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Biohydrogen production by dark fermentation of milk processing wastewater using *Eschirichia coli* as pure strain

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Abstract

This study focuses on the optimization of biohydrogen production through dark fermentation of milk processing waste water using the facultative anaerobic strain *Escherichia coli* (a strain easier to control compared to strict anaerobic bacteria) under mesophilic conditions (37°C). The effect of the type of culture medium and substrate concentration were evaluated, along with attempts to enhance biohydrogen production through physicochemical pretreatments of the substrate. Milk processing wastewater was used as the substrate and various physicochemical pretreatments were tested, including ultrasonic treatment, thermal shock at 80°C, 90°C, and 100°C, and chemical pretreatments with *NaOH*, *$Ca(OH)_2$*, *HCl*, and *CH_3COOH*. Biohydrogen production was modeled using the modified *Gompertz* and *Modified logistic* equations.

Results show that the optimum culture medium and substrate concentration were physiological saline at 0.43 g COD/L, reaching 15 mL of H_2 34,88 mL/ g COD and The optimal pH for biohydrogen production was found to be 6.5. The substrate thermal pretreatment at 90°C was found to be the most effective, increasing biohydrogen production by 140%, while pretreatment at 100°C led to lower hydrogen yields. Ultrasonic pretreatment also improved hydrogen production, with the best results achieved after 20 minutes of treatment. Chemical pretreatments showed limited effects on biohydrogen production. Kinetic modeling revealed that both the modified *Gompertz* and *Modified logistic* models accurately described hydrogen production, with an optimal thermal pretreatment temperature of 90°C showing the highest biohydrogen production rates.

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Keywords**:** Enzymatic pretreatment**,** physicochemical pretreatment, biohydrogen, *lactase*, BHP test.

Abbreviations

MPWW : Milk processing waste water.

TS : Total solids.

TVS : Total volatile solids.

S : Substrate.

I : Inoculum.

gVS $_{added}$: Gram volatile suspended added.

TCOD : Total chemical oxygen demand.

SCOD : Soluble chemical oxygen demand.

TKN : Total Kjeldahl nitrogen.

U5 : Ultrasonic pretreatment for 5 minutes.

U10 : Ultrasonic pretreatment for 10 minutes.

U20 : Ultrasonic pretreatment for 20 minutes.

T80 : Thermal pretreatment at 80°C.

T90 : Thermal pretreatment at 90°C.

T100 : Thermal pretreatment at 100°C.

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1. Introduction

As global energy demand continues to rise, there is increasing interest in renewable and sustainable energy alternatives, and biohydrogen has emerged as one of the most promising candidates. Biohydrogen is produced through biological processes, such as dark fermentation, offering multiple advantages over traditional fossil fuels. It is not only a clean energy source but also has a high energy content and a low environmental footprint, making it an attractive solution in the context of the ongoing energy crisis and climate change challenges (Hallenbeck & Ghosh, 2009)(Azeitona, 2012). The potential to produce biohydrogen from renewable feedstocks, including agricultural and industrial waste, further enhances its sustainability and aligns with the goals of circular economy strategies (Yin et al., 2020).

Among the various microorganisms capable of producing biohydrogen, Escherichia coli (E. coli), a facultative anaerobe, has received considerable attention. E. coli can survive and grow in both aerobic and anaerobic conditions, providing greater flexibility in fermentation processes compared to strict anaerobic bacteria, which often require more complex environmental controls. Furthermore, E. coli is relatively easy to manipulate genetically, allowing for the optimization of biohydrogen production pathways and enabling higher yields under mesophilic conditions (37°C) (Rittmann & Herwig, 2012)(Syakina & Jahim, 2016). Its ability to efficiently metabolize a range of substrates, including organic waste, positions E. coli as a suitable candidate for biohydrogen production in both laboratory and industrial settings.

Dark fermentation, the process of biohydrogen production in the absence of light, has become one of the most studied methods for biohydrogen production. This process relies on anaerobic microorganisms to convert organic substrates, such as sugars, carbohydrates, and food industry waste, into hydrogen gas (Cesaro & Belgiorno, 2015). Whey, a byproduct of the dairy industry, is

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particularly attractive as a substrate due to its high organic content, abundant availability, and relatively low cost. Dairy waste, including whey, represents a significant environmental challenge due to its high biochemical oxygen demand (BOD), and its conversion into biohydrogen offers a dual benefit: the waste is valorized while simultaneously producing a renewable energy source (Bai et al., 2016). However, the efficiency of biohydrogen production from whey depends on various factors, including the type of culture medium, substrate concentration, pH, and the pretreatment of the substrate to improve its availability for microbial metabolism.

The formulation of the culture medium plays a crucial role in optimizing the growth and metabolic activity of microorganisms. The right medium ensures the availability of necessary nutrients and supports the desired metabolic pathways for biohydrogen production. Several studies have demonstrated that a carefully selected culture medium enhances the rate and efficiency of biohydrogen production, making it one of the critical factors in both laboratory experiments and large-scale production (Ito et al., 2019). For example, media such as minimal ISP9 or complex nutrient solutions are commonly used, depending on the growth requirements of the microbial strain used (Siddique et al., 2014).

