mol glucose respectively (Table 1.2).

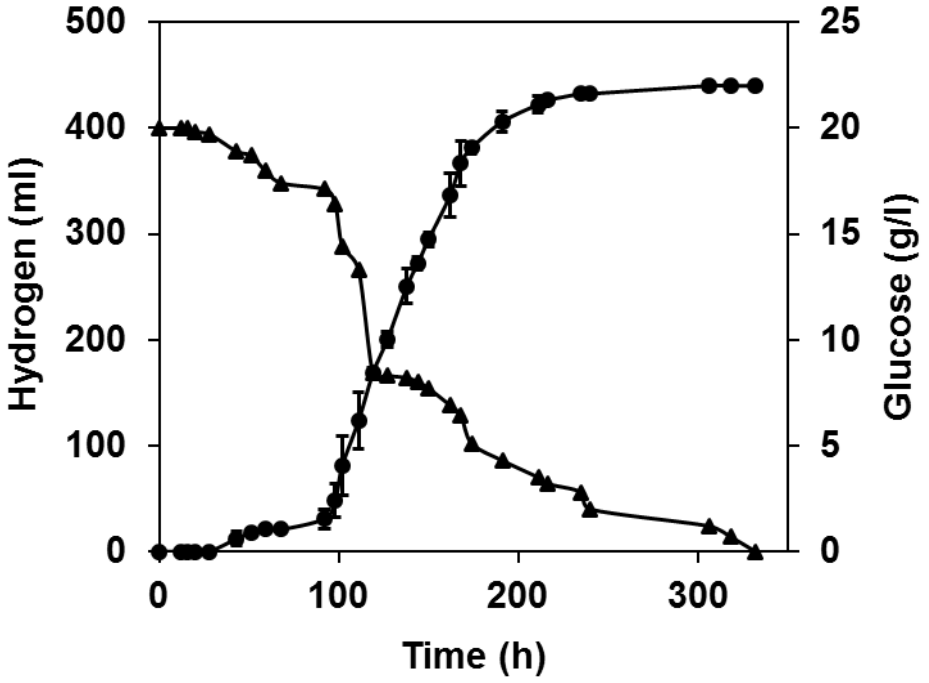

Figure 1.2 Batch culture of psychrotolerant G088 strain closely related to *Polaromonas rhizosphaerae* using glucose as substrate. Hydrogen production (●) and remaining amount of carbon source in the container (▲). Each data point represents the average of triplicate experiments ± SD.

1.3.2.2 Fructose

The fermentation of fructose by the G088 strain had duration of 386 h, of which 50 h corresponded to the lag-phase (Figure 1.3). Unlike glucose, where its consumption started at 20 h, the consumption of fructose began at 40 h, and hydrogen production started 21 h later with a volume of 51.33 ml of hydrogen after having consumed 2.8 g/l of the available substrate. Moreover, the maximum hydrogen volume was 388.1 ml, achieved after 330 h of fermentation; at this point fructose was not detected. The maximum yield reached was 1.37 mol H_2/ mol

fructose and the maximum hydrogen production rate using fructose was 29 ml/l/h (Table 1.1).

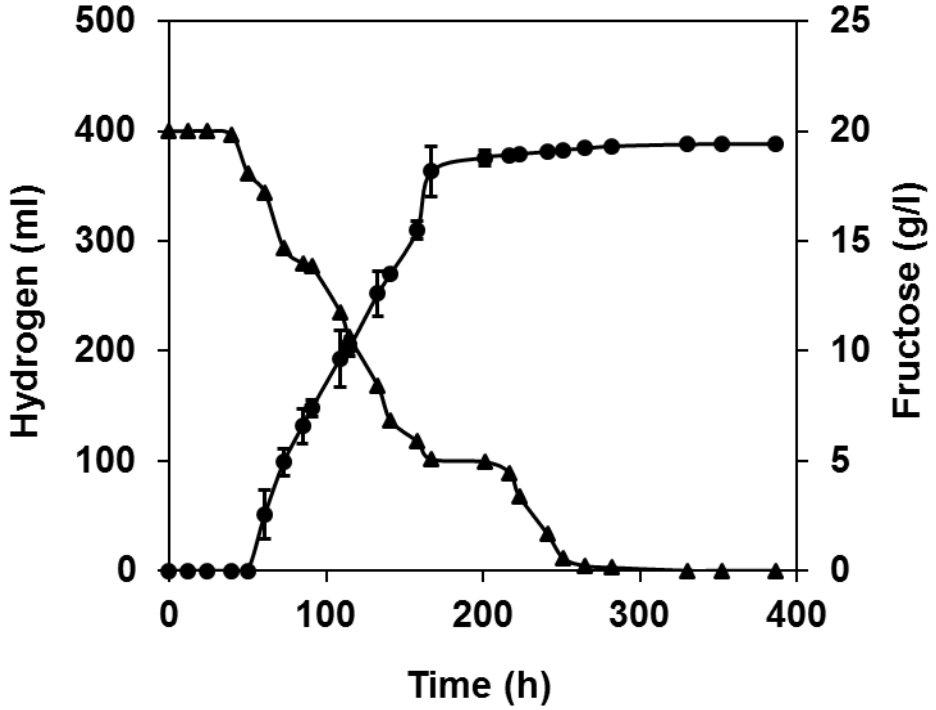

Figure 1.3 Batch culture of psychrotolerant G088 strain closely related to *Polaromonas rhizosphaerae* using fructose as substrate. Hydrogen production (●) and remaining amount of carbon source in the container (▲). Each data point represents the average of triplicate experiments ± SD.

1.3.2.3 Galactose

The galactose fermentation presented a lag-phase of 44 h, nonetheless hydrogen production started at 69 h with a volume of 5.66 ml (Figure 1.4). After 279 h of

cultivation, approximately the 50% of available galactose was consumed by the G088 strain. The maximum production rate was obtained at 206 h with 7.9 ml/l/h, which is approximately 1/6.3 of the production rate using glucose. On the other hand, the hydrogen yield was 1.32 mol H_2/ mol galactose (Table 1.1). Furthermore, almost at the end of the fermentation at 568 h, the maximum hydrogen production was measured, registering a volume of 293.3 ml. In addition, the galactose started to be consumed from the beginning of the fermentation, and 2.7 g/l of galactose still remained in the culture medium after 592 h of culture (Figure 1.4).

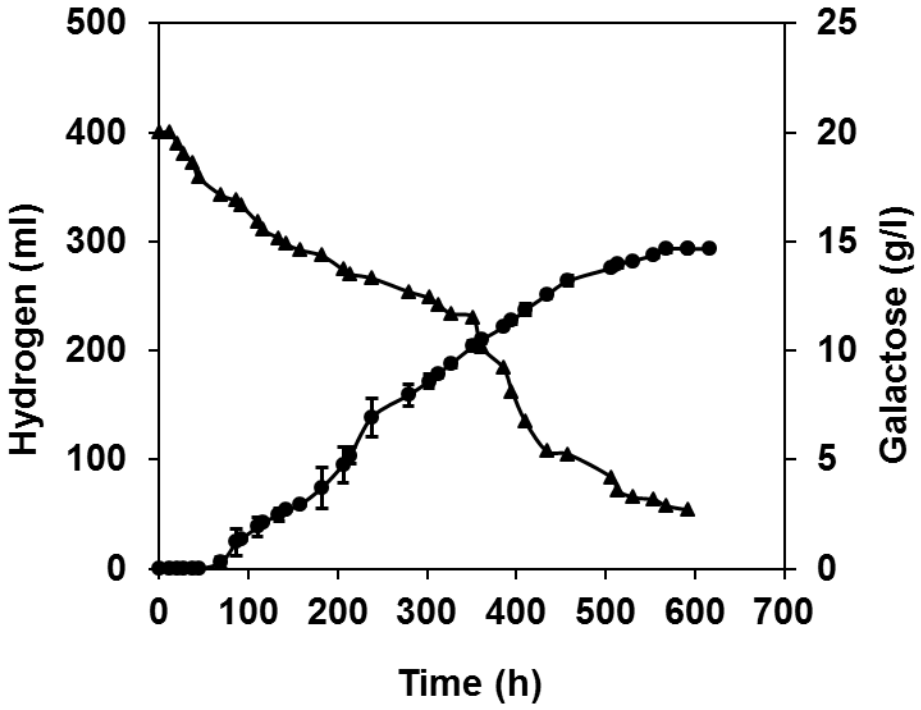

Figure 1.4 Batch culture of psychrotolerant G088 strain closely related to *Polaromonas rhizosphaerae* using galactose as substrate. Hydrogen production (●) and remaining amount of carbon source in the container (▲). Each data point represents the average of triplicate experiments ± SD.

1.3.3 Fermentation of disaccharides

Sucrose and lactose are typical disaccharides, the first one is obtained from molasses, a by-product of the sugar industry that is obtained as a thick syrup sugar extraction [27], and the latter is obtained from cheese whey, a by-product generated during cheese production, which represents an 85-90% of the total volume of processed milk [21]. Therefore, we evaluated the potential use of these carbohydrates as raw material for hydrogen production.

1.3.3.1 Sucrose

The fermentation of sucrose lasted for about 350 h. Due to the lag phase of 20 h (Fig. 1.5); hydrogen production began 34 h after the cultivation started. Hydrogen production attained 201.7 ml in 351 h. This production represents approximately the 50% the hydrogen produced from glucose. The maximum hydrogen production rate (14.1 ml/l/h) was observably lower than using glucose (50.1 ml/l/h). Furthermore, the yield obtained in this culture was 1.7 mol H_2/ mol sucrose (Table 1.1).

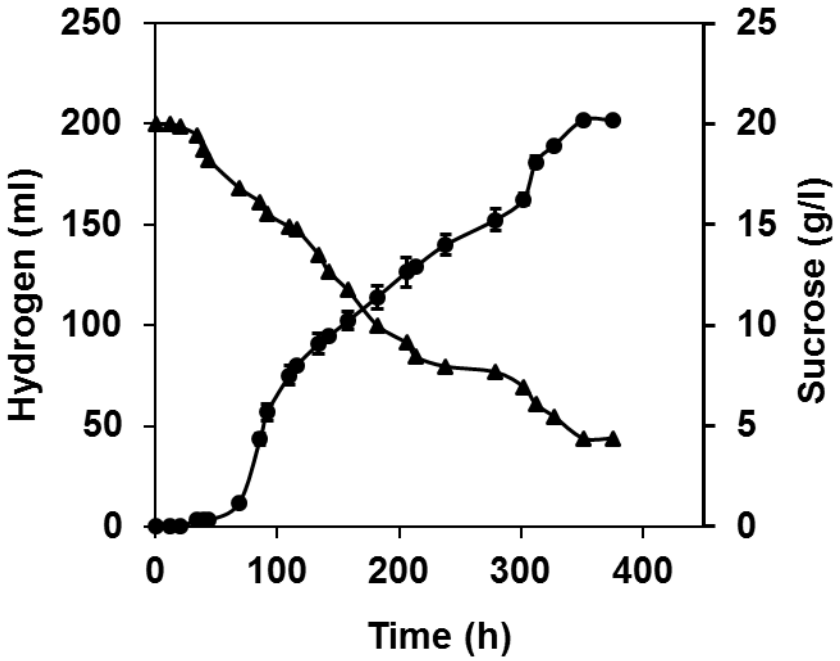

Figure 1.5 Batch culture of psychrotolerant G088 strain closely related to *Polaromonas rhizosphaerae* using sucrose as substrate. Hydrogen production (●) and remaining amount of carbon source in the container (▲). Each data point represents the average of triplicate experiments ± SD.

