

Trace Metal Effect on Hydrogen Production Using *C. acetobutylicum*

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Abstract: This study was carried out to study the effect of trace metal addition for hydrogen production. The results show that this bacterial need a selected nutrient with selected concentration. The optimum parameters for cultivation were at initial pH of 7.0 and temperature of 30°C. Trace metal addition to the new medium showed that only iron enhanced the H₂ yield. The results showed that 25 mgL⁻¹ FeSO₄·7H₂O enhanced the hydrogen yield from 391 mLg⁻¹ to 408 mLg⁻¹ glucose utilized with biomass concentration of 1.38 gL⁻¹. Y_{P/X} of 292 mLg⁻¹ L⁻¹, Y_{X/S} of 0.276 and Y_{H₂/S} of 0.0364, gave the maximum hydrogen productivity of 81.6 mL L⁻¹ h⁻¹. Magnesium and calcium addition were better for growth and not for hydrogen production. Biomass concentration increases as trace metal increases in the fermentation medium. The highest biomass concentration was 1.65 gL⁻¹ at 1000 mgL⁻¹ FeSO₄·7H₂O, 1.47 gL⁻¹ at 1000 mgL⁻¹ MgSO₄·7H₂O and 1.73 gL⁻¹ at 500 mgL⁻¹ CaCl₂·2H₂O, suggested that all trace metal are necessary for biomass production.

Key words: Biohydrogen, batch fermentation, glucose, trace metal

INTRODUCTION

Fermentative H₂ bio-production processes are not only environment friendly, but also lead to open a new avenue for the utilization of renewable energy resources which are inexhaustible^[1]. The fermentative H₂ production is of higher capacity and lower cost than photosynthetic. The purpose of biohydrogen production studies is to develop commercially practical H₂ production processes through modern biotechnology. In this case, the critical factor is to find the means to enhance the hydrogen yield. Since nutrient availability and metabolites accumulation may often result in growth limitation and consequent inhibition of hydrogen production, there is need to obtain the precise quantification of the extent to which each phenomenon contributes to overall growth and hydrogen production behavior.

Bio-hydrogen production requires essential micronutrients for bacterial metabolism during fermentation. Sodium, magnesium, zinc and iron are all important trace metals affecting hydrogen production. Among them iron is an important nutrient element to form hydrogenase or other enzymes which almost all biohydrogen production needs fundamentally^[2]. According to literature, magnesium ion is an activator of many kinases and synthetases. In cytoplasm there are

10 kinds of enzymes involved in glycolysis and most of these enzymes need magnesium ion as cofactors such as hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and enolase, etc.^[3]. Thus, the aim of this study was to study the effect of trace metal like Iron, Magnesium and Calcium addition on hydrogen production by fermentative bacterial *C. acetobutylicum* NCIMB13357.

MATERIAL AND METHODS

Microorganism and culture conditions: *C. acetobutylicum* NCIMB 13357 was purchased from a British culture collection, NCIMB Ltd. Scotland, UK. The bacterium was cultivated in anaerobic condition in Reinforced Clostridial Medium (RCM) for 24 h at 30°C. Liquid medium of RCM was used for inoculum preparation. The growth of culture in RCM was monitored by measuring an optical density at 600nm using a spectrophotometer. Only inoculum with Optical Density (OD) values greater than 0.4-0.6 after 18 h. cultivation was used as inoculum. An inoculum of 10% v/v was used throughout this study. Batch fermentation was carried out at a working volume of 100 mL in 500 mL rector bottles at 30°C, each medium was seeded with a 10% inoculum inside anaerobic cabinet and sparged with nitrogen gas (99.9%). All the bottles

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were tightly closed. The culture pH was not controlled during fermentation and the initial pH was fixed to 7.0 before sterilization process. To quantify H₂, gas produced during fermentation was recorded at the end of its production. The evolved gas was collected in a gas collection inverted cylinder and the volume of evolved gas was measured at room temperature by the water displacement method Morimoto *et al.*^[4] in a graduated cylinder that had been filled with water of pH 3 or less in order to prevent dissolution of the gas components

Cultivation medium: New medium we formulated in our lab to be used for hydrogen production and for the bacterium species we used in this study have the following composition in (g L⁻¹): glucose (5), Yeast Extract (5), L-Cystine. HCl (1.0) and bacteriological agar (0.5). Batch experiments were conducted with 500 mL Scotch bottle with working volume of 100 mL (90% culture medium and 10% inoculum). Different concentrations of trace metal were used to find out its effect on fermentative bacteria we used in this study, ranging from 0.0 to 1000 mgL⁻¹ for FeSO₄.7H₂O and MgSO₄.7H₂O and from 0.0 to 500 mgL⁻¹ for CaCl₂.2H₂O. All of these data were the average (mean) of three trials.