In addition to culture medium selection, physicochemical pretreatments have been shown to significantly enhance biohydrogen production by improving the hydrolysis of complex organic materials, making them more accessible for microbial conversion. Thermal pretreatment, for instance, involves exposing the substrate to high temperatures, which helps break down complex carbohydrates into simpler sugars, thereby improving microbial fermentation efficiency (Pagliaccia et al., 2016). Ultrasonic pretreatment, which uses high-frequency sound waves to disrupt macromolecular structures, has also been shown to improve hydrogen yield by enhancing substrate degradation (Prabakar et al., 2018). Chemical pretreatments, including alkaline or acid shock, can further enhance biohydrogen production by disrupting the cellular structure of the substrate, releasing more fermentable sugars (Pachapur et al., 2019).

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Although each pretreatment method has its advantages, there is no one-size-fits-all solution. The efficiency of each method depends on the specific properties of the substrate and the microbial strain used. For example, while thermal pretreatment at moderate temperatures (e.g., 90°C) can be highly effective, higher temperatures may lead to the degradation of essential nutrients and the formation of fermentation inhibitors (Pagliaccia et al., 2016). Ultrasonic treatments can increase hydrogen yields, but the duration and intensity of the treatment must be carefully controlled to avoid the generation of inhibitory compounds such as furans (Prabakar et al., 2018).

This study aims to explore the optimization of biohydrogen production from whey using E. coli through dark fermentation. We investigate the effects of various physicochemical pretreatments on the substrate, as well as the influence of culture medium, substrate concentration, and pH on the production process. By analyzing biohydrogen production data with kinetic models, we aim to gain insights into the fermentation dynamics and identify parameters that can improve the scalability and efficiency of the process for industrial applications. This research will contribute to a better understanding of how to optimize biohydrogen production systems and facilitate their transition from laboratory-scale experiments to large-scale industrial operations.

In summary, biohydrogen production from renewable waste substrates, such as whey, represents a promising avenue for sustainable energy production. By optimizing various factors, including culture media, pretreatment methods, and fermentation conditions, it is possible to enhance hydrogen yields and create more efficient and economically viable biohydrogen production systems. Through this study, we aim to advance the knowledge base needed to scale up biohydrogen production and contribute to the development of alternative, environmentally friendly energy solutions.

2. Material and Methods

2.1. Milk processing waste water (substrate)

Milk processing wastewater was used as the substrate and was collected from the 'NUMIDIA' dairy industry in Constantine, Algeria. To prevent any biological degradation, this waste was stored at 4°C.

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2.2.Inoculum

The pure culture of *Escherichia coli* was brought from the Constantine University Hospital (CHU), and the colonies were added to physiological saline at 37°C. After 48 hours, 13 out of 15 tubes appeared turbid. A volume of 5 mL of *E. coli* was added to 100 mL of peptone medium and stored in an incubator at 37°C.

2.3. Chemical reagents

Following chemical reagents were used for chemical pretreatment (acid and alkaline) operations: sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$), chloride acid (HCl); acetic acid (CH_3COOH). All the reagents are pure to analysis grade and purchased from Sigma Aldrich (Darmstadt, Germany).

2.4.Culture medium

The culture medium plays a crucial role in supporting the growth and metabolism of microorganisms, including bacteria such as *Escherichia coli*, Peptone Medium, the minimum ISP9 medium and physiological saline solution were prepared to provide essential nutrients, helps maintain specific growth conditions

The peptone medium (PM) was prepared and sterilized for the cultivation of the pure Escherichia coli culture under mesophilic anaerobic conditions (37°C) in sterile 125 mL serum bottles sealed with rubber caps reinforced with aluminum. The composition of the PM is shown in Table 2.2. (Poladyan et al., 2020).

Table 1: Composition of Peptone Medium

Constituant	Concentrations	unit
Peptone	20	g/L
K_2HPO_4	2	g/L
NaCl	5	g/L
Glucose	2	g/L
Yeast extract	5	g/L
pH=6.5	/	/

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The minimum ISP9 medium was used as the fermentation medium for the pure culture. It is a medium composed of the strictly necessary chemical elements for the bacterial growth of *Escherichia coli*, in a form that can be utilized by bacteria with no specific requirements. The composition of the ISP9 medium is shown in Table 2.

Table 2: Composition of ISP9

Constituants	Concentrations	Unit
$(NH_4)SO_7$	2.64	g/L
KH_2PO_4	2.38	g/L
K_2HPO_4	5.65	g/L
MgSO4	1	g/L
saline Solution	1	mL
PH= 7.4	/	/

Tableau 3 : saline solution Composition

Constituants	Concentrations	Unit
$CuSO_4$	0.64	g/L
$FeSO_4$	0.11	g/L
$MnCl_2$	0.79	g/L
$ZnSO_4$	0.15	g/L

Physiological saline solution was used for cultivation and as the fermentation medium for the pure culture. It is a sterile aqueous medium prepared by dissolving sodium chloride in distilled water at a concentration of 9%.