1.3.3.2 Lactose

In these cultures, the lag-phase lasted 39 h. The biohydrogen production started after 44 h of culture (Fig. 1.6). Unlike cultures using sucrose, the fermentation using lactose took about 10 h to produce the first 9.3 ml. However the exponential phase lasted about 138 h and the maximum hydrogen production achieved by G088 strain was 91.7 ml after 302 h of fermentation. On the other hand, the highest production rate and yield were 8.8 ml/l/h and 1.5 mol H_2/ mol hexose, respectively (Table 1.1).

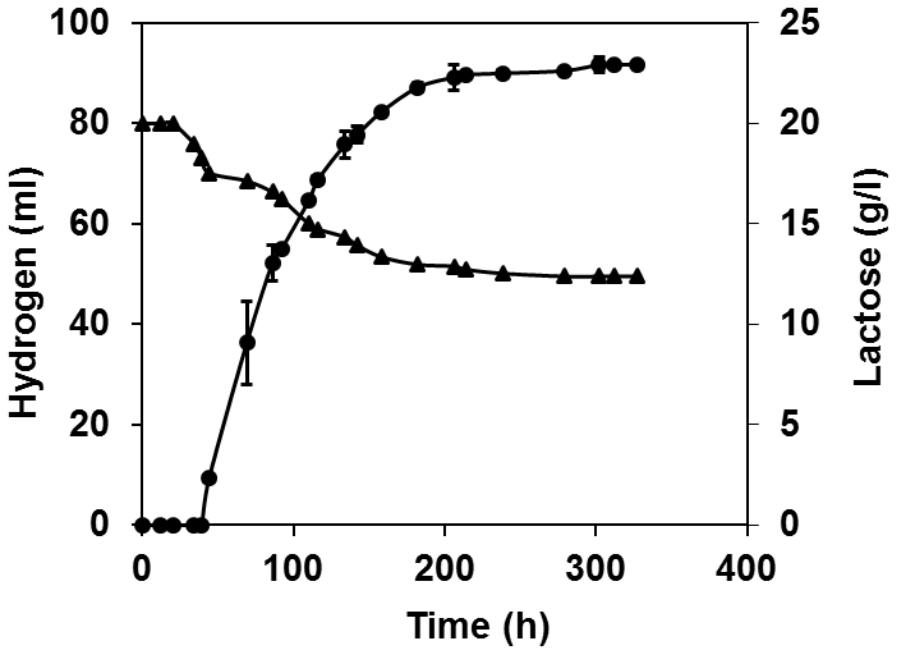

Figure 1.6 Batch culture of psychrotolerant G088 strain closely related to *Polaromonas rhizosphaerae* using lactose as substrate. Hydrogen production (●) and remaining amount of carbon source in the container (▲). Each data point represents the average of triplicate experiments ± SD.

1.3.4 Comparison of hydrogen production

Table 1.1 shows that using lactose as a substrate for G088 strain resulted in poor hydrogen production (91.7 ml) and a low production rate (8.8 ml/l/h), which is comparable to the maximum hydrogen production rate of 7.9 ml/l/h achieved during galactose catabolism. In the case of galactose, the low hydrogen production rate could be attributed to a deficiency in galactose transportation into the cell, or due to catabolic repression of various enzymes involved in the galactose conversion to

glucose-6-phosphate [28]. On the other hand, the low production rate using lactose could be associated to an incomplete biodegradation into glucose and galactose; in this regard, the lactose fermentation showed a yield of 1.5 mol H_2/ mol lactose which is considerably lower than the hydrogen yield attained by the fermentations with the other five substrates. Glucose is an easily biodegradable carbon source, and as expected, the highest hydrogen production capability was achieved using this hexose as substrate with the highest values of hydrogen volume (439.8 ml), hydrogen production rate (50.1 ml/l/h) and yield (1.7 mol H_2/ mol glucose). Sucrose can be biodegraded into glucose and fructose, thereafter, these monosaccharides enter the glycolysis pathway and continue their degradation [29]. On the other hand, fermentation using fructose presented the best hydrogen volume (388.1 ml) and production rate (29 ml/l/h) after glucose, while the culture with xylose achieved a total hydrogen volume (349.9 ml) lower than the volumes attained by the fermentations with glucose and fructose. However, the analysis of variance (ANOVA) showed that there was no significant difference ($p < 0.05$) between glucose, which is the substrate with the highest hydrogen production, in comparison with xylose and fructose.

1.3.5 Fermentative metabolites

Hydrogen formation is accompanied with production of volatile fatty acids or solvents during an anaerobic digestion process. Table 1.2 shows the excreted metabolites found in the culture at the end of the cultivations. In each fermentation with a different single substrate, the presence and concentration of metabolites

varied. For instance, a higher concentration of ethanol was detected in the culture with xylose, followed by the culture with glucose, while in cultures using lactose it was not detected. In the case of the production of butyric acid, the fermentation with glucose achieved the highest concentration (2.51 g/l), and for the rest of the carbohydrates used a concentration lower than 0.9 g/l was registered. According to reaction stoichiometry in strict anaerobes, a bioconversion of 1 mol of glucose into acetate would produce 4 moles of hydrogen. Whereas facultative microorganisms, convert one mole of glucose into butyrate and produce only 2 moles of hydrogen. Therefore, the yield of 1.7 mol H2/mol glucose attained in this study indicates that most of the glucose was converted to butyric acid instead of acetic acid. On the other hand, the acetic acid presence on the fermentation with fructose was remarkably high, with 2.31 g/l, while on the other cultures the concentration of this acid was shown to be below of 0.8 g/l. However, the hydrogen yield by fructose was lower compared the attained using glucose. Moreover, the highest concentration of propionic acid was detected on the fermentation with xylose as a substrate (2.02 g/l), followed by fermentation using glucose. As for the remaining fermentations, the concentrations of this metabolite were in small quantities. According to Hanaki et al. [30] propionic acid is believed to be the most toxic volatile acid appearing in the anaerobic process, however, its toxic effect acts upon the methanogenic population in mixed cultures, [30]. On the other hand, succinic acid was found on the fermentations using glucose, galactose, lactose and sucrose, being the fermentation with galactose the one that attained the highest concentration (4.92 g/l). In addition, the presence of other solvents was detected, such as butanol and acetone. In the case of butanol, the highest concentration of

1.54 g/l was detected in the cultures with glucose, followed by the cultures with fructose (1.33 g/l). On the other hand, acetone was detected only in the cultures with glucose and fructose at 1.5 g/l and 0.54 g/l, respectively.

Table 1.2 Fermentative metabolites produced by the psychrotolerant strain G088 at the end of each fermentation with different substrates in g/l.

Metabolite	Glucose	Xylose	Fructose	Galactose	Lactose	Sucrose
Succinic acid	3.88	0	0	4.92	0.21	0.18
Lactic acid	0	0	0	3.85	0	0
Acetic acid	0.67	0.1	2.31	0.42	0.18	0.8
Propionic acid	0.91	2.02	0.08	0.09	0.11	0.08
Butyric acid	2.51	0.85	0.65	0.09	0.85	1
Butanol	1.48	0	1.33	0	0.27	0.3
Ethanol	0.5	1.22	0.1	0.18	0	0.15
Acetone	1.5	0	0.54	0	0	0

1.4. Discussion

The Polar Regions, such as Antarctica, represent a vast source of novel psychrophilic and psychrotolerant microorganisms. Psychrophilic bacteria and their enzymes are of commercial interest because their possibility of use at low temperatures [9]. However, their application in biohydrogen production has just begun. Organic materials and residues currently constitute a large source of biomass, which includes agricultural crops and their waste by-products, wood and wood waste, food processing waste, aquatic plants, algae and effluents produced during the human activities [25]. Consequently production of biohydrogen from renewable resources would become a major and attractive future source of energy. In accordance, glucose, xylose, fructose, galactose, sucrose, and lactose were

explored as substrates because they are available in large amounts on the materials mentioned above.

Only within the past few years has it been recognized that psychrophilic microorganisms and their products or enzymes provide a large reservoir of potentially novel biotechnological exploitation [31]. However, hydrogen production by these microorganisms has not been extensively explored, representing a new alternative in the biohydrogen production field. To our knowledge there is only one report on the use of psychrophilic bacteria isolated from Antarctica for biohydrogen production, which was reported by our research group, where the capability of hydrogen production at 25°C by 14 psychrophilic strains was evaluated [9]. The results showed that all psychrophilic strains produced biohydrogen from 34.8 to 253.3 ml. Maximum hydrogen production and production rates of 253.3 ml and 16.64 ml/l/h, respectively were attained by the GA051 strain, which is closely related to *Janthinobacterium agaricidamnosum*, whereas the maximum yield of 1.57 mol H_2/ mol glucose was for the GA024 strain closely related to the *Polaromonas jejuensis* strain JS12-13 with an identity of 99%. Another study by Debowsky et al. [10] assessed the biohydrogen production at 20°C using 12 psychrophilic strains, out of which, 9 were Gram-negative bacteria belonging to the class Gammaproteobacteria *Rahnella aquatilis* (RA1-RA9) and 3 Gram-positive bacteria belonging to the phylum Firmicutes: *Carnobacterium maltaromaticum* (CM), *Thrichococcus collinsii* (TC) and *Clostridium algidixylanolyticum* (CA) isolated from underground water and demersal lake water samples. The highest total hydrogen volume of 66.93 ml was attained by the RA7 strain; however, in our work the highest total hydrogen volume was 439.8 ml using the G088 strain with

glucose as substrate, which is 6.8 times higher than RA7. Nevertheless, the RA7 fermentation was carried out using a complex carbon source as cheese whey. In comparison to RA7, hydrogen production by the G088 strain, using lactose as a substrate, is 1.4 times higher than RA7 hydrogen production, despite the substrate for RA7 had a high content in carbohydrate, protein and fat. Thus, it is necessary to compare the hydrogen production of G088 using cheese whey as carbon source.