Analytical methods: The gas composition was determined by gas chromatography (Shimadzu Co., Kyoto, GC-8A) under the following conditions: column: Porapack-Q, carrier gas: Nitrogen, flow rate: 33 mL min⁻¹, column temperature: 50°C, injection temperature: 100°C, detector temperature: 50°C, detector: Thermal Conductivity Detector (TCD). The soluble glucose concentration was measured at the end of each batch experiment for the calculation of the amount of glucose consumed by DNS method modified by Miller^[5] using spectrophotometer (UV 1601 IPC, Shimadzu corporation- Japan) optical density (OD_{550nm}). Individual batch experiments were observed until the hydrogen production from each bottle stopped. All of these data were the average (mean) of three trials.

RESULTS AND DISCUSSION

Effect of Iron: Gas analysis showed that produced gas has only two gases with 65% for H₂ and the rest was for CO₂. The results shown in Fig. 1 shown that the hydrogen yield was enhanced from 391-408 mLg⁻¹ glucose utilized, by addition of 25 mgL⁻¹ FeSO₄.7H₂O to the culture medium, then started to decrease for

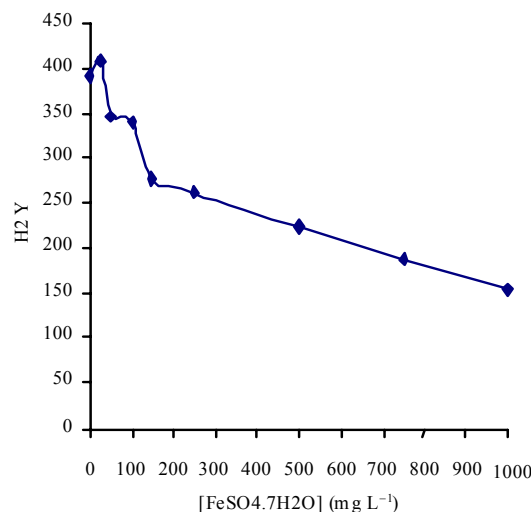


Fig. 1: Effect of [FeSO₄.7H₂O] addition on H₂ Y (mLg⁻¹ utilized)

Table 1: Results of FeSO₄.7H₂O addition to fermentation medium on glucose consumption and H₂ P (mLL⁻¹ h⁻¹) by *C. acetobutylicum* NCIMB 13357

[FeSO ₄] (mgL ⁻¹)	f pH	Glucose consumed (%)	H ₂ P
0.0	4.45	87	77.5
25	4.46	89	81.6
50	4.45	90	70.0
100	4.46	91	70.0
150	4.48	93	58.5
250	4.50	94	55.0
500	4.57	95	61.0
750	4.76	97	42.0
1000	4.86	98	34.0

[Glucose]: 5 gL⁻¹, Inoculum size 10% (v/v) I pH. 7.0. Temperature 30°C

further increase of iron addition to culture medium. From this observation, it was apparent that increasing the iron concentration in a culture medium more than the optimum concentration would decrease the H₂ yield within the concentration range investigated. Iron supplementation up to 25 mgL⁻¹ FeSO₄.7H₂O, significantly enhanced the H₂ yield by 4% over that produced by control (0.0 mgL⁻¹) (Table 1). Between 25 and 50 mgL⁻¹ FeSO₄.7H₂O, further decreased of H₂ yield from 408 to 347 mLg⁻¹ glucose utilized, which then decreased to 154 mLg⁻¹ glucose utilized at 1000 mgL⁻¹ FeSO₄.7H₂O.

The results shown in Fig. 2 reveal that iron addition to fermentation medium have affected positively on two ways, the first was on hydrogen production by addition 25 mgL⁻¹ of FeSO₄.7H₂O but was negatively for further increased and the second way was for biomass which was increased as the iron

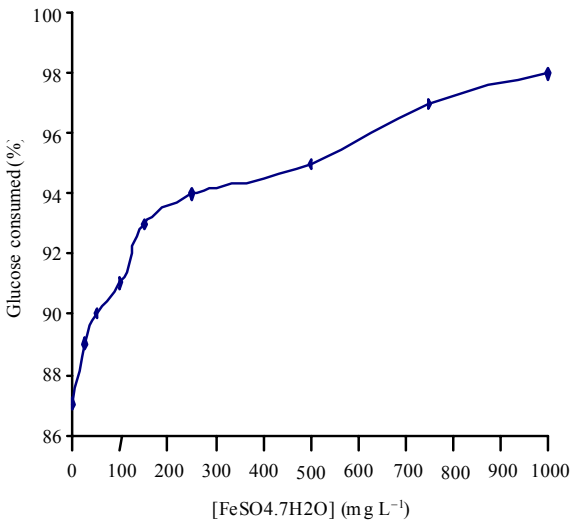


Fig. 2: Effect of [FeSO₄.7H₂O] addition on. Glucose consumption (%)