2.5.Procedure of physicochemical pretreatment

Four different physicochemical strategies for substrate were tested, ultrasonic pretreatment for 5, 10 and 20 min using ultrasonic bath (BTX-600) at 25 kHz, thermal pretreatment was a heat shock

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at 80, 90 and 100°C for 30 minutes. Chemical pretreatment was alkaline shock to pH 11 with NaOH and $Ca(OH)_2$ and acid shock to pH 2 with HCl and CH_3COOH.

2.6.Experimental procedure for biohydrogen production (BHP test)

In this research study the experimental procedure implemented is represented in Figure 2. Hydrogen production experiments were carried out in 100 mL glass bottles under batch conditions with 67 mL, it was filled with of inoculum, substrate solution, water and culture medium.

The calculation of substrate volume was based on TVS of milk processing waste water Initial pH was fixed to 5.5 using NaOH(1N) and HCL (1N) sterilized solutions, all batch tests were performed in triplicate. The bottles were then capped with a rubber stopper, removed the air contained using an inert gas (N_2) to ensure anaerobic conditions and incubated under mesophilic temperature of 37°C until the end of biogas production. The biogas generated was collected from the headspace of bottle.

After determining the culture medium and pH corresponding to the maximum yield of hydrogen production, the best parameters are applied on de physicochemical pretreatment and applied to the substrate before BHP test and investigated biohydrogen production enhancement.

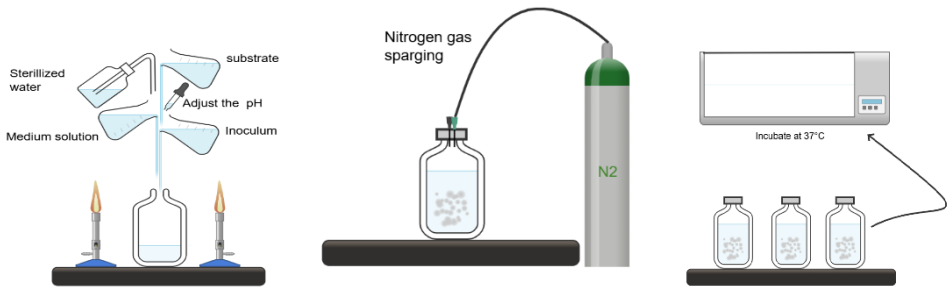

Figure 1. Schematic representation of BHP test experiments.

2.7. Analytical methods

The characteristics of the substrate and inoculum, including total solids (TS) content, total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), carbohydrates,

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and alkalinity, were analyzed following the Standard Methods (APHA, 2012). pH measurements were taken using a Thermo Scientific Orion 3-star pH meter. All analyses were performed in duplicate, and the average results were used to present reproducible findings.

The volume of biogas was intermittently measured using a hydrochloric acid solution (pH 3) for removal. A 20 g/L KOH solution was used to absorb the generated CO2 volume. The remaining biogas components (H_2 and H_2S) were analyzed with a portable gas analyzer (GA 5000; Geotech, Leamington, UK) equipped with infrared and electrochemical sensors.for determine the parameters of H_2 production, the cumulative H_2 production values of the optimum test fitted using two models : modified Gompertz equation (equation (1)) and modified Logistic equation (BOUCHAREB, 2020).

$$H(t) = H_{max} \exp(-\exp(\frac{R_m e}{H_{max}}(\lambda - t) + 1)) \quad (1)$$

$$H(t) = \frac{H_{max}}{1 + \exp(\frac{4R_m}{H_{max}}(\lambda - t) + 2)} \quad (2)$$

Where (dimensionless and equivalent to 2.718)(Rambabu et al., 2021) :

$H(t)$ is cumulative hydrogen production at time t (mL H_2), H_{max} is the maximum hydrogen production at the end of production process (mL H_2), R_m is the maximum hydrogen production rate during all production process (mL H_2/d), λ is Lag time constant (d), e is a mathematical constant.

3. Results and discussion

3.1.Bacterial Growth of E. Coli

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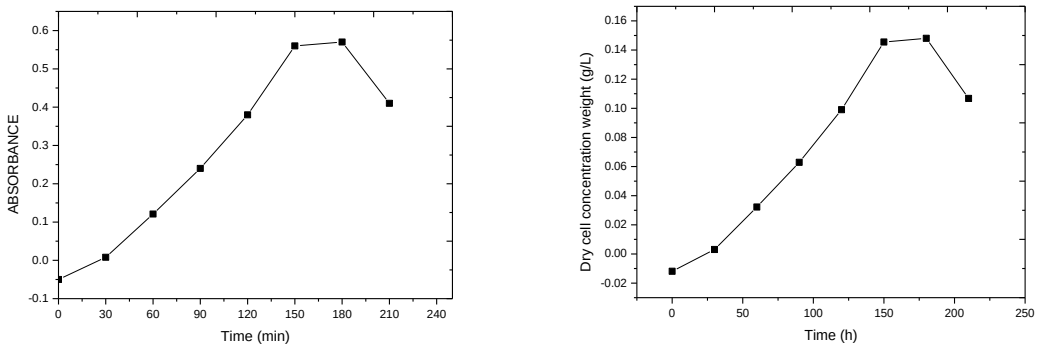