Current fermentative biohydrogen production processes are carried out mainly at mesophilic (24-40°C), thermophilic (40-65°C) or hyperthermophilic (>80°C) temperatures [5]. In addition, glucose, sucrose and starch mixtures are the most commonly used substrates [1]. Although these processes generate high yields, they have the disadvantage of requiring large amounts of energy to maintain the optimum process temperature. Table 1.3 shows the hydrogen production yields at different temperatures using different substrates and microorganisms. For example Chin et al. [32] assessed hydrogen production using *Clostridium acetobutylicum* at 37°C obtaining a yield of 2.0 mol H_2/ mol glucose, while Mizuno et al. [33] employed *Clostridium sp.* at 35°C reaching a hydrogen yield of 0.85 mol H_2 /mol glucose. Ishikawa et al. [34] reported the hydrogen production by a *Escherichia coli* MC13-4 strain that overproduce hydrogen because of its deficiency in lactate production, reached a yield of 1.2 mol H_2/ mol glucose at 37°C. By comparing these results, it is clear that the yield of 1.7 mol H_2/ mol glucose achieved by the psychrotolerant G088 strain lies between the yields obtained under conditions of mesophilic temperature. However, our process has the advantage of requiring approximately 15°C lower than the aforementioned processes. Moreover, Lo et al. [35] examined hydrogen production with seven hydrogen-producing pure strains at

37°C with xylose as a substrate; those results showed that *Clostridium butyricum* CGS5 was the best hydrogen producer on xylose, with a yield of 0.73 mol H_2/ mol xylose. Whereas the yield reached by the G088 strain is 1.73 times higher than that reported above. In this regard, other substrates such as fructose have been evaluated under mesophilic conditions as shown by Wu et al. [36] where the maximum yield attained by anaerobic sludge at 37°C was 0.56 mol H_2/ mol fructose; this result is considerably lower compared to the yield of 1.3 mol H_2/ mol fructose obtained by G088 strain.

Table 1.3 Comparative hydrogen production yields at different temperatures using different substrates and microorganisms.

Microorganism	T (°C)	Working volume (ml)	Culture type	Substrate	Biohydrogen production (ml)	Maximum hydrogen yield (mol H_2/ mol substrate)	Ref
Thermotoga maritima	80	100	Batch	Glucose	NR	4	[37]
Caldicellulosiruptor saccharolyticus	70	1000	Batch	Sucrose	NR	3.3	[38]
Thermotoga elfii	65	1000	Batch	Glucose	NR	3.3	[38]
***Thermoanaerobacterium saccharolyticum* YS485**	55	8	Batch	Cellobiose	23.4[a]	0.87	[39]
Clostridium acetobutylicum	37	850	Batch	Glucose	NR	2.0	[32]
***Escherichia coli* MC13-14**	37	20	Batch	Glucose	NR	1.2	[34]
***Clostridium butyricum* CGS5**	37	150	Batch	Xylose	163	0.73	[35]
Anaerobic sludge	37	3860	Continuous	Fructose	26[b]	0.56	[36]
Clostridium sp.	35	2300	Continuous	Glucose	53.4[b]	0.85	[33]
***Rhanella aquatilis* strain 7**	20	500	Batch	Cheese whey	66.93	NR	[10]
G088	20	110	Batch	Glucose	439.8	1.7	This study

G088	20	110	Batch	Fructose	388.1	1.3	This study
G088	20	110	Batch	Xylose	349.9	1.4	This study

NR: Not reported.
a mmoles H2
b %H2 content

As mentioned before, biohydrogen production can also be carried out at thermophilic and hyperthermophilic temperatures. The high temperatures provide some advantages, such as low viscosity, better mixing, less risk of contamination, and higher reaction rates [1]. Nevertheless, these attributes are overshadowed by the high-energy consumption required in these processes. Nowadays the most desired processes are those in which hydrogen can be produced with the minimum amount of energy input.

The hydrogen yields from cultures of thermophiles and hyperthermophiles are reported in a range between 0.87 and 4.0 mol H2/mol hexose [40] (Table 3). The minimum yield corresponds to a published report by Shaw et al. [39], in which a culture of *Thermoanaerobacterium saccharolyticum* YS485 at thermophilic conditions (55°C), reached a yield of 0.87 mol H2/ mol hexose. In addition to this, there are studies regarding hydrogen production with hyperthermophiles. For example Van Niel et al. [38] reported a hydrogen yield of 3.3 mol H_2/ mol sucrose using either *Caldicellulosiruptor saccharolyticus* on sucrose (70°C) or *Thermotoga elfii* on glucose (65°C). The highest yield was obtained by *Thermotoga maritima* at 80°C using glucose as a substrate with 4 mol H_2/ mol glucose reported by Schröder et al. [37]. Contrary to these studies our operating temperature was 20°C,

which allowed us to obtain a considerable yield at temperatures close to room temperature.

Currently, the cost of hydrogen generated from biological processes is very high, and one of the key aspects in biohydrogen production is the use of a feasible substrate. In this regard, potential substrates intended for sustainable biohydrogen production must be not only be abundant and readily available but also cheap and highly biodegradable such as agro industrial and food waste, which meet all these requirements [41]. In this respect, the psychrotolerant G088 strain showed high hydrogen production using glucose (439.83 ml), fructose (388.16 ml) and xylose (349.9 ml) as a substrate. These monosaccharides form part of a wide variety of agricultural residues, and hence, these results suggest that the biohydrogen production with G088 strain can be coupled to the use of lignocellulosic feedstock. Therefore, the utilization of wastes to generate hydrogen energy could reduce the costs of production, making hydrogen gas more available and cheaper.

1.5 Conclusions

Biohydrogen has gained attention due to its potential as a sustainable alternative to the conventional methods for hydrogen production. However, the current processes demand external energy input to maintain the optimal fermentation temperature, which represents a disadvantage for the main reason that the most desired processes are those in which the hydrogen can be produced with the minimum amount of energy demand. This study has shown the feasibility of the psychrotolerant G088 strain, isolated from Antarctica and which is closely related

to Polaromonas rhizosphaerae, to produce biohydrogen at low temperature. The yields obtained in this study are comparable to those reported for mesophilic and thermophilic microorganisms. On the other hand, glucose, xylose and fructose are the best substrates for biohydrogen production by the G088 strain. In consequence, this strain could be used for the exploitation of agro industrial waste as carbon source for hydrogen production, since it contains these three monosaccharides in large amounts. Additional investigations are necessary to find the optimal conditions to operate the biohydrogen production process using complex substrates with psychrotolerant microorganisms.

1.6 References

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Chapter 2

Optimization of hydrogen production by the psychrophilic strain G088

Abstract

In this study, the response surface method with a Box-Behnken design was applied to evaluate the effect of temperature, pH and glucose concentration to optimize hydrogen production by psychrophilic strain G088 ([EU636029], which is closely related to *Polaromonas rhizosphaerae* [EF127651]). Biohydrogen production was performed in 120 ml serological bottles with a production medium containing 2.75 g/l tryptone, 0.25 g/l yeast extract and glucose concentration in the range of 10-40 g/l, pH 3.0-8.0 and temperature 13-37ºC. According to the mathematical model, temperature 26.30ºC, pH 6.2 and glucose 25.31 g/l were the optimum conditions for a predict hydrogen production (HP) of 503.34 ml of H_2, a hydrogen production rate (HPR) and hydrogen yield (HY) of 37.91 ml/l/h and 1.93 mol H_2/ mol glucose, respectively. The linear effect of pH and quadratic effect of temperature, pH and glucose concentration were the most significant terms affecting HP. Otherwise HPR was affected only by the quadratic terms of temperature and glucose concentration, while HY was affected by the linear and quadratic effect of the three factors. The optimal production conditions were experimentally tested by triplicate corroborating the correspondence with the predicted value by the mathematical model. The experimental values for hydrogen production, yield and rate under these conditions were 513±12.48 ml, 36.5±4.10 ml/l/h and 1.81±0.04 mol H_2/ mol glucose, respectively.

Alvarez-Guzmán CL, Balderas-Hernández V, González-García Raul, Ornelas-Salas JT, Vidal-Limón AM, Cisneros-de la Cueva S, De León Rodríguez A. Optimization of hydrogen production by Psychrophilic strain G088. International Journal of Hydrogen Energy. Accepted.

2.1 Introduction

In recent years, the problem of air pollution has reached limits of considerable dangerousness both on human health and for the environment that surrounds them. This situation is a direct consequence of the continuous fossil fuel combustion by energy systems [1]. A possibility to avoid the production of non-carbon neutral green-house gasses, such as carbon dioxide, is the development and continuous investigation of alternative biofuels [2]. Hydrogen energy systems appear to be one of the most effective solutions and can play a significant role in providing better environment and sustainability [3] owing to its clean, renewable and high energy yielding (122 kJ/g) nature. When hydrogen is used as a fuel, it generates no pollutants but produces water. Furthermore, in comparison with fossil fuels, hydrogen has a higher energy yield [4].

Hydrogen can be produced by a number of different processes including electrolysis of water, thermocatalytic reformation of hydrogen rich substrates and various biological processes. To date, hydrogen is mainly produced by electrolysis of water and steam reformation of methane. These processes are very energy intensive [2]. Biohydrogen production, unlike their chemical or electrochemical counterparts, is catalyzed by microorganisms in an environment at ambient temperature and pressure [5]. Biological method mainly includes photosynthetic hydrogen production and fermentative hydrogen production. The efficiency of photosynthetic hydrogen production is low, and it cannot be operated in the absence of light, while fermentative hydrogen production can produce hydrogen all day long without light using various kinds of substrates such as organic wastes and

has a higher feasibility for industrialization. Thus fermentative hydrogen production is more feasible process and widely used [6]. Currently, the study on microbiological aspects of fermentative biohydrogen production is still in the early stage of development. Most of the current research is focusing on the mesophilic or thermophilic and most rarely at psychrophilic temperature ranges [7].

Psychrophilic microorganisms are distinguished from mesophiles by their ability to grow at low temperatures. These microorganisms isolated from cold environments have received little attention especially in comparison to thermophiles. Their advantages on both ecological and economical values may not yet have been realized extensively [8]. The application of psychrophilic microorganisms offers numerous advantages, such as, both high enzymatic activities and catalytic efficiencies in the temperature range of 0-20ºC. This low temperature range prevents the risk of microbial contamination, also allows renouncing on expensive heating/cooling systems, thus constituting a considerable progress towards the saving of energy [8]. A possible disadvantage when using obligatory psychrophilic microorganisms may arise from the requirement of cooling rather than heating. Considering this, it is important to find facultative psychrophilic bacteria able to grow at ambient temperature. On this subject, some authors [7, 9] have reported the fermentative hydrogen production at temperatures in a range of 20-25ºC using psychrophilic bacteria.

Dark fermentation is a very complex process, and can be influenced by different factors such as operation temperature, pH and substrate concentration affecting the activity of some essential enzymes, such as hydrogenases, for fermentative hydrogen production. The effects of these factors on fermentative hydrogen

production have been widely studied on mesophilic and thermophilic hydrogen bacteria [6, 10], however, to date, there are not studies addressing the effect of these factors on hydrogen production by psychrophilic bacteria. In this study, the effects of temperature, pH and glucose concentration on hydrogen production performance were investigated to identify the proper conditions for hydrogen production via response surface methodology (RSM) by the psychrophilic strain G088 isolated from Antarctica.

2.2 Materials and methods

2.2.1Strain and Culture media

In this study, the psychrophilic G088 strain [EU636050] closest relativity of this strain according to NCBI is *Polaromonas rhizosphaerae* [EF127651] [11] was used. The strain was grown routinely in YPG agar plates [0.25 g/l Bacto-tryptone (Difco), 0.25 g/l yeast extract (Difco), 0.25 g/l glucose (Sigma)] and maintained at 4ºC. Biohydrogen production experiments were done in a rich production medium containing 2.75 g/l Bacto tryptone (Difco), 0.25 g/l and glucose in a concentration range of 10-40 g/l.