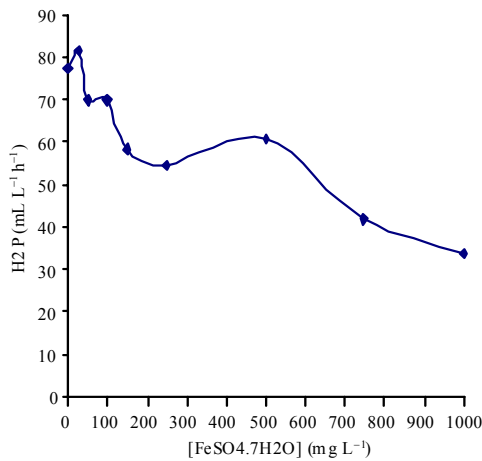


Fig. 3: Effect of [FeSO₄.7H₂O] addition on H₂ P

concentration increased and reached themaximum at 1000 mgL⁻¹ of FeSO₄.7H₂O. This increased in glucose consumption percentage was not enhanced the bacterial productivity but was used as shown in Fig. 4 for bacterial growth which was enhanced from 1.34-1.65 gL⁻¹ at 1000 mgL⁻¹. The increase of biomass was not shared with increasing of hydrogen production with only hydrogen yield of 154 mlg⁻¹ utilized glucose at 1000 mgL⁻¹. Final pH was depend on the produced biomass and as biomass increased, the bacterial metabolites increased, that would affect on bacterial productivity of hydrogen with maximum of 81.6 mL L⁻¹ h⁻¹ to decrease with increasing of FeSO₄.7H₂O in culture medium as shown in Fig. 3,

Table 2: Results of FeSO₄.7H₂O addition to fermentation medium on hydrogen production by *C. acetobutylicum* NCIMB 13357

FeSO ₄ .7H ₂ O (mgL ⁻¹)	Y ¹ _{P/S}	Y ² _{P/S}	[Biomass]	Y _{P/X}	Y _{X/S}	Y _{H₂/S}
0.0	340	391	1.34	292	0.27	0.034
25	364	408	1.38	296	0.28	0.036
50	312	347	1.38	251	0.28	0.031
100	309	340	1.39	245	0.28	0.030
150	258	277	1.41	196	0.28	0.024
250	246	262	1.44	182	0.29	0.022
500	214	225	1.53	147	0.31	0.020
750	183	189	1.59	119	0.32	0.016
1000	151	154	1.65	92	0.33	0.014

Y¹_{P/S} (H₂ mL g⁻¹ glucose supplied) (mLg⁻¹), Y²_{P/S} (mLg⁻¹) (Utilized): (H₂ mL g⁻¹ glucose utilized), [Biomass] (gL⁻¹). Biomass production g L⁻¹ culture, Y_{P/X} (mLg⁻¹ L⁻¹): (H₂ mL g⁻¹ Biomass L⁻¹), Y_{X/S}: (Biomass production per g glucose supplied), Y_{H₂/S}: (conversion of H₂ (mL) to H₂ (g) g⁻¹ glucose utilized) [Glucose] .5 gL⁻¹, inoculum size 10% (v/v), I pH. 7.0, Temperature 30°C

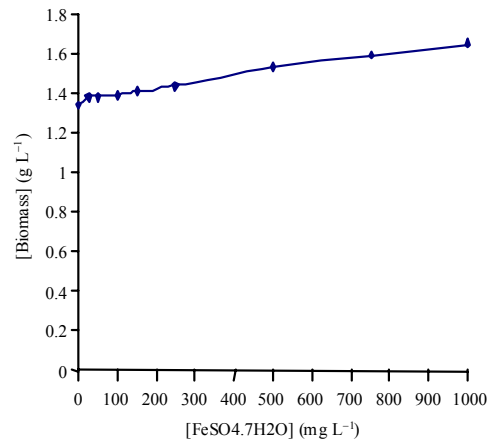


Fig. 4: Effect of [FeSO₄.7H₂O] addition on. [Biomass]

suggested that the iron concentration adversely affect on the bacterial metabolism. These results showed that even a small amount of iron greatly affected production and that the major effect of additional iron was to enhance the rate of production. It's well-known that iron is an indispensable element for bacterial multiplication. Undoubtedly, an appropriate amount of iron is beneficial to increasing the activity of bacteria.

Obtained data shown in Table 1 and 2 were used to calculate different yields like Y_{P/X} (mLg⁻¹ L⁻¹): (H₂ mL g⁻¹ Biomass L⁻¹), Y_{X/S}: (Biomass production per g glucose supplied) and Y_{H₂/S} (conversion of H₂ (mL) to H₂ (g) per g glucose utilized). These results showed that increasing the iron concentration affect inversely on hydrogen production if used more than the proper concentration and was better for bacterial biomass suggested that the effect was related on the enzymes responsible for hydrogen evolution.