Figure 2 Bacterial growth curve of the *Escherichia coli* strain

The liquid culture was inoculated onto nutrient agar; after 24 hours, colony appearance was observed. One colony was then enriched in physiological saline, where the bacterium grew aerobically under mesophilic conditions (37°C) at pH 7.0. The cell growth was monitored by spectrophotometry at 600 nm, and the results are shown in Figure 3.30.

As shown in Figure 3.30, the shape of the curve is similar to typical bacterial growth curves, with an adaptation phase, an exponential growth phase, a stationary phase, and a decline phase. The culture was sampled after 30 minutes of growth and considered as the inoculum for all experiments conducted in this section.

3.2.Substrate characterization

The physicochemical characteristics of milk processing waste water are summarized in Table 1. MPWW characteristic values indicate that the used substrate is rich in biodegradable organic matter which favorites biological processes and the high percentage of TVS/ TS presents the possible potential of the substrate for biogas valorization.

Table 4. Physicochemical characteristics of MPWW

Parameter	Value	Unit
pH	4.68	-
Alkalinity pH6	0	mgCaCO3/L
Alkalinity pH4	1297	$mgCaCO_3/L$
TVA	7820	$mgCaCO_3/L$
VFA	1297	$mgCaCO_3/L$
TS	49.24	g/L
TVS	43.33	g/L
TVS/ TS	87.97	%
TCOD	56	g/L

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SCOD	44.8	g/L
SCOD/TCOD	80	%
Carbohydrates	92.2	g/L
Proteins	39	g/L
TKN	6,24	g/L
Turbidity	4170	NTU

The results show that this substrate MPWW has an acidic character at a high turbidity containing a large amount of degradable organic matter 44,8 g/L of COD and 92.2 g/L of total sugars, a high percentage of SCOD 80% which promotes microbial growth; in addition to that a percentage of TVS/TS 97.30% shows the ease of biogas production by bacterial culture.

3.3. Effect of physicochemical pretreatment

The physicochemical characteristics of milk processing wastewater before and after pretreatment are reported in Table 3. According to Table 3, all pretreatments cause an increase in COD and TVS values, only low acid pretreatment results in a decrease in TVS value 38.97 g/L. After heat pretreatment of MPWW the pH value was almost constant, and the high percentages of soluble COD and TVS increased to 93.4% and 90.8% respectively, after heat pretreatment at 90°C, COD increased with increased duration of ultrasound pretreatment. The variation of these parameters after 5 min ultrasound pretreatment is not significant, as this duration is not sufficient for a remarkable destruction of complex sugars. Ultrasonic pretreatment can destroy chemical bonds or generate free radicals. The ultrasound processing mechanism is based on the acoustic cavitation; ultrasound provides a huge power density in a short time and promotes chemical reactions that also helps to solubilize the substrate therefore parameters such as pH, alkalinity, total sugars remain almost constant, while the TS and TVS were varied and increased. The acid pretreatment resulted in a value of TVS higher than that of the basic pretreatment, almost no change of carbohydrates and proteins, these variations allow strongly to dissolve the substrate, making it easier to consume and digest by micro-organisms.

Table 5. Physicochemical characteristics of substrate before and after physicochemical pretreatments.

Charecteristics	**Before treatment**	**After pretreatment**									
		Thermal (°C)			Ultrasoic (min)			Acid		Alkaline	
		80	90	100	5	10	20	HCl	CH_3COOH	NaOH	$Ca(OH)_2$
pH	4.68	5.1	5.6	5.9	4.8	4.2	4.7	2	2.6	11	11
TCOD (gO_2/L)	56	160	107	160	128	151	160	80	80	144	58
SCOD(gO_2/L)	44.8	149	100	143	121	129	139	57	62	135	55

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SCOD/TCOD (%)	80	93.1	93.4	89.5	94.5	85.4	86	71.2	77.5	93.7	94.8
TS (g/L)	49.24	57.8	60.19	58.9	61	59	62	60.61	57.48	50.35	55.2
TVS (g/L)	43.33	52.4	54.66	53.2	60	57	51	54.86	53.87	38.97	51
TVS/TS (%)	87.9	90.6	90.8	90.3	98.3	96.6	82.2	90.5	93.7	77.3	92.4
Proteins (g/L)	39	56	98	31	54	48	29	52	22	47.1	28.5
Carbohydrates(g/L)	92.2	92	91.2	89.2	87	91	84	87.2	90.3	82	80
Alkalinity pH6 ($mgCaCO_3/L$)	0	0	0	0	0	0	0	0	0	/	/
Alkalinity pH4 ($mgCaCO_3/L$)	1297	1107	983	1202	958	964	880	963	1028	1189	768

3.4.Effect of the Type of Culture Medium and Organic Load Concentration on the Biochemical Potential for Biohydrogen Production

Bio hydrogen production through dark fermentation of raw whey using *E. coli* culture was carried out using two different culture media: ISP9 and physiological saline, while simultaneously varying the concentration of the organic load.