2.2.2 Biohydrogen production experiments

To conduct the hydrogen production by the G088 strain, preinocula were grown in rich production medium under anaerobic conditions. Cells were harvested, centrifuged, washed and inoculated into 120 ml anaerobic serological bottles

(Prisma, DF, Mex) containing 110 ml of production medium with glucose as substrate. Production medium was supplemented with 1 ml/l of trace elements solution (0.015 g/l $FeCl_3.4H_2O$, 0.00036 g/l $Na_2.MoO_4.2H_2O$, 0.00024 g/l $NiCl_2.6H_2O$, 0.0007 g/l $CoCl_2.6H_2O$, 0.0002 g/l $CuCl_2.2H_2O$, 0.0002 g/l Na_2SeO_3, 0.01 g/l $MgSO_4$). The cultures were started with an optical density at 600 nm wavelength (OD_{600nm}) of 0.1. The initial glucose concentration, temperature and pH were fixed according to the experimental design described below (Table 1).

2.2.3 Experimental design

A three-factor Box-Behnken design was used to study the effect of temperature, pH and glucose concentration on hydrogen production. Cumulative hydrogen production (HP), hydrogen production rate (HPR) and hydrogen yield (HY) were chosen as the response variables, while temperature (X_1), initial pH (X_2) and glucose concentration (X_3) were chosen as three independent variables. A total of 15 runs were performed. Table 1 lists the actual (X_i) levels of each variable. For statistical calculations, the relation between the coded values and actual values was described by Eq. (1).

$$x_i = X_i - X_0/\Delta X_i\,,\ \ i = 1,2,3,\dots,k \qquad (1)$$

Where x_i is the coded value of the variable X_i, X_i= the actual value of the i^{th} independent variable, X_0= the actual value of X_i at the center point and ΔX_i= step change value. The complete quadratic model represented in Eq. (2) was used to

build surfaces graphs for the model for each response variable and to predict the optimal conditions.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 \quad (2)$$

Where Y is the predicted response; X_1, X_2 and X_3 are the temperature, initial pH and glucose concentration, respectively. β_0 is the intercept term, β_1, β_2 and β_3 are the linear coefficients, β_{12}, β_{13} and β_{23} are the interactive coefficients, and β_{11}, β_{22}, β_{33} are the quadratic coefficients. The optimal levels of the selected variables were obtained by solving the regression equation and by analyzing the surface plot. Analysis of variance (ANOVA) was used to determine the quality of the fit of quadratic model expressed by the coefficient of determination R^2, and its statistical significance was checked by the *F*-test.

2.2.4 Analytical methods

The amount of hydrogen produced was measured by water displacement method with NaOH 1N in an inverted burette connected to serological bottles with rubber tubing and a needle and validated by gas chromatography and using a thermal conductivity detector (Agilent Technologies Wilmington, DE, USA) as described elsewhere [12]. Samples of 1 ml where taken at different times during fermentation, then were diluted and filtered through a 0.22 µm membrane (Millipore, Bedford, Massachusetts, USA) [9]. Concentrations of glucose and metabolites such as

succinic acid, lactic acid, acetic acid and 2,3-butanediol were analyzed by High Performance Liquid Chromatography (HPLC, Infinity LC 1220, Agilent Technologies, Santa Clara CA, USA) using a Refraction Index Detector, a column Phenomenex Rezex ROA (Phenomenex Torrance, CA, USA) at 60ºC, and 0.0025 M H_2SO_4 as mobile phase at 0.41 ml/min. Ethanol, butyric acid and propionic acid were analyzed in a Gas Chromatograph (GC, 6890N Network GC System, Agilent Technologies Wilmington, DE, USA) using a flame ionization detector (Agilent Technologies Wilmington). The column used was a capillary column HP-Innowax with the following dimensions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness (Agilent, Wilmington, DE, USA). Temperatures of the injector and flame ionization detector (FID) were 220 and 250ºC respectively. Helium was used as carrier gas at a flow rate of 25 ml/min. The analyses were performed with a split ratio of 5:1 and a temperature program of 25ºC for 10 min to 280ºC,and was maintained at this temperature for a final time of 10 min [9].

2.3 Results and discussion

Among the biological hydrogen production methods, fermentative hydrogen production is considered the most favorable due to some outstanding advantages such as high hydrogen production rate, low energy requirement, relative easy operation, and high sustainability [13]. However, fermentative hydrogen production is influenced by different factors, such as temperature, initial pH and substrate concentration. The relative importance of these three variables for hydrogen production using a psychrophilic strain was investigated.

The optimum levels of the independent variables and the effect of their interactions were determined by the Box-Behnken design and response surface methodology (RSM).

2.3.1 Effect of temperature, pH and glucose concentration on cumulative hydrogen production (HP)

To find the optimum hydrogen production conditions by the psychrophilic strain G088, 15 experiments were carried out according to the experimental design described in Table 2.1. The cumulative hydrogen production ranged from 12 to 520 ml of hydrogen (H_2) (Table 2.1). Under low initial pH, hydrogen production was negatively affected, and the lowest hydrogen volume (12 ml) was achieved using a temperature of 13ºC, pH 5.5 and a glucose concentration of 10 g/l (Table 2.1, exp. 14).

Table 2.1. Experimental design for optimizing fermentative hydrogen production by the psychrophilic strain G088 and the corresponding experimental results.

Run	Factor X_1 (ºC)	Factor X_2 (-)	Factor X_3 (g/l)	HP (ml)	HPR (ml/l/h)	HY (mol H_2/ mol glucose)
1	13	3.0	25	13	1.23	0.2
2	13	5.5	40	24	1.59	0.96
3	13	8.0	25	36	2.72	1.16
4	37	5.5	40	27	5.11	0.46
5	25	5.5	25	489	34.16	1.75
6	37	3.0	25	44	15.15	0.75
7	37	8.0	25	57	18.93	1.93
8	25	3.0	40	29	2.39	0.29
9	25	5.5	25	516	38.82	1.88

10	25	5.5	25	520	35.98	1.77
11	25	3.0	10	42	1.51	0.54
12	25	8.0	10	146	14.48	1.79
13	25	8.0	40	254.43	35.03	1.11
14	13	5.5	10	12	0.9	0.91
15	37	5.5	10	28	9.46	1.62

***X_1*= Temperature, *X_2*= *pH, X_3*= Glucose concentration, HP= Hydrogen production, HPR= hydrogen production rate, HY= Hydrogen yield**

On the other hand, maximum hydrogen production of 520 ml H_2 was achieved using the same temperature of 25ºC, pH of 5.5 and a sugar concentration of 25 g/l (Table 2.1, exp. 10). Table 2.2 shows the analysis of variance (ANOVA); factors evidencing *P*-values of less than 0.05 were considered to have significant effects on the response variable. The linear effect of pH (X_2) and the quadratic effect of temperature (X_1^2), pH (X_2^2) and glucose concentration (X_3^2) were highly significant, indicating that these factors had a great influence on hydrogen production. The second order Eq. (3) was obtained from Eq. (2) in terms of coded factors to fit the experimental data of hydrogen production.

$$HP = 508.33 + 8.87X_1 + 45.68X_2 + 13.30X_3 - 2.50X_1X_2 - 3.25X_1X_3 + 30.36X_2X_3 - 282.97{X_1}^2 - 187.86{X_2}^2 - 202.61{X_3}^2 \quad (3)$$

Where HP is the cumulative hydrogen production response (ml H_2) and X_1, X_2 and X_3 are the coded values of temperature (ºC), pH (-) and glucose concentration (g/l) respectively. The *p* value (0.0014) of the model was lower than 0.05 (Table 2.2), indicating that the model fitness was significant. The high value of regression

coefficient (R^2) suggests that the regression model was an accurate representation of the experimental data, which can explain 97.71% variability of the dependent variable. Also, the model has an adequate precision value of 13.0, this suggest that the model can be used to navigate the design space. The precision value is an index of the signal to noise ratio and a value >4 is an essential prerequisite for a model to have a good fit [10].

Table 2. 2 ANOVA of the fitting model for hydrogen production (HP).

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	$5.272x10^5$	9	58579.73	23.70	0.0014
X_1	630.12	1	630.12	0.25	0.6351
X_2	16692.39	1	16692.39	6.75	0.0483
X_3	1415.92	1	1415.92	0.57	0.4832
X_1X_2	25	1	25	0.010	0.9238
X_1X_3	42.25	1	42.25	0.017	0.9011
X_2X_3	3686.31	1	3686.31	1.49	0.2764
X_1^2	$2.957x10^5$	1	$2.957x10^5$	119.61	0.0001
X_2^2	$1.303x10^5$	1	$1.303x10^5$	52.72	0.0008
X_3^2	$1.516x10^5$	1	$1.516x10^5$	61.32	0.0005
Residual (error)	12358.49	5	2471.70		
Pure error	568.67	2	284.33		
Total	$5.396x10^5$	14			

***R^2*=0.9771; CV=33.33%, SS, sum of squares, DF, degrees of freedom, MS, mean square; Adj *R^2*=0.9359**

A hydrogen production of 511.53 ml H_2 was estimated from Eq. (3) at temperature of 25.17ºC, initial pH of 5.81 and glucose concentration of 25.62 g/l. The three dimensional (3D) response surface plot, which is the graphical representation of

the regression equation Eq. (3) is showed in figure 2.1A with the value of initial glucose concentration being kept constant at its optimum level and varying the other two variables within the experimental range. As shown in figure 2.1A, in the design boundary, the response surface plot had a clear peak and the corresponding contour plot had a clear highest point, which indicates that the maximum response could be attained inside the design boundary. This figure shows a marked linear increase in hydrogen production as the temperature increases until 25ºC, and then decreased with a further increase to 37ºC. Temperature is one of the most important factors that influence the activities of hydrogen-producing bacteria, affecting the kinetics of cell growth and enzymatic reactions. This factor has been widely studied on biohydrogen production, and most of the optimal temperatures fall into the mesophilic or thermophilic range, whose favorable ranges are 30-40 and 45-55ºC, respectively. For instance Chiu-Yue et al. [14] assessed the hydrogen production using sewage sludge microflora at temperatures of 30-55ºC and reported the highest hydrogen content occurring at 50ºC. The same effect was observed by Hanqing et al. [15] who examined the hydrogen production by mixed bacterial flora in a temperature range of 20-55ºC and determined that the optimum hydrogen production was achieved at 55ºC. Wang et al. [16] studied the effect of temperatures in the same range as Hanqing et al. (20 to 55ºC) using mixed cultures, the results showed that the hydrogen production increased with increasing temperatures from 20 to 40ºC reaching a maximum hydrogen volume of 269.9 ml at 40ºC. In this study, we observed a similar effect; when temperature increased from 13 to 25ºC, a maximum hydrogen

volume of 520 ml was attained, while increasing temperature to 37ºC resulted in a reduced hydrogen production (Fig. 2.1).