Table 3: Results of MgSO₄.7H₂O addition to fermentation medium on glucose consumption (%) and H₂ P (mLL⁻¹ h⁻¹) by *C. acetobutylicum* NCIMB 13357

[MgSO ₄ .7H ₂ O] (mgL ⁻¹)	f pH	Glucose consumed (%)	H ₂ P
50	4.50	89	52
100	4.51	90	52
200	4.50	91	56
500	4.48	94	65
750	4.52	96	53
1000	4.54	98	42

[Glucose] 5 gL⁻¹, inoculum size 10% (v/v), I pH. 7.0, Temp 30°C

Table 4: Results of MgSO₄.7H₂O addition to fermentation medium on hydrogen production by *C. acetobutylicum* NCIMB 13357

[MgSO ₄ .7H ₂ O] (mgL ⁻¹)	Y ¹ _{P/S}	Y ² _{P/S}	[Biomass]	Y _{P/X}	Y _{X/S}	Y _{H₂}
0.0	340	391	1.34	292	0.27	0.034
50	227	255	1.34	190	0.27	0.022
100	227	252	1.37	184	0.27	0.022
200	246	270	1.39	194	0.28	0.024
500	277	295	1.41	209	0.28	0.026
750	233	243	1.43	170	0.29	0.020
1000	183	187	1.47	127	0.29	0.018

Y¹_{P/S} (H₂ mL g⁻¹ glucose supplied) (mLg⁻¹), Y²_{P/S} (mLg⁻¹) (Utilized): (H₂ mL g⁻¹ glucose utilized), [Biomass] (gL⁻¹). Biomass production g L⁻¹ culture, Y_{P/X} (mLg⁻¹ L⁻¹): (H₂ mL g⁻¹ Biomass L⁻¹), Y_{X/S}: (Biomass production per g glucose supplied), Y_{H₂/S}: (conversion of H₂ (ml) to H₂ (g) g⁻¹ glucose utilized) [Glucose] .5 gL⁻¹, inoculum size 10% (v/v), I pH. 7.0, Temp 30°C

Effect of Magnesium: The results described below indicated that the hydrogen production rate and yield was enhanced with the addition of MgSO₄.7H₂O over a range of supplemented medium from 50-500 mgL⁻¹ but not with control at 0.0 mgL⁻¹, then decreased for further increasing of magnesium concentration with maximum H₂ yields of 295 mLg⁻¹ glucose utilized, obtained at MgSO₄.7H₂O concentration of 500 mgL⁻¹. The results shown in Fig. 7 demonstrate that the maximum H₂ productivity of 65 mLL⁻¹ h⁻¹ was obtained at MgSO₄.7H₂O concentration of 500 mgL⁻¹ and this was lower than the control of 77.5 mLL⁻¹ h⁻¹ of control at 0.0 mgL⁻¹. Table 3 and 4 shows the obtained results from MgSO₄.7H₂O addition to formulated medium on hydrogen production by the bacterium species we used in this study.

The results shown in Fig. 6 demonstrated that in supplemented culture, glucose consumed increased from 89% at 50 mgL⁻¹ to 94% at 500 mgL⁻¹ of MgSO₄.7H₂O and to 98% at 1000 mgL⁻¹ but that consumption was used for bacterial growth not for hydrogen production and that clear in Fig. 8, which demonstrated that as the concentration of magnesium increased the final biomass concentration increased with maximum of 1.466 gL⁻¹ at 1000 mgL⁻¹, suggested that this metal supported the bacterial growth, but not

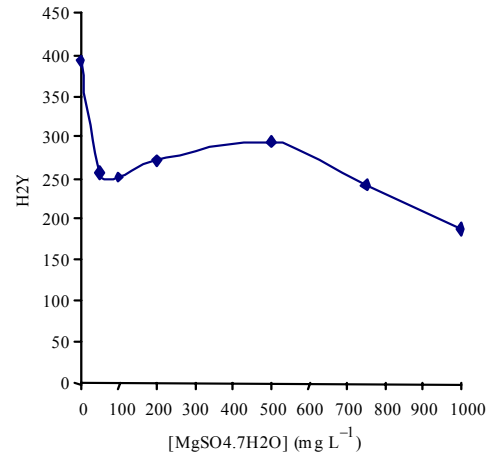


Fig. 5: Effect of [MgSO₄.7H₂O] addition on. H₂ Y (mLg⁻¹ utilized)

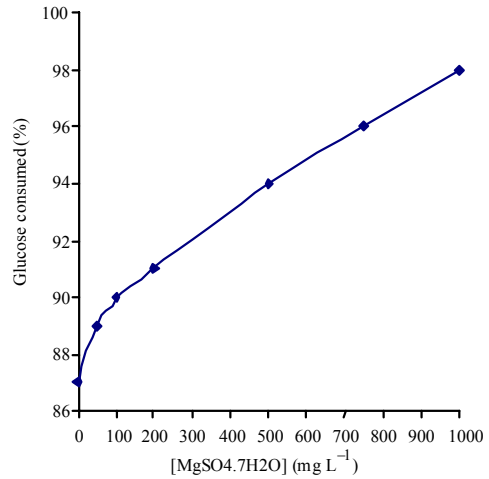


Fig. 6: Effect of [MgSO₄.7H₂O] addition on. Glucose consumption (%)

hydrogen production by *C. acetobutylicum* NCIMB 13357.