Figure 3.31 shows the effects of the type of nutrient medium and the effect of the treated organic load. As shown in the figure, using the minimal ISP9 medium, the best cumulative production of 3 mL of H_2 was obtained at an organic load concentration of 0.43 g COD/L. Meanwhile, using physiological saline, the maximum cumulative production resulted in 15 mL of H2 at the same substrate concentration.

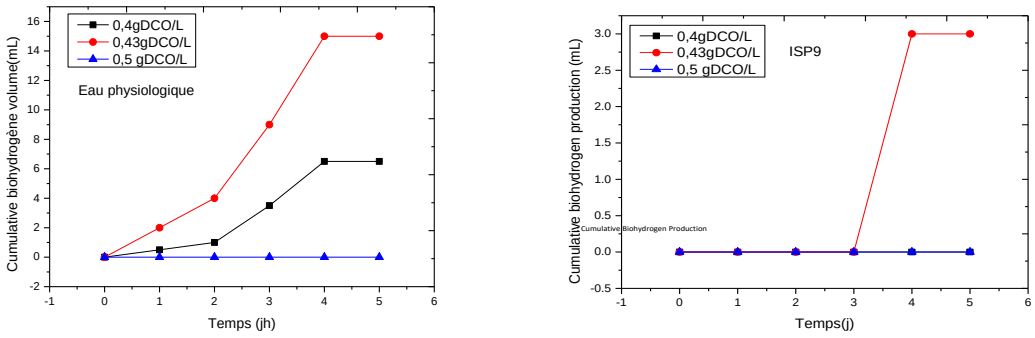

Figure 3: Cumulative Biohydrogen Production during Dark Fermentation of Whey as a Function of the type of Medium and Organic Load

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In the presence of physiological saline, an organic load concentration of 0.5 g COD/L resulted in zero production, whereas when the concentration was 0.4 g COD/L, a production of 6 mL of H_2 was achieved. These results indicate that fermentation with a low substrate dose generates a small amount of hydrogen, which can be explained by the limited amount of substrate. However, when the substrate dose is excessive, it can inhibit bacterial growth, leading to reduced biohydrogen production (Poladyan et al., 2020).

3.5. Effect of pH

3.6. The effect of the initial pH on cumulative biohydrogen production through dark fermentation of raw whey at an organic load of 0.43 g COD/L using pure *E. coli* culture was carried out using physiological saline as the medium.

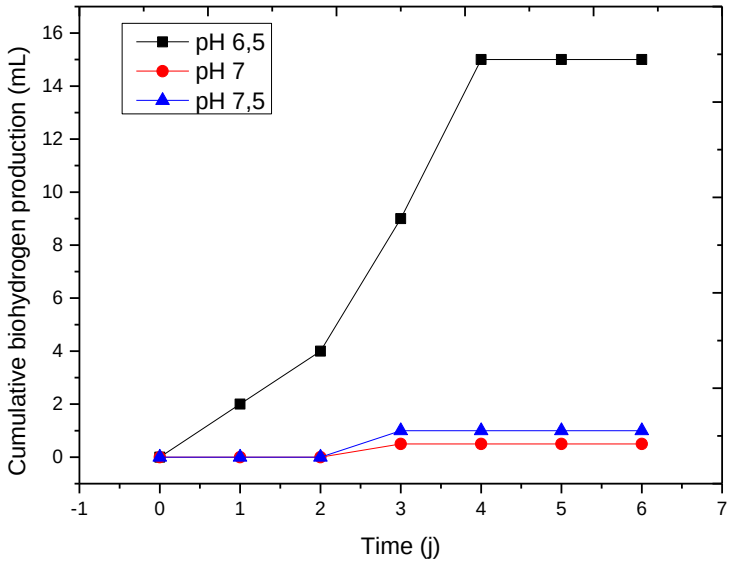

Figure 4 *Cumulative Biohydrogen Production during Dark Fermentation of Whey as a Function of pH at 0.43 COD/L in the Presence of Physiological Saline*

The initial pH value was varied within the activity range of the *E. coli* strain from [6.5 to 7.5]. The study showed that the optimal pH for biohydrogen production is 6.5. A similar result was obtained for biohydrogen production through dark fermentation of glucose

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using the pure *E. coli* strain by evaluating the effect of initial pH in the range of [5.5 to 7.5], where the optimal value of 6.5 was also observed (Akroum-Amrouche et al., 2019).