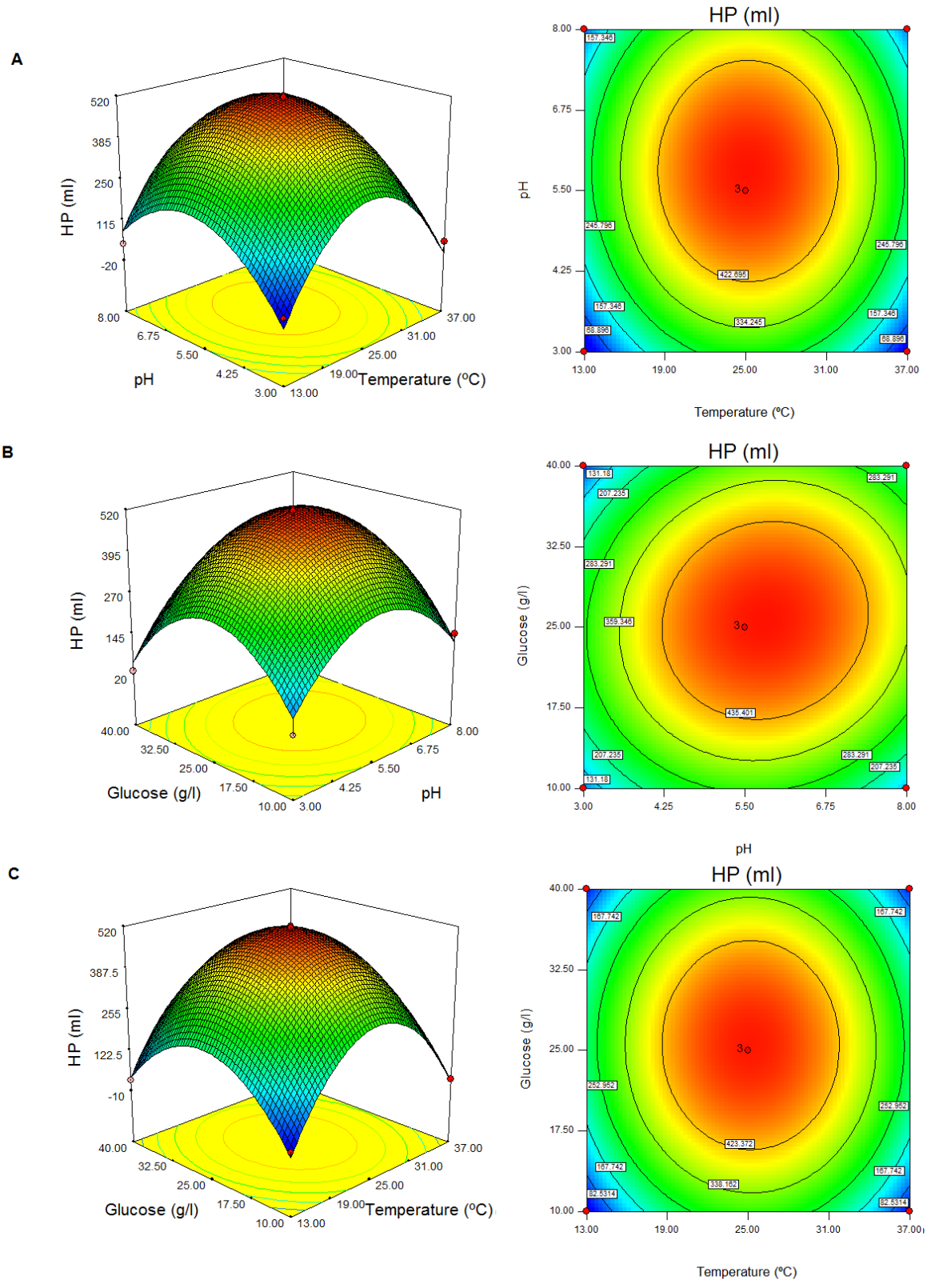

Figure 2.1 Response surface plot and contour plot for hydrogen production rate (HP).

These results may seem incongruous since it has been established that psychrophilic bacteria are able to grow well at low temperatures. However, despite being described as “cold-loving” most of them grow above 20ºC. Hence, they are actually cold-tolerant rather than “cold-loving”. In this respect, there are reports of psychrophiles with a maximum growth temperature greater than 30ºC. Witter [17] gave an extensive survey on this subject, showing that most of the psychrophilic bacteria have a wide range of growth temperatures, in some cases ranging from -5 to 45ºC. In this study, it was determined that the highest hydrogen volume is achieved at 25ºC while a marked decrease in cumulative hydrogen was observed at 37ºC. This could be attributed to the negative effect of high temperatures on the expression of the hydrogenases, which are the most important enzymes in molecular hydrogen production during dark fermentation. This effect was measured by Quist and Stokes [18] who studied the effect of temperature on the induced synthesis of hydrogenase in psychrophilic bacteria, and determined that cell suspensions of psychrophilic strain 82 cannot be induced at the moderate temperature of 25ºC. However, this strain grows optimally at 30ºC and has a maximum growth temperature of 35ºC. The authors assume that at 25ºC and higher temperatures, general protein synthesis is not affected and the microorganism retains most of its biochemical characteristics. Nevertheless, at this temperature range the bacteria lose its ability of gas producer during sugar fermentation. In G088 fermentations, the decrease in hydrogen production at 37ºC could be attributed to the thermolability of its hydrogenases at temperatures above of 25ºC.

In respect to initial pH, the results showed that this factor had a noticeable impact on hydrogen production. As it can be seen in fig. 2.1B, hydrogen production was favoured as initial pH increasing from 3 to 5.5, and then decreased with further increase to 8.0. This was expected, since it has been reported that both pH extremes; low and high, tend to have inhibitory effects on the bacteria. At low initial pH values longer lag periods are observed while, high initial pH values such 8.0 decrease the lag periods, but have a lower yield of hydrogen production [19]. Regarding to initial substrate concentration, the increasing glucose concentration caused an increment on cumulative hydrogen. It is logical to assume that hydrogen production increases with substrate concentration. Glucose is the fundamental resource for hydrogen production; this monosaccharide is fermented via the Embden-Meyerhof-Parnas (EMP) pathway to pyruvate. Pyruvate oxidation to acetyl coenzyme A requires ferredoxin (Fd) reduction. Reduced Fd is oxidized by hydrogenase, which generates Fd and releases electrons as molecular hydrogen [20]. When the cell is exposed to a large amount of glucose, a greater amount of electrons is generated. Therefore a greater hydrogen production is achieved in order to maintain the redox balance of the cell. On the other hand, it has been shown that an excessive substrate concentration inhibits hydrogen production. Wang and Wan [6], Skonieczny and Yargeau [21] and Chen et al. [22] saw that the total hydrogen production increased as the substrate concentration increased, and then decreased when the substrate concentration was elevated further. This can be attributed to the pH drop caused by the high acid production, substrate inhibition or both. This effect was observed during hydrogen production by G088 as can be seen in figure 2.1C when substrate concentration increases, hydrogen

production increases until a maximum point. However, an increment to 40 g/l led to a noticeable decrease in hydrogen production.

2.3.2 Effect of temperature, pH and glucose concentration on hydrogen production rate (HPR)

A highest HPR of 38.82 ml/l/h (Table 2.1) was attained at 25ºC, pH of 5.5 and glucose concentration of 25 g/l (Table 2.1, run. 9). Whereas, the lowest HPR of 0.9 ml/l/h occurred under conditions of 13ºC, initial pH of 5.5 and glucose concentration of 10 g/l (Table 2.1, run 14). The analysis of variance ANOVA (Table 2.3), showed that the parameters which affect HPR, are the quadratic effect of temperature (X_1^2) and glucose concentration (X_3^2) (Table 2.3). The quadratic model in terms of coded factors (Eq. 4) was obtained from Eq. (2) to fit the experimental data of hydrogen production rate.

$$HPR = 36.32 + 5.87X_1 + 6.37X_2 + 2.80X_3 + 0.57X_1X_2 - 0.077X_1X_3 + 4.89X_2X_3 - 17.37{X_1}^2 - 9.44{X_2}^2 - 13.50\,{X_3}^2 \quad (4)$$

Where HPR is the hydrogen production rate (ml/l/h) and X_1, X_2, and X_3 are the coded values of temperature (ºC), initial pH (-) and glucose concentration (g/l). The *p* value (0.0449) of the model was lower than 0.05 (Table 2.3) indicating that the model fitness was significant. The high value of regression coefficient (R^2= 0.9007) suggests that the regression model was an accurate representation of the experimental data, which can explain 90.07% variability of the dependent variable.

Moreover, the model has an adequate precision value of 6.383 that suggests that the model can be used to navigate the design space.

Maximum HPR of 38.31 ml/l/h was estimated from Eq. (4) at temperature of 27.1ºC, initial pH of 6.47 and glucose concentration of 27.6 g/l. 3D response surface plot (Fig. 2.2) based on Eq. (4) shows the effects of temperature and pH on hydrogen production rate at substrate concentration being constant at its optimal value.

Table 2. 3 ANOVA of the fitting model for hydrogen production rate (HPR).

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	2619.43	9	291.05	5.04	0.0449
X_1	275.42	1	275.42	4.77	0.0808
X_2	324.87	1	324.87	5.62	0.0639
X_3	62.72	1	62.72	1.09	0.3452
X_1X_2	1.31	1	1.31	0.023	0.8862
X_1X_3	0.024	1	0.024	4.517×10^{-4}	0.9845
X_2X_3	95.75	1	95.75	1.66	0.2544
X_1^2	1114.19	1	1114.19	19.28	0.0071
X_2^2	329.12	1	329.12	5.70	0.0627
X_3^2	673.05	1	673.05	11.65	0.0190
Residual (error)	288.94	5	57.79		
Pure error	11.03	2	5.52		
Total	2908.37	14			

R^2=0.9007; CV=51.30%, SS, sum of squares, DF, degrees of freedom, MS, mean square; Adj R^2=0.7218