Obtained data shown in Table 3 and 4 were used to calculate different yields like Y_{P/X} (mLg⁻¹ L⁻¹): (H₂ mL g⁻¹ Biomass per L), Y_{X/S}: (Biomass production per g glucose supplied) and Y_{H₂/S} (conversion of H₂ (mL) to H₂ (g) per g glucose utilized). These results showed that the presence of magnesium in fermentation medium was adversely affected on hydrogen production but was better for bacterial growth.

Effect of calcium addition: The results shown in Table 5 and 6 indicated that the addition of CaCl₂.2H₂O to fermentation medium didn't enhance the hydrogen

Table 5: Effect of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ addition to fermentation medium on glucose consumption and hydrogen H_2 P ($\text{mL L}^{-1} \text{h}^{-1}$) by *C. acetobutylicum* NCIMB13357

[$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$] (mgL^{-1})	f pH	Glucose consumed (%)	H_2 P
0.0	4.45	87	77.5
10	4.46	88	49
50	4.46	88	50
100	4.38	90	59
150	4.39	93	58
300	4.41	94	52
500	4.48	96	45

[Glucose]: 5 gL^{-1} , Inoculum size 10% (v/v), I pH. 7.0, Temperature 30°C

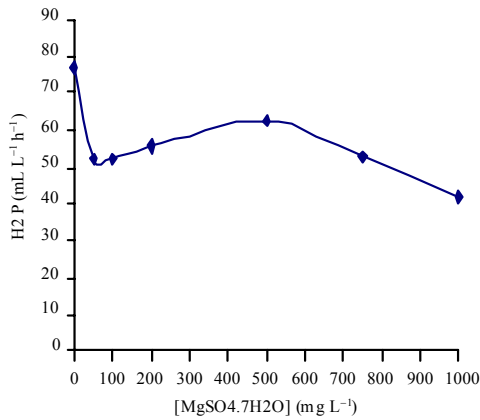


Fig. 7: Effect of [$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$] addition on H_2 P

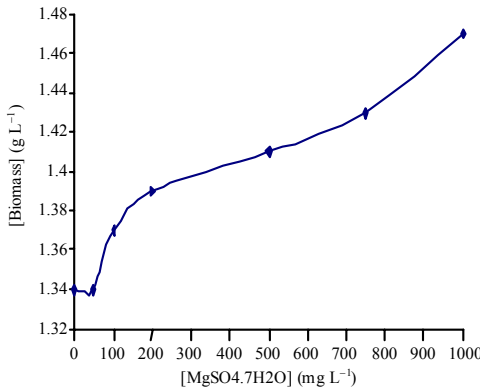


Fig. 8: Effect of [$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$] addition on. [Biomass]

production compared with the control. The results shown in Fig. 9 and 11, indicated that the addition of 100 mgL^{-1} of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to the new medium lead to increase in both H_2 production yield and hydrogen productivity over supplemented culture medium but not with control at 0.0 mgL^{-1} . The highest hydrogen yield in supplemented culture was 287 mLg^{-1} glucose utilized and that was lower than 391 mLg^{-1} glucose utilized, obtained at 0.0 mgL^{-1} . The results shown in

Table 6: Results of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ addition to fermentation medium on hydrogen production by *C. acetobutylicum* NCIMB13357

[$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$] (mg L^{-1})	$Y^1_{P/S}$	$Y^2_{P/S}$	[Biomass]	$Y_{P/X}$	$Y_{X/S}$	Y_{H_2}
0.0	340	391	1.34	292	0.27	0.034
10	214	243	1.34	181	0.27	0.022
50	221	251	1.38	182	0.28	0.022
100	258	287	1.43	201	0.27	0.026
150	172	185	1.47	126	0.29	0.014
300	147	156	1.58	99	0.32	0.014
500	135	141	1.73	82	0.35	0.012

$Y^1_{P/S}$ ($\text{H}_2 \text{ mL g}^{-1}$ glucose supplied) (mLg^{-1}), $Y^2_{P/S}$ (mLg^{-1}) (Utilized): ($\text{H}_2 \text{ mL g}^{-1}$ glucose utilized), [Biomass] (gL^{-1}). Biomass production g L^{-1} culture, $Y_{P/X}$ ($\text{mLg}^{-1} \text{L}^{-1}$): ($\text{H}_2 \text{ mL g}^{-1}$ Biomass L^{-1}), $Y_{X/S}$: (Biomass production per g glucose supplied), $Y_{H_2/S}$: (conversion of H_2 (mL) to H_2 (g) g^{-1} glucose utilized) [Glucose] $.5 \text{ gL}^{-1}$, inoculum size 10% (v/v), I pH. 7.0, Temperature 30°C

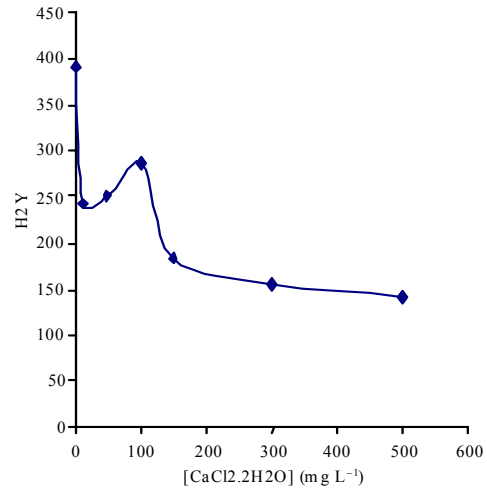


Fig. 9: Effect of [$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$] addition on. H_2 Y (mLg^{-1} utilized)