3.7. Effect of Substrate Pretreatment on Biohydrogen Production Potential

To enhance the fermentative biohydrogen production by *E. coli*, several attempts at physicochemical substrate pretreatment were carried out.
The substrate pretreatments were performed using the same method mentioned in our study(Del Angel-Acosta et al., 2021).

- ***Effect of Thermal Pretreatment***

The effects of thermal pretreatments were conducted on raw whey at temperatures of 80°C, 90°C, and 100°C. Figure 3.33 illustrates the cumulative volumes of biohydrogen produced by the fermentation of thermally pretreated whey at these temperatures as a function of time. These treatments helped improve biohydrogen production, with 15 and 36 mL produced from the second to the sixth day at 90°C, then plateauing at 36 mL after the seventh day, which corresponds to a 140% improvement rate. In contrast, hydrogen production was 18.5 mL and 10 mL with substrate pretreatment at 100°C and 80°C, respectively.

The thermal treatment at 90°C resulted in the best biohydrogen production because this temperature appears to be ideal for limiting hydrolysis without completely destroying the whey molecules. In the case of a pretreatment at 80°C, the temperature is high enough, but it is not sufficient for complete hydrolysis of the substrate to achieve maximum production. On the other hand, a pretreatment at 100°C caused excessive hydrolysis, leading to the complete destruction of the substrate and the generation of fermentation inhibitors (Pagliaccia et al., 2016), as well as the evaporation of water present in the whey, which is a precursor for biohydrogen production (Boboescu et al., 2016)(Rodríguez-reyes et al., 2021).

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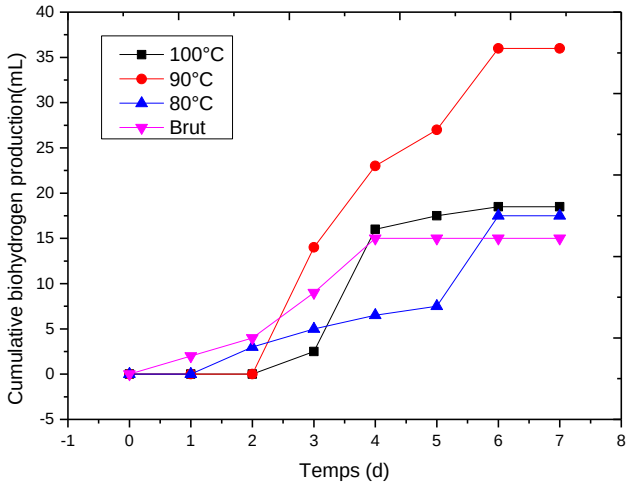

Figure 5 Cumulative Biohydrogen Production during Dark Fermentation of Thermally Pretreated Whey as a Function of Treatment Temperature at pH 6.5 and 0.43 COD/L in the

- ***Effect of Ultrasonic Pretreatment***

Figure 3.34 shows the evolution of the cumulative biohydrogen volume as a function of time and the duration of ultrasonic pretreatment. The results indicate that all three pretreatments improve biohydrogen production within a range of 15.5 to 25.5 mL, with this improvement increasing proportionally with the duration of the treatment, varying between 3.33% and 70%. The maximum hydrogen production was achieved with a 20-minute ultrasonic pretreatment, which helps reduce the time required for the hydrolysis phase of the substrate and facilitates substrate consumption for hydrogen production. A 10-minutes ultrasonic pretreatment improves hydrogen yield by 26.66%, but does not exceed the improvement achieved with the same pretreatment duration of 20 minutes, as 10 minutes is not sufficient for complete hydrolysis of carbohydrates. However, very long ultrasonic pretreatment durations can lead to the production of inhibitory compounds such as furans and phenolic compounds (Rodríguez-reyes et al., 2021)(Prabakar et al., 2018). Ultrasonic pretreatment of the substrate for 5 minutes results in a cumulative hydrogen production almost similar to that obtained by fermenting the raw substrate, as the 5-minute

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duration has very little impact on the macromolecules of the substrate (carbohydrates, lipids, proteins, etc.).