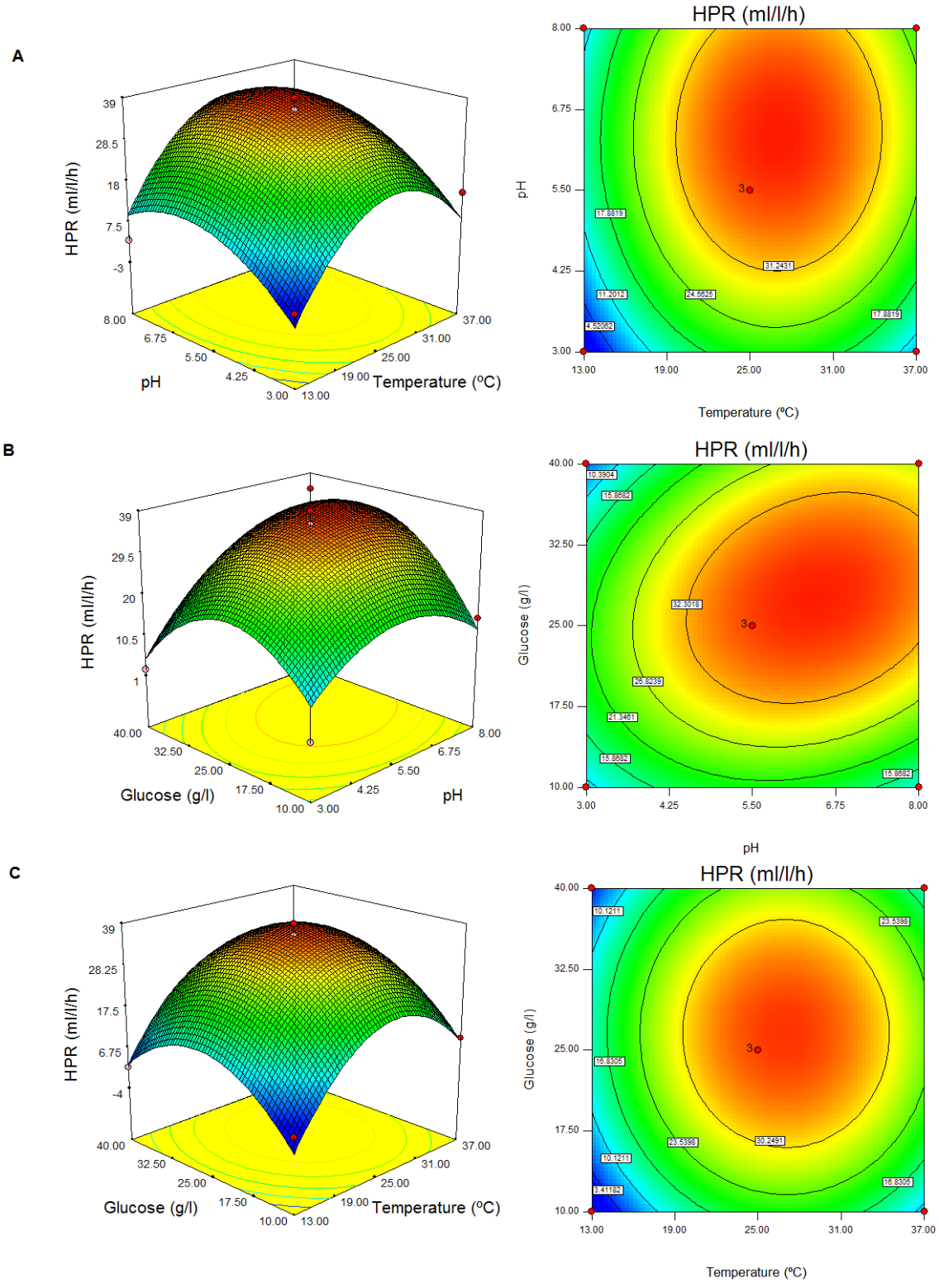

Figure 2. 2 Response surface plot and contour plot for hydrogen production rate (HPR).

The response surface of HPR had a net peak, which means that the HPR could be achieved inside the design boundary. In figure 2.2A HPR increases as the temperature increases from 13 to 25ºC and then drops when temperature increases to 37ºC, which indicates that G088 strain has an optimum temperature at 25ºC. In this respect, psychrophilic bacteria are generally considered to be inactive under psychrotolerant conditions [7]. This assumption is related to the definition of psychrophile; which describes that the optimum growth temperature for these microorganisms is at low temperatures. However, various authors have emphasized the problem that has existed over the appropriate definition of a psychrophile; this is because various microbial species that can grow at 0ºC can also grow at temperatures well into the upper range at which mesophiles grow. This group of bacteria considered psychrotolerant usually has higher growth rates at temperatures above 20ºC. In this study, as mentioned before, the lowest HPR was registered at low temperature (13ºC), while the highest rate was attained at 25ºC. At present day, there are few reports regarding to hydrogen production using psychrophilic bacteria [7, 9, 23] and to our knowledge this is the first study that evaluate different operational conditions for hydrogen production using a psychrophilic microorganism. Upadhyay and Stokes [24] described earlier some properties of anaerobic psychrophilic bacteria, including some fermentation characteristics. They reported that 28 of their 32 strains were able to ferment one or more sugar (glucose, sucrose and lactose). The fermentations were much slower at 0ºC than at 20ºC. Their final results were available in 2 days at 20ºC, whereas at least 7 days and as long as 29 days were required at 0ºC for visible fermentation. A similar effect was observed using G088 strain, indicating that

fermentation by psychrophilic bacteria is favoured by the increase in temperature. However, further increase in temperature to 37ºC resulted in a decrease in the HPR. This is consistent with the fact that the active site and adjoining regions of psychrophilic enzymes remains flexible at low temperatures, however, the increased conformational flexibility is accompanied by increased thermolability at higher temperatures. Our data suggest that growth at 37ºC could decrease hydrogen production by psychrophilic G088, since a sub-population of hydrogenase enzyme could have unpaired active site conformation. As shown by Friedrich and Friedrich [25], the *HoxTS* hydrogenase from psychrotolerant *Alcaligens eutrophus* remains stable at least for 5h at 37ºC, when shifted from 30ºC. Moreover, the expression of recombinant hydrogenase from *Clostridium acetobutylicum* is increased at lower temperatures of 20ºC, while its specific activity is inhibited at 30ºC [26], suggesting that an increasing temperature can exert detrimental effects over hydrogenase expression and catalysis. In addition, this temperature effect on hydrogen production rate is also observed in mesophilic and thermophilic bacteria. As an example, Mu et al. [27] reported the hydrogen production at temperatures at mesophilic range (33-41ºC) using mixed anaerobic cultures. The results showed that an increase in temperature from 33 to 39ºC resulted in an increase in hydrogen production rate from 2112 ± 23 to 3922 ± 45 ml/g-VSS/d. However, at a higher temperature of 41ºC, the hydrogen production rate was slightly decreased.

Initial glucose concentration also plays an important role on the production rate. Low initial glucose concentration results in the low rate of the fermentation steps, and fermentation time increases as starting substrate concentration increases [28].

In this study, the highest HPR was achieved at moderate high initial glucose concentration of 25 g/l. As it can be seen in figure 2.2B, HPR increases with an increment in glucose concentration; however, an increase prompted a decrease in the HPR. This is consistent with previous studies that reported the same effect. For instance, Wang and Wan [6] evaluated the effect of temperature, initial pH and glucose concentration on hydrogen production by mixed cultures and reported that hydrogen production rate increased with increasing temperatures, initial pH and glucose concentrations to the optimal levels, and then decreased with further increase. Moreover, substrate concentration effect on hydrogen production rate is related directly to pH since an increment in substrate concentration leads to a rise on soluble metabolite production such as volatile fatty acids which tends to accumulate and inhibit hydrogen production. Low pH gives poor hydrogen production rates across the entire substrate concentration span. This is somewhat expected, as low pH tends to have an initial inhibitory effect on the bacteria causing a longer lag phase and lower rate of production [21].

2.3.3 Effect of temperature, pH and glucose concentration on hydrogen yield (HY)

The highest hydrogen yield of 1.93 mol H_2/ mol glucose was achieved under mesophilic conditions of 37ºC, initial pH of 8.0 and 25 g/l of glucose (Table 2.1, run 7). The analysis of variance ANOVA (Table 2.4) showed that the linear effect of temperature (X_1), pH (X_2), glucose concentration (X_3), the interaction between temperature and glucose concentration (X_1X_3), and the quadratic effect of

temperature (X_1^2), pH (X_2^2) and glucose concentration (X_3^2) are significant factors in hydrogen production yield. The second order Eq. (5) was obtained from Eq. (2) in terms of coded factors to fit the experimental data of HY.

$$HY = 1.80 + 0.19X_1 + 0.53X_2 - 0.26X_3 + 0.055\,X_1X_2 - 0.30\,X_1X_3 - 0.11\,X_2X_3 - 0.37\,{X_1}^2 - 0.42{X_2}^2 - 0.45\,{X_3}^2 \quad (5)$$

Where HY is the hydrogen production yield response (mol H_2/ mol glucose) and X_1, X_2 and X_3 are the actual values of temperature (ºC), initial pH and glucose concentration (g/l) respectively. The *p* value (0.0032) of the model was lower than 0.05 (Table 2.4), indicating that the model fitness was significant. The high value of regression coefficient (R^2) suggests that the model was an accurate representation of the experimental data, which can explain 96.80% variability of the dependent variable.

Table 2. 4 ANOVA of the fitting model for hydrogen yield (HY).

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	5.09	9	0.57	16.79	0.0032
X_1	0.29	1	0.29	8.68	0.0320
X_2	2.22	1	2.22	65.75	0.0005
X_3	0.52	1	0.52	15.44	0.0111
X_1X_2	0.012	1	0.012	0.36	0.5751
X_1X_3	0.37	1	0.37	10.86	0.0216
X_2X_3	0.046	1	0.046	1.37	0.2943
X_1^2	0.50	1	0.50	14.80	0.0120
X_2^2	0.66	1	0.66	19.56	0.0069

X_3^2	0.73	1	0.73	21.70	0.0055
Residual (error)	0.17	5	0.34		
Pure error	$9.8x10^{-3}$	2	$0.4.9x10^{-3}$		
Total	5.26	14			

***R^2*=0.9680; CV=16.08%, SS, sum of squares, DF, degrees of freedom, MS, mean square; Adj *R^2*=0.9103**

In addition, the model has an adequate precision value of 10.42, this suggest that the model can be used to navigate the design space. A HY of 1.96 mol H_2/ mol glucose was estimated from Eq. (5) at temperature of 27.1ºC, initial pH of 6.0 and glucose concentration of 22.0 g/l. The interactive effect of temperature and glucose concentration on hydrogen production yield is shown in figure 2.3C with pH value fixed at its optimum value. The figure shows that the response surface plot had a clear peak, which means that the hydrogen yield could be achieved inside the design boundary. It can be seen that the influence of temperature produces a linear effect on hydrogen yield, which increases as temperature rises until an optimal point at 25ºC and when temperature increases to 37ºC, HY decreases.