Fig. 10 reveal that the highest hydrogen productivity of $59 \text{ mL L}^{-1} \text{h}^{-1}$ obtained at 100 mgL^{-1} of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and that was lower than $77.5 \text{ mL L}^{-1} \text{h}^{-1}$ obtained at 0.0 mgL^{-1} and successive increases in $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ lead to decreased in both hydrogen yield and productivity over supplemented culture whereas the results shown in Fig. 10 it might be indicated that the presence of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in fermentation medium facilitate the bacterial growth by increasing the percentage of consumed glucose. The results shown in Fig. 12 indicating an excellent utilization efficiency of the carbon source was for bacterial growth and obtained in supplemented culture was at 500 mgL^{-1} of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ with maximum of 1.73 gL^{-1} . Obtained results suggested that calcium is important for bacterial growth but not for hydrogen production by *C. acetobutylicum* NCIMB13357.

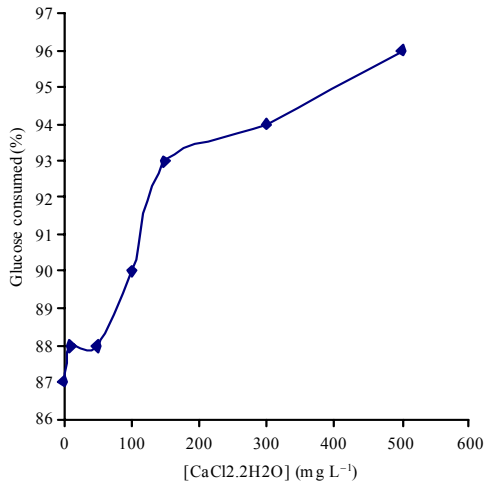


Fig. 10: Effect of [CaCl₂.2H₂O] addition on. Glucose consumption (%)

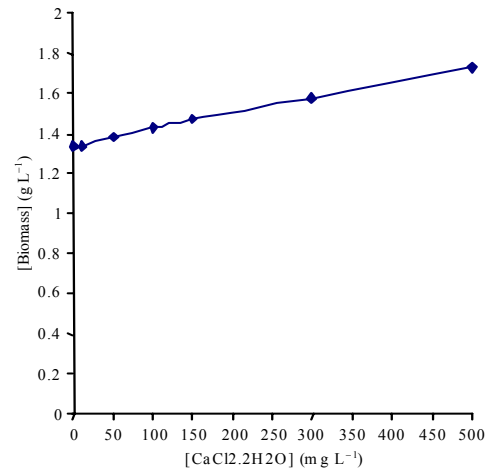


Fig. 12: Effect of [CaCl₂.2H₂O] addition on. [Biomass], [Glucose]: 5 gL⁻¹, inoculum size 10% (v/v) I pH. 7.0. Temperature 30°C

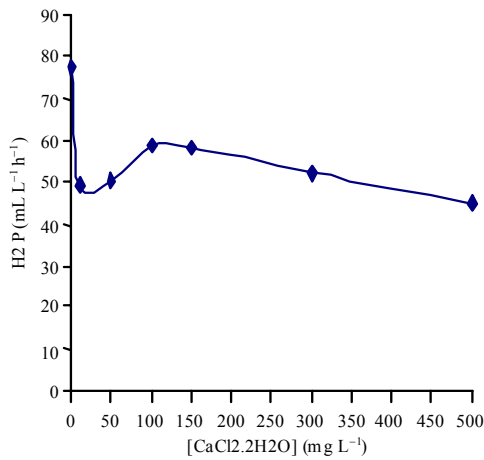


Fig. 11: Effect of [CaCl₂.2H₂O] addition on H₂ P

The results shown in Table 5 and 6 were used to calculate different yields like $Y_{P/X}$ (mLg⁻¹ L⁻¹): (H₂ mL g⁻¹ Biomass per L), $Y_{X/S}$: (Biomass production g⁻¹ glucose supplied) and $Y_{H_2/S}$ (conversion of H₂ (mL) to H₂ (g) per g glucose utilized). These results showed that calcium addition to the fermentation medium was inhibited the bacterial production of hydrogen but as well as magnesium addition, it was better for growth suggested that this trace metal affected in such way on the enzyme cascade for hydrogen production even if was in small amount in fermentation medium.

Iron is present in cytochromes and ferridoxins, enzyme cofactor and is important in regulation of secondary metabolism and also plays a role in excretion of primary metabolites^[2]. All processes of biological

hydrogen production are fundamentally dependent upon the presence of hydrogenases. It is hypothetically possible that the quantity or inherent activity of these enzymes could limit the overall process. However, even though the catalytic activity of the various enzymes is enormously different from each other, there is no evidence for the quantity of the hydrogen-producing enzyme being the limiting factor in any system currently under study^[6].