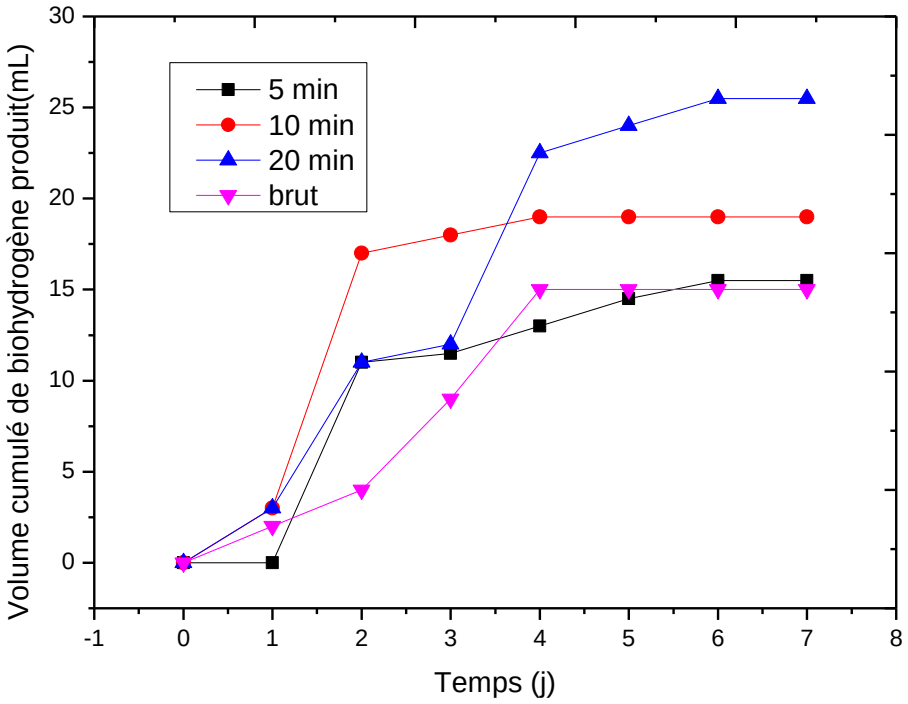

Figure 6 Cumulative Biohydrogen Production during Dark Fermentation of Ultrasonically Pretreated Whey as a Function of Treatment Duration at pH 6.5 and 0.43 COD/L in the Presence of Physiological Saline

- ***Effect of Chemical Pretreatment***

Figure 3.35 shows biohydrogen production and cumulative production during fermentation of substrates pretreated with two chemical treatments, acidic and basic, as a function of time. The results show a stabilization of the total cumulative volume after acidic pretreatment at 14.5 mL after 6 days. At the end of fermentation, the maximum production value achieved by chemical shock with the weak base $Ca(OH)_2$ was 2.5 mL. No studies on this specific aspect have been published.

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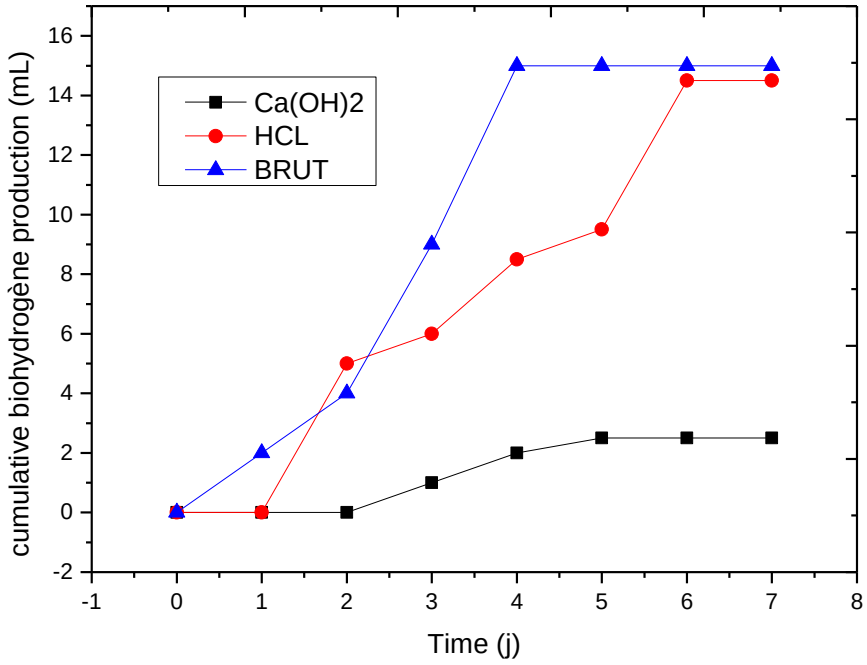

Figure 7 Cumulative Biohydrogen Production during Dark Fermentation of Chemically Pretreated Whey as a Function of Treatment Temperature at pH 6.5 and 0.43 COD/L in the Presence of Physiological Saline

The comparative histogram shown in Figure 3.36 clarifies the differences in biohydrogen production rates depending on the pretreatment. It is clear that physical pretreatment methods improve biohydrogen production, whereas chemical pretreatment has an undesirable effect on biohydrogen production. It is possible that physical pretreatments do not affect the culture as much as chemical pretreatments, since the latter introduce inhibitory chemicals into the pure culture within the fermentation medium.

All thermal pretreatments (80°C, 90°C, and 100°C) increase the biochemical potential for biohydrogen production from whey, with 90°C seeming to be the optimal temperature that resulted in the highest cumulative biohydrogen value. Similarly, ultrasonic pretreatments (5, 10, and 20 minutes) led to slight increases in the biochemical hydrogen potential of whey. Furthermore, for chemical pretreatments, none of the acids or alkalis used were effective.