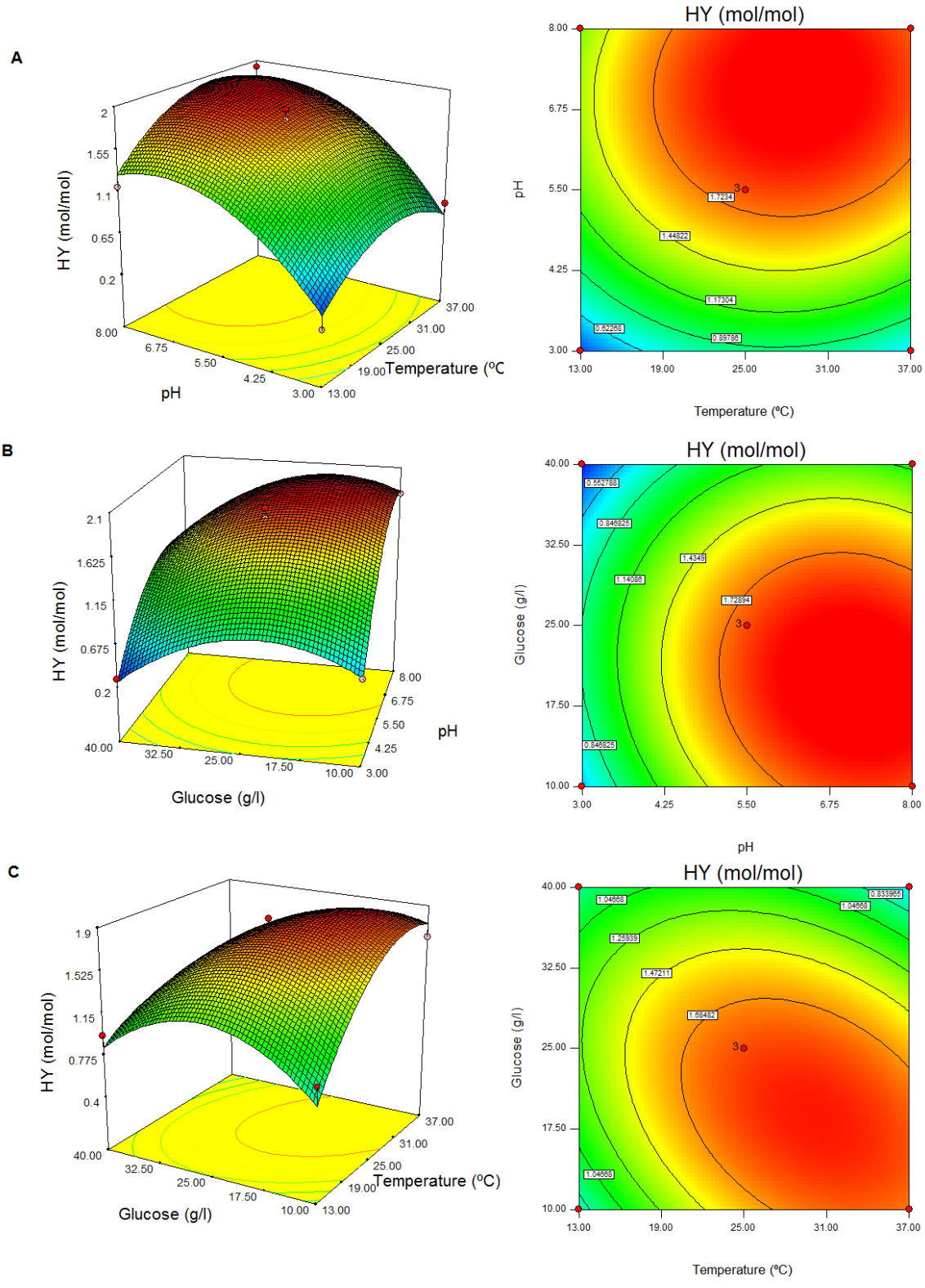

Figure 2.3 Response surface plot and contour plot for hydrogen production rate (HY).

On the other hand, the data shows that a decrease in glucose concentration lead to an increase of hydrogen yield. In this study, hydrogen yield was affected negatively when initial glucose concentration increased. Xing et al. [29] observed the same effect and reported that when initial concentration of glucose was increased gradually (from 12 to 20 g/l), hydrogen yield and hydrogen production rate decreased. Wang and Wan [30] saw that hydrogen yield in batch test increased as the glucose concentration increased from 1 to 2 g/l. However, they observed a decrease on hydrogen yield when glucose concentration increased from 2 to 300 g/l glucose. These results suggest that while the lower substrate concentration is, more complete the metabolism is. In addition, it has been established, that under substrate-limited conditions, regulation of the enzymes systems may allow cells to form products with high energy efficiency. On the other hand, when substrate is sufficient, cells may prefer pathways leading to less toxic products in order to maximize cell growth. This may lead to a lower energetic efficiency of substrate conversion [30].

2.3.4 Fermentative metabolites

Dark fermentation of organic compounds such as carbohydrates generates biomass, hydrogen, CO_2, and soluble metabolites like volatile fatty acids and solvents. The metabolites produced in the cultures performed here are shown in the Figure 2.4. As noted, glucose was readily converted into the aqueous products of succinic, lactic, propionic, acetic and butyric acids, as well as solvents like ethanol and 2,3-butanediol. The main metabolite at 25ºC was the ethanol (30.7-

58.3%), whereas at low temperature (13ºC), the most abundant metabolite was 2,3-butanediol (13.7-64.9%). Lactic acid was other frequent metabolite; it was frequent in almost all cases in a range of up to 39% of total metabolites. On the other hand, at mesophilic temperature (37ºC), propionic acid was present in a higher proportion of 12.1% at pH of 8 and 14.2% at pH of 3. From hydrogen perspective volatile fatty acids, acetate and butyrate are the desirable soluble products since hydrogen generation occurs via those reactions. Meanwhile, the presence of other reduced compounds implies that part of the hydrogen generated was not released as gas. High ethanol and 2,3-butanediol percentages could be attributed to the accumulation of the undissociated organic acids, since these are able to penetrate the cell membrane whereby they then dissociate. As a result, the cell shifts its chemical reactions to solventogenesis in order to produce neutral solvents to prevent any further pH drop [31].

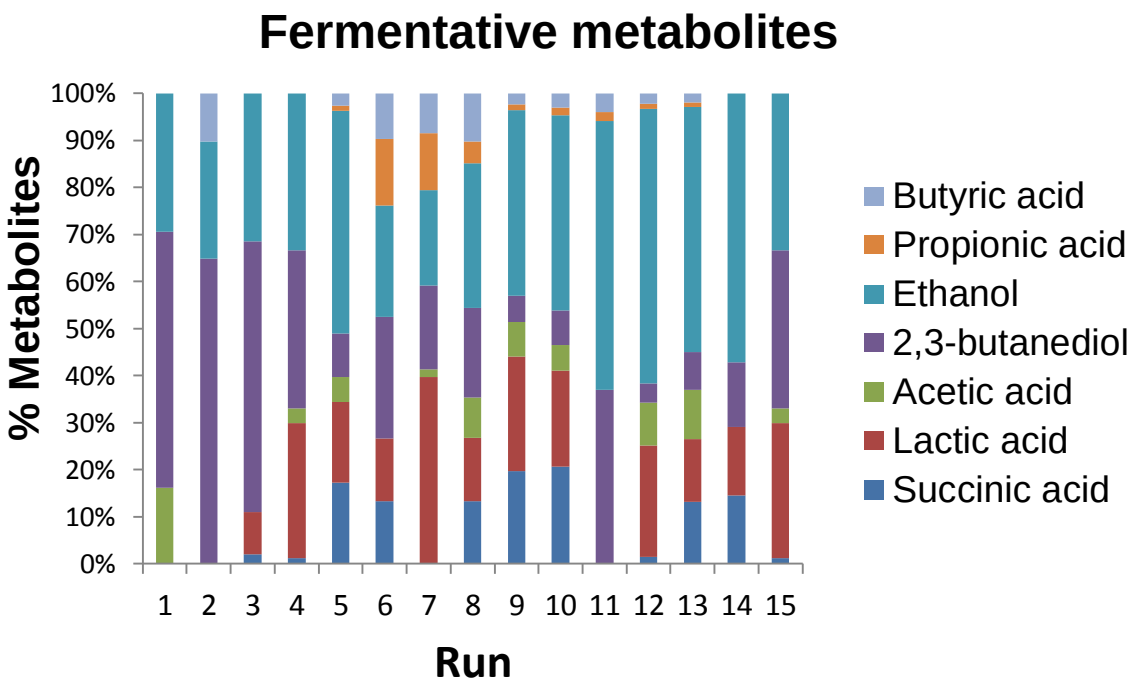

Figure 2.4 Fermentative metabolites measured at the end of fermentation.

2.3.5 Validation of optimum conditions

The optimal conditions of temperature, pH and glucose concentration from the statistical model to obtain simultaneously the highest three response variables were 26.30ºC, initial pH of 6.24 and 25.31 g/l of glucose. To confirm the validity of the statistical model, three replicates of batch experiments were performed under these optimal conditions. The predicted responses were a hydrogen production volume of 503.37 ml, a hydrogen production rate of 37.91 ml/l/h and a hydrogen yield of 1.93 mol H_2/mol glucose. The experimental values of HP, HPR and HY obtained were 513±12.48 ml of H_2, 36.5±4.10 ml/l/h and 1.81±0.03 mol H_2/ mol glucose, respectively. These results were close to those estimated by the response surface methodology corroborating the validity of the model. The hydrogen

production parameters obtained by psychrophilic strain G088 under temperature conditions of 20ºC, pH 6.8 and 20 g/l of glucose in a previous study [23] were 439.8 ml H_2 and 1.7 mol H_2/mol glucose. In, this study, we improved the cumulative hydrogen production, and yield, even when the optimum conditions were close to those of the previous study. Nevertheless, the application of psychrophilic bacteria on hydrogen production is relatively new compared to mesophiles and thermophiles which are microorganisms widely studied on this subject. Thus, more investigation is needed in order to understand this group of microorganisms with high potential application on fermentative hydrogen production.

2.4 Conclusions

Effect of temperatures, initial pH and glucose concentrations on fermentative hydrogen production using the psychrophilic strain G088 was investigated through response surface methodology with a Box-Behnken design in order to optimize the hydrogen production by psychrophilic strain G088. Maximum values of HP, HPR and HY were obtained under conditions of 26.30ºC, initial pH of 6.24 and glucose concentration of 25.31 g/l. The quadratic effect of temperature, pH and glucose concentration had impact on fermentative hydrogen production. To date, in the literature there are not studies addressing the optimum conditions for hydrogen production using psychrophilic bacteria. In our knowledge, this is the first study showing a tight study on the statistical effect of operational conditions on hydrogen production using psychrophilic microorganisms.

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Supplementary material 1

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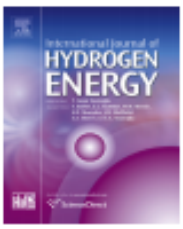

Biohydrogen production by the psychrophilic G088 strain using single carbohydrates as substrate

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ABSTRACT

The production of biohydrogen by psychrophilic G088 strain ([EU636029]), closely related to *Polaromonas rhizosphaerae* ([EF127651]) was evaluated using xylose, glucose, fructose, galactose, lactose or sucrose as a carbon source. Biohydrogen production was performed in 120 ml serological bottles with a production medium containing 2.75 g/l tryptone, 0.25 g/l yeast extract, and 20 g/l of each carbohydrate. Results showed that the G088 strain produced biohydrogen using all the evaluated substrates, ranging from 91.7 to 439.8 ml for lactose and glucose, respectively. However, glucose was the substrate with the highest consumption rate, accompanied by the maximum values of biohydrogen production rate and a biohydrogen yield of 50.1 ml/l/h and 1.7 mol H_2/mol glucose, respectively. Analysis of the secreted metabolites showed that ethanol and organic acids were the main by-products. Our results demonstrate that the G088 strain has potential to be used for developing new biotechnological processes for biohydrogen production.