There is thus little information on how the iron concentration influences hydrogen production via fermentative bacteria. In this study, in order to convert glucose to high-value hydrogen efficiently, it was investigated that the role of ferrous ion concentration on anaerobic bacteria for hydrogen production. Liu and Shen^[7] mentioned that the external iron concentration affect the fermentation of anaerobic bacteria in term of hydrogen production. While others like Dabrock *et al.*^[8] reported that the iron concentration did not have affect on hydrogen production. The result of present study agreed with Peguin and Soucaile^[9] they reported that iron concentration above 25 μmol L⁻¹ was none limiting and the metabolism of *C. acetobutylicum* was normally acidogenic and molecular hydrogen was one of the metabolites and with Schoenhet *et al.*^[10] they demonstrated that growth of *C. pasteurianum* in batch culture was growth limited when the iron concentration of the medium was less than 10 μmol L⁻¹, while Dabrock *et al.*^[8] reported that iron concentration up to 10 μmol L⁻¹ were growth limiting for *C. pasteurianum* but did not cause a decrease of hydrogenase specific activity, a decrease of acid production and an increase

of solvent production. In our study, the maximum H₂ yield was obtained at 25 mgL⁻¹ FeSO₄.7H₂O. This iron concentration was higher than the 10 and 25 μmol L⁻¹ below which the growth of clostridia was limited by the iron concentration^[8,10]. It may be concluded that the effect of the iron concentration on the hydrogen production of clostridia may be caused by not only the iron concentration of the external as limiting factor for the growth but also other environment conditions which iron concentration affected.

In order to find out the conditions that could make the bacterium to be affected by the presence of iron in fermentation media, Junelles *et al.*^[11] pointed out that the hydrogenase specific activity was decreased, carbon and electron flow were affected under iron limitation. Whereas Mongi *et al.*^[12], they stated that at initial pH, the bacterial needed for iron was variant. They reported that at initial pH of 7.0, 1000 mgL⁻¹ of FeSO₄.7H₂O was sufficient and gave maximum production rate but at initial pH of 8.5, the needed was about 25 mgL⁻¹ of FeSO₄.7H₂O, suggested that iron supplementation depend in both initial pH, microorganisms and another factors that affected by iron concentration. Our results regarding the bacterial growth agreed with Junelles *et al.*^[11], they reported that iron limitation affect the growth and the endproducts of fermentation of *Clostridium acetobutylicum*. They found under iron limitation (0.2 mgL⁻¹) (in this study 5 mgL⁻¹) at a pH of 4.8, glucose was fermented to butanol as the major fermentation end product and the final conversion yield of glucose into butanol could be increased from 20-30% by iron limitation. They reported also that the Hydrogenase specific activity was decreased by 40% and acetoacetate decarboxylase specific activity by 25% under iron limitation. Concluding that under iron limitation the effect will be on carbon and electron flow in addition to hydrogenase.

Regarding the reported yield, the results of present study was more other reported yield like Lee *et al.*^[13], using sucrose and mixed microorganisms. They found the maximum hydrogen production yield of 131.9 mLg⁻¹ sucrose supplied was obtained at the iron concentration of 800 mgL⁻¹ FeCl₂ and with Zhang and Shen^[14] they reported that 200 mgL⁻¹ FeSO₄.7H₂O gave maximum production of hydrogen by mesophilic bacteria. All of these value suggested that the effect depend on the bacterial species and weather pure or mixed culture and also to the medium components and to iron source. For the majority of that researcher they were using mixed culture but for pure culture the conditions is different and also depends on the bacterial species they used. Yang and Shen^[2] they were studying

the effect of ferrous iron concentration on anaerobic bio-hydrogen production from soluble starch, mentioned that the bio-hydrogen production requires essential micronutrients for bacterial metabolism during fermentation. They found that Sodium, Magnesium, Zinc and Iron are all important trace metals affecting hydrogen production among them iron is one of the important nutrient elements to form hydrogenase or other enzymes which almost all biohydrogen production needs fundamentally. But for the bacterium we used in this study, the addition of iron was enhanced the bacterial productivity of hydrogen and that was concentration dependent and the addition of sodium was affect adversely on both the hydrogen production and bacterial growth. It was reported by Liu and Shen^[7] that the lag phase time was affected by the iron concentration when the Fe⁺² concentration was less than 20 mgL⁻¹ and the maximum hydrogen production increased as the iron concentration increased from 1.2-10 mgL⁻¹ then decreased for further increased of iron concentration. Their results showed that 10 mgL⁻¹ was optimum for bacteria (mixed) they used to produce hydrogen. For its medium it was found that 25 mgL⁻¹ of FeSO₄.7H₂O ≈ (5 mgL⁻¹ iron) was sufficient for *C. acetobutylicum* NCIMB13357 to maximize the bacterial productivity of hydrogen.