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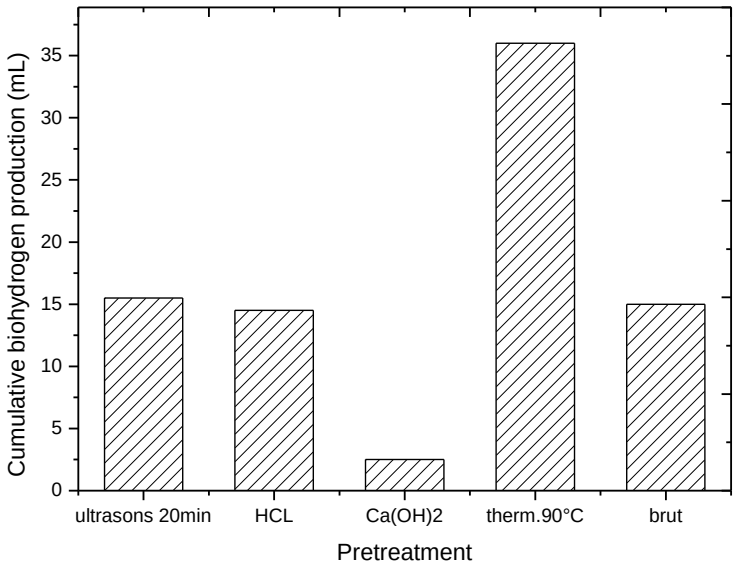

Figure 8 Comparative histogram of the cumulative biohydrogen production by the dark fermentation of pretreated lignocellulosic biomass (LS) with various physicochemical pretreatments.

3.8. Kinetic modeling of hydrogen production

Kinetic modeling of the dark fermentation of LS for biohydrogen production provides essential information on the theoretical characteristics of maximum biohydrogen production yield. This analysis is a crucial tool for studying the scale-up of experimental batch fermentations to industrial production (Wang & Wan, 2009). Experimental data were fitted with two different kinetic models: the *modified Gompertz model* and the *modified logistic model*, to study kinetic parameters such as the adaptation time of hydrogen-producing cultures (λ), the maximum hydrogen yield potential (Hmax), and the maximum hydrogen production rate (Rm).

Table 6 Kinetic parameters for biohydrogen production by dark fermentation of raw and heat-pretreated LS using pure E. coli culture.

Pretreatement	EXPERIMENTAL	Modified *Gompertz* Model				Modified de *Logistic* Model			
	H_{max}	H_{max}	R_m	λ	R^2	H_{max}	R_m	λ	R^2

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	(mLH_2)	(mLH_2/gVS)	(mL H_2)	(mL H_2/j)	(j)		(mL H_2)	(mL H_2 /j)	(j)	
/	15	45,55	18,09374	5,1632	0,0903	0,9629	16,1994	5,9095	1,3765	0,9799
Thermic 90°C	36	109,32	37,3955	11,5031	0,0006	0,9796	35,8718	11,5455	2,0686	0,9654

As shown in Table 3.12, both models effectively describe biohydrogen production, which is clearly reflected in their R^2 values greater than 0.96. The hydrogen production potential and the hydrogen production rate increased with the thermal pretreatment of the substrate at 90°C. Maximum hydrogen production rates of 37.3955 mL H2 and 35.8718 mL H2 were obtained by thermal pretreatment at 90°C in the modified *Gompertz* and *modified logistic* models, respectively. Furthermore, the models predicted a drastic reduction in the fermentation adaptation phase due to the thermal pretreatment at 90°C. Remarkably, the *Gompertz* model estimated a shorter adaptation phase than the modified logistic model. In general, biohydrogen production occurs between the adaptation phase and the stationary phase of the bacterial growth curve (Swathy et al., 2020). The modeling of biohydrogen production via fermentation is not a difficult technique and is an effective way to scale up fermentation technology from the laboratory scale to industrial scale to meet the growing energy demand.

Kinetic analysis on the dark fermentation of MPWW on biohydrogen supplies the essential information on the theoretical maximum biohydrogen production yield characteristic. This analysis is an imperative tool which serves to study the scale-up of the experimental batch mode fermentation into commercial production (Pal et al.,2018). Experimental data was fitted with two different kinetics model: *Gompertz model* and *modified logistic model*, to study the Kinetic parameters such as lag phase time of hydrogen production (λ), maximum potential for hydrogen yield (H_{max}), and maximum hydrogen production rate (Rm).

The results from section D show that biohydrogen production using pure *E. coli* strain is applicable, but the productivity of this pure culture remains low compared to other strains

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mentioned in the literature, such as the Clostridium family (Lee et al., 2021). On the other hand, fermentation using pure cultures presents a significant drawback in ensuring sterilization conditions. Most studies conducted on biohydrogen production via pure cultures have cited synthetic solutions of simple sugars as substrates, serving to understand the biochemical mechanisms involved (Baeyens et al., 2020).

4. Conclusion

Biohydrogen production by pure E. coli is feasible, but the productivity remains low compared to other strains like *Clostridium* species. Fermentation with pure cultures, however, presents challenges in ensuring sterilization. The study highlights the importance of physicochemical pretreatments and kinetic modeling in optimizing biohydrogen production, with potential for scaling up from laboratory to industrial production to meet growing energy demands.

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