Este artículo puede ser consultado en la siguiente liga:

http://www.sciencedirect.com/science/article/pii/S0360319916308709

Supplementary material 2

Bacterias
amantes del frío para producir biocombustibles

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Sin duda alguna, la demanda global de energía y la preocupación por el cuidado ambiental son los temas más citados en el siglo XXI. El petróleo y el gas natural junto con el carbón, combustibles fósiles, no sólo son nuestras principales fuentes de energía, también son las materias primas para una gran variedad de materiales y productos hechos por el hombre, desde la gasolina y el diésel hasta químicos y petroquímicos. Lo que la naturaleza nos dio como un regalo, formado a través de millones de años, a un ritmo alarmante. Las reservas de combustibles fósiles se han reducido de manera significativa y se volverán aún más costosos de lo que imaginamos. Por lo tanto, necesitamos buscar nuevas soluciones para el abasto energético y otras opciones de fuentes de energía (Olah, 2005).

El hidrógeno es el elemento más abundante en el universo, sin embargo, en la Tierra raramente se encuentra en estado libre. A diferencia del carbón, el petróleo o el gas natural, el hidrógeno no puede obtenerse de la naturaleza por trabajos de minería o de extracción, como en el caso de los recursos fósiles, sino que se trata de un 'transportador de energía'

o, mejor dicho, un vector energético. Por tal razón un gran número de investigadores se dedican a encontrar y desarrollar procesos sustentables para la obtención de este gas.

Dicho elemento es ampliamente reconocido como el recurso energético limpio y eficiente del futuro. Tiene mayor contenido de energía por unidad de peso que cualquier otro combustible conocido y es el único que no está químicamente unido al carbono; además, su combustión sólo libera agua como desecho final. Por lo tanto, la combustión de hidrógeno no contribuye al efecto invernadero, agotamiento del ozono ni produce lluvia ácida. Existen diversos métodos para la obtención del gas. Dentro de este contexto, la producción biológica de hidrógeno aparece como una alternativa prometedora a los métodos actuales de obtención de energía y es un tema de investigación en diversos países.

Microorganismos al rescate del planeta Tierra

En la actualidad la producción biológica de hidrógeno incluye principalmente los métodos de biofotólisis y fermentación oscura en los cuales se aprovechan las vías metabólicas de algas y bacterias para la generación del biocombustible. Las algas y cianobacterias son las encargadas de realizar la biofotólisis, son capaces de descomponer el agua en oxígeno e hidrógeno dirigiendo una parte de la energía solar a este proceso. Asimismo, las bacterias anaerobias, tal como su nombre lo indica, en ambientes libres de oxígeno utilizan sustratos ricos en carbohidratos para generar energía, una parte de esta es disipada en forma de hidrógeno con el fin de mantener el equilibrio electroquímico en la célula (Das y Vezirolu, 2001).

La producción biológica de hidrógeno o 'biohidrógeno' es un método atractivo debido a que es llevado a cabo en condiciones de presión y temperatura ambiente y no es energéticamente intensivo, a diferencia de los procesos de obtención de combustibles fósiles. Una ventaja del hidrógeno fermentativo es que utiliza un amplio rango de fuentes de carbono como materia prima, no necesita luz, por lo general se obtienen altos rendimientos y se puede acoplar al uso de residuos orgánicos industriales.

La producción de hidrógeno fermentativo es llevada a cabo por bacterias anaerobias estrictas, es decir, que no pueden desarrollarse en presencia de oxígeno, o por bacterias anaerobias facultativas, las cuales pueden vivir tanto en presencia como en ausencia de oxígeno. La mayoría de los estudios reportados tienen como protagonistas bacterias del género *Clostridium*, aunque también se han utilizado *Enterobacter sp.* y *Escherichia coli*, las cuales son aisladas principalmente de los residuos generados por la actividad humana.

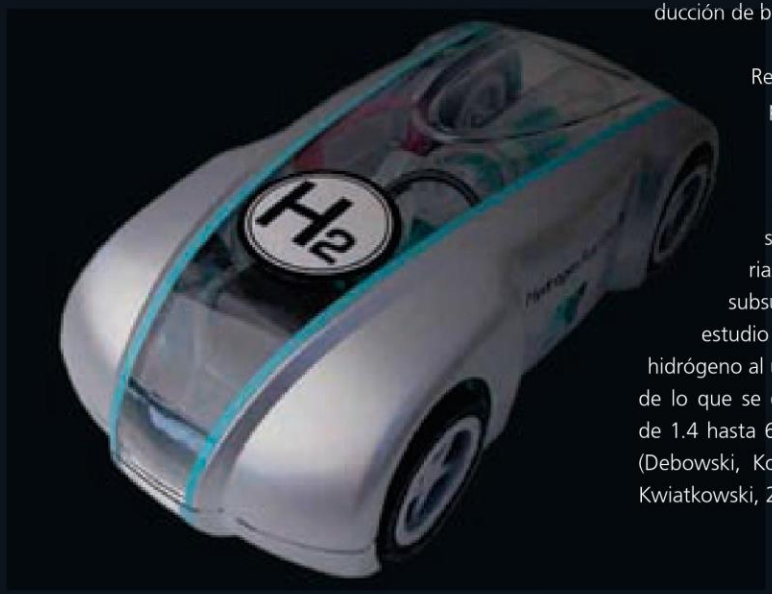
También se han realizado trabajos para optimizar los procesos de obtención de hidrógeno para mejorar las cepas productoras de hidrógeno a nivel genético, un buen ejemplo es la cepa sobreproductora de hidrógeno *E. coli* WDHL, a la cual le fue removido el gen *hycA* —responsable de reprimir la formación de formiato—, molécula procedente de la degradación de glucosa a partir de la cual *E. coli* produce hidrógeno. Además, esta cepa también carece del gen *lacI*, lo que le da la capacidad de consumir la lactosa presente en suero de leche, subproducto de producción de queso que representa un contaminante (Rosales, Razo, Ordoñez, Alatriste y De León, 2010).

Se han buscado microorganismos productores de hidrógeno en una amplia variedad de entornos, hoy en día la mayoría de los estudios reportan el uso de bacterias mesófilas (capaces de crecer a temperaturas de 25 a 40 °C) y termófilas (capaces de crecer a temperaturas de 40 a 70 °C). Sin embargo, se ha subestimado a las bacterias que crecen a temperaturas menores a éstas, lo cual se puede atribuir a que las bacterias fermentativas son, de manera ge-

neral consideradas inactivas bajo condiciones frías. Un par de grupos de investigación han confirmado que la producción de hidrógeno también se puede llevar a cabo a bajas temperaturas.

¡Bacterias al extremo!

El humano es un animal mesófilo, por lo general nos gusta habitar las regiones cálidas y somos incapaces de soportar grandes variaciones de temperatura, excepto con algunas medidas especiales que no involucran adaptaciones fisiológicas o bioquímicas. Por lo tanto, consideramos los ambientes fríos como "extremos", de manera particular aquellos en los cuales la temperatura no supera los 0 °C. Aunque creemos haber colonizado todos los rincones de nuestro planeta, la mayor parte de éste se encuentra frío y deshabitado por los humanos, la razón de esto es que más de 70 por ciento de la Tierra está cubierta por agua de mar, cuya temperatura permanece a 2 °C. Si incluimos las capas de hielo polar, entonces encontraremos que más de 80 por ciento de la biosfera de la Tierra está permanentemente fría (Russell, et al.,1990). Así que ¿en realidad somos los colonizadores más exitosos o debemos otorgar este título a aquellos organismos que son más capaces de hacer frente a estas temperaturas?



Esta reflexión nos confirma que la vida en el planeta Tierra es variada, compleja y sorprendente. Las bacterias psicrófilas se definen como microorganismos capaces de crecer a temperaturas que van desde los 0 a 20 °C, en el caso de las bacterias psicrófilas estrictas, mientras que las facultativas pueden crecer desde 0 a 30 °C; las temperaturas óptimas de crecimiento son 15 y 20 °C, respectivamente. Estas bacterias y sus enzimas son de interés biotecnológico debido a la posibilidad de aplicarlas en procesos a bajas temperaturas, así como de interés científico por la relación entre la estructura protéica y la estabilidad térmica de las enzimas bacterianas (Alvarado, et al., 2015).

Una fría alternativa para la producción de biohidrógeno

Las bacterias psicrófilas representan un nuevo concepto en el campo de la producción de biohidrógeno, por lo tanto, aún no son tan conocidas en esta área, ya que a la fecha son escasos los reportes en donde se les considera productoras de hidrógeno. No obstante, estos microorganismos son muy interesantes, y no nos sorprenderá que en unos cuantos años incremente el número de reportes donde estas bacterias aparezcan como protagonistas de la producción de biohidrógeno.

Recientemente, en 2014, se publicó el primer trabajo referente a la producción de biohidrógeno por bacterias psicrófilas realizado por un grupo de investigación en Polonia, donde se reporta el aislamiento de bacterias psicrófilas de muestras de agua de subsuelo y del fondo de un lago. En este estudio se probó la capacidad para producir hidrógeno al utilizar suero de leche como sustrato, de lo que se obtuvieron volúmenes que van desde 1.4 hasta 66.93 mililitros (mL) de biohidrógeno (Debowski, Korzeniewska, Filipkowska, Zielinski y Kwiatkowski, 2014).

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Egresada de la carrera de Ingeniería Bioquímica por parte del Instituto Tecnológico de Durango. En la actualidad estudia la Maestría en Biología Molecular en el Instituto Potosino de Investigación Científica y Tecnológica A.C. y trabaja en el tema de producción de biohidrógeno por microorganismos psicrófilos aislados de la Antártica.

El segundo estudio fue publicado por nuestro grupo de trabajo, en donde se reporta la producción de biohidrógeno por 14 bacterias psicrófilas aisladas de muestras colectadas de la Antártica, utilizando glucosa como sustrato. Los resultados mostraron que las 14 cepas son productoras, con volúmenes de producción que van desde 34.8 hasta 253.3 mL de biohidrógeno (Alvarado, et al., 2015). Estos últimos experimentos fueron realizados a 25 °C, por lo tanto, estas bacterias pueden ser nuevos microorganismos con potencial para ser empleados en procesos de producción de hidrógeno a temperatura ambiente, con la ventaja de no requerir grandes cantidades de energía para mantener la producción de hidrógeno como en el caso de los procesos actuales que requieren de temperaturas superiores a los 30 °C. Además, en este estudio se reportó que algunos de estos microorganismos generan etanol como subproducto en una concentración de hasta 6.8 g/L, este hallazgo es también relevante ya que existe la posibilidad de desarrollar procesos usando a estos microorganismos en procesos simultáneos de producción de hidrógeno y etanol, el cual, hoy en día es considerado como uno de los biocombustibles más importantes.

Con base en lo anterior, estas bacterias congeladas pueden contribuir a obtener energía de manera sustentable, pero todavía queda un largo camino por recorrer, por lo tanto, es necesario seguir investigando para desarrollar novedosos procesos de producción de hidrógeno mediante bacterias amantes del frío que en un futuro no muy lejano puedan aplicarse a escala comercial.

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