Iron supplementation in hydrogen fermentation media is important because hydrogen producing Clostridia possess the metalloenzymes, hydrogenase and NADH-ferredoxin reductase, which mediate hydrogen formation. Santangelo *et al.*^[15] they presented evidence from hydrogenase purified and characterized from two clostridial species showed that these enzymes contain iron clusters and a unique type of Fe-S centre termed the H₂ cluster. The H₂ cluster has been proposed as the site of H₂ oxidation and H₂ evolution^[16]. The iron clusters serve to transfer electrons between the hydrogen cluster and the external electron carrier^[17]. Therefore, it was expected that supplementing with iron would facilitate hydrogenase biosynthesis and hydrogen evolution, whereas iron deprivation could severely affect the enzyme's biosynthesis and function which would then shift the metabolic pathway to alcohol production^[18].

The magnesium requirement of bacteria is principally the result of a specific requirement of the ribosomes, it also functions as enzyme cofactor and is present in cell walls and membranes. It was reported by Bahl and Gottschalk^[19] that solvent not produced in magnesium limited medium maintained at low pH, whereas Jones and Woods^[20] reported that high yield of solvent were obtained in magnesium limited system. Magnesium is one of the most abundant elements

within microorganism. It is indispensable for many cell reactions and functions. According to literature magnesium ion is an activator of many kinases and synthetases. In cytoplasm there are 10 kinds of enzymes involved in glycolysis and most of these enzymes need magnesium ion as cofactors such as hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and enolase, etc.^[3].

Magnesium is not only a cofactor of many enzymes but also components of cell wall and cell membrane. The Mg-chelated species of adenine nucleotides are the true substrates for cellular phosphate transfer reactions. Thus, Mg is a cofactor for many enzymatic reactions, particularly kinases and phosphatases. Glycolysis involves a sequence of reaction converting glucose into pyruvate with the production of ATP. The first step in the glycolysis cascade is to phosphorylate glucose, thus trapping it in the cell. Phosphorylation is achieved in the presence of ATP as the phosphate source and a kinase enzyme (hexokinase in the case of this six-carbon sugar). The magnesium interacts with the oxygen of the triphosphate chain, thereby weakening the phosphorous-oxygen bond and making it more susceptible to cleavage reaction required for phosphoryl transfer. Buchanan *et al.*^[21] indicated the addition of $MgSO_4 \cdot 7H_2O$ resulted in an increase in hydantoinase activity. Our results was disagreed with Mandal and Mandal^[22] they reported that magnesium enhanced biogas production from organic wastes for an optimum value of 45 mg for 300 cm³ slurry but the presence of magnesium inside the fermentation medium was better for bacterial growth not for biogas production and that was clear when it is added even small amount to the fermentation medium suggested that each bacterium species need selected requirements with selected concentration to produce such products with maximum productivity. It was also reported by Mandal and Mandal^[22] that pure metallic magnesium was toxic at high concentration for biomethanation process.

The idea of Calcium addition came from the reports showing that addition of divalent metal ions (especially, Ca⁺²) enhanced sludge granulation and cell aggregation^[23]. Morgan *et al.*^[24] proposed that Ca⁺² is probably a constitution of extracellular polysaccharides known to play a major role in formation of biofilm and thereby enhances cell granulation and settling ability. Yu *et al.*^[24] also showed that addition of low concentrations of Ca⁺² to an UASB reactor enhanced the adsorption, adhesion and multiplication steps of sludge granulation. Calcium is present in bacterial spores and is required as a cofactor (and for stability) of

alpha-amylase and many proteases. It is usually expelled from the cytoplasm and can have an important structural role in the cell proteins and extracellular polymer material, capsule, etc.^[3]. These observations showed that calcium addition to fermentation medium was important to enhance the bacterial growth and that it was obtained in this study. Our results were disagreed with Yu *et al.*^[24] who found that at Ca²⁺ concentration of 150-300 mg L⁻¹, hydrogen production by mixed culture was maximum. Thus, addition of 100 mg L⁻¹ of $CaCl_2 \cdot 2H_2O$ was enough to retain a sufficient amount of biomass but not for biohydrogen production. Addition of calcium to the fermentation medium was adversely affect on bacteria for hydrogen production therefore the new medium it was formulated by using *C. acetobutylicum* NCIMB13357 for hydrogen production should have only iron as trace element with selected concentration and the others they were tested in this study were better for growth not for hydrogen production

CONCLUSION

Hydrogen production by *C. acetobutylicum* NCIMB13357 was enhanced in the presence of iron in fermentation medium and that was concentration dependent, while the addition of $MgSO_4 \cdot 7H_2O$ or $CaCl_2 \cdot 2H_2O$ was inhibited the hydrogen production. Maximum hydrogen yield of 408 mLg⁻¹ obtained by addition of 25 mgL⁻¹ $FeSO_4 \cdot 7H_2O$. Increasing the iron concentration resulted to reduce the bacterial production of hydrogen. All trace metals tested was better for the bacterial growth with maximum biomass concentration was obtained at maximum concentration of each.

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