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Orthogonal optimization of *Carboxydothermus hydrogenoformans* culture medium for hydrogen production from carbon monoxide by biological water-gas shift reaction

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ABSTRACT

The objective of the present study was to investigate the optimal nutritional requirements for hydrogen production from carbon monoxide by biological water-gas shift (WGS) reaction with *Carboxydothermus hydrogenoformans* using orthogonal layout methods. Cultures of *C. hydrogenoformans* on the medium as formulated by the strain supplier (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) unexpectedly showed a large content in inorganic material. This was confirmed by the rather elevated levels of calcium and phosphorus contained in the grown biomass. As an excessive production of those minerals may interfere with the growth and catabolism rates as well as clog up biofilm-based reactors, it was desirable to minimize the mineral accumulation during growth, while keeping at maximal levels both the H_2 yield and the specific H_2 production rate (SHPR). PO_4^{3-}, HCO_3^-, Ca^{2+} and Mg^{2+} were considered as potentially the major factors of the mineral accumulation. The experiments were designed according to the Taguchi's orthogonal method, using the above factors at three levels, and considering the culture mineral content, the H_2 yield and the SHPR as optimization criteria. Optimal concentrations of PO_4^{3-}, HCO_3^-, Ca^{2+} and Mg^{2+} were determined as (mM): 1.0, 5.0, 0.1 and 0.5, respectively. Under those culture conditions, Ca + P content decreased from 55.6 ± 1.8 to 9.5 ± 3% while the highest H_2 yield at 90.9 ± 1.2% and SHPR at 0.85 ± 0.06 mol H_2 g^{-1} VSS d^{-1} were achieved in bottle batch tests at 100% CO headspace atmosphere, neutral pH, a temperature of 70 °C, and an agitation of 100 rpm.

1. Introduction

The industrial hydrogen demand is expected to grow, due for instance on the short term, to substantial additional hydrogen requirements to produce higher quality gasoline from low-grade oil, or to produce liquid fuels from coal [1], or on the long term, to the hydrogen economy. At present, over 95% of the hydrogen produced is derived from fossil fuels such as coal, oil and natural gas, thus is not sustainable [2]. To be sustainable, hydrogen production has to be based on renewable energy using solar, wind, and biomass sources [3]. For instance, hydrogen can be extracted from carbohydrate-rich substrates (including industrial and municipal organic wastes, agriculture residues, biosolids, even energy crops) using dark anaerobic fermentation processes which would use similar hardware to that used currently in industrial

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methane fermentation, so bioreactor technologies are readily available [2,4–7]. Yet the H_2 yield of carbohydrates' dark fermentation is barely above 0.5 mol H_2/C-mol (compared to a stoichiometric H_2 potential of 2 mol/C-mol). Additionally a significant portion of biomass is difficultly and/or slowly biodegradable by microorganisms, due to its refractory and polymeric nature. When the organic residue is relatively dry (e.g. woodchips, bug wood …) or non-biodegradable (bark, plastic, rubber ….), it might be more appropriate to use thermochemical conversion techniques such as gasification, which results in a synthesis gas (syngas) mainly composed of carbon monoxide (CO), CO_2 and hydrogen. Syngas can be used directly to power industrial boilers, gas turbines or fuel cells to make electricity. As well syngas can be upgraded into H_2 using catalytic water-gas shift reaction. Catalyzed chemical processes are well established. They normally involve high pressure and/or temperature, may be problematic when impurities are present and tend to have low product specificity [8]. To circumvent these disadvantages and use milder treatments with minimal chemical and energy, biologically-mediated water-gas shift (WGS) reaction could be used to convert the carbon monoxide contained in syngas into hydrogen. Biological WGS reaction is catalyzed by an increasing number of carboxydotrophic microorganisms using CO as a preferred electron donor [9] and could become a cost-effective technology for upgrading syngas into hydrogen. Those microbes contain an intricate enzymatic system initiated by the oxidation of CO to CO_2 by a CO-dehydrogenase (CODH) [10–12] and closed by the reduction of two protons to form H_2 [13]. *Carboxydothermus hydrogenoformans* is one of those CO-oxidizing (carboxydotrophic) bacterial species that have been isolated and are able of forming H_2 from CO [12,14–17]. *C. hydrogenoformans* is an extreme-thermophilic anerobic bacterium that grows fast on CO as the sole carbon and energy source (doubling time 2 h) at optimal temperature of 70 °C. Microbial growth by conversion of CO to H_2 and CO_2 using *C. hydrogenoformans* (DSM6008) was first shown by Svetlichny in 1991 [14], then studied in more details by Henstra and Stams [16,17], with different medium compositions as listed in Table 1. Cultures of *C. hydrogenoformans* on the medium as formulated by the strain supplier (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ) unexpectedly showed after enrichment, based on the ratio of volatile suspended solids (VSS) to the suspended solids (SS) and elemental analysis, a large content in inorganic material, i.e. 93.6 ± 3% of cell dry weight (dr. wt). This was confirmed by the rather elevated levels of calcium and phosphorus contained in the grown biomass. As an excessive production of those minerals may interfere with the growth and catabolism rates as well as clog up biofilm-based reactors, it is desirable to minimize the accumulation during growth, while keeping at maximal levels both the H_2 yield and bioactivity rate. Alike any fermentation activity, biohydrogen production requires a medium formulation which could satisfy the elemental requirements for cell growth and catabolite production [2,18–22]. Because of the large number of nutritional components that are potentially essential and the number of interactions to evaluate, it is impractical to choose a design method including all the factors that need to be considered. However, a variety of experimental design methods can be used to investigate optimum parameters for maximum yield and rate; among them is the Taguchi's method [23–26]. The Taguchi method is based on a fractional factorial design and allows an experiment to be complete with only a fraction of all possible experimental combinations of variable values [27]. The method determines the effect of variables and noise factors by a «signal to noise ratio » which equally considers the effectiveness with which objectives have been attained and the degree of variability that has been experienced. As a result, with the aid of the range and variance analysis, the key factors that have significant effects on a response can be identified and the best factor levels for a given process can be determined from the pre-determined factor levels [24,25,28,29]. While some nutrients at a sub-

Table 1 – Composition of culture medium and bioactivity results for *C. hydrogenoformans* as reported in literature.

Reference	DSM[a]	Svetlichnyi 1991 [14]	Svetlichnyi 2001 [12]	Henstra 2004 [16]
Component (mmol/L)				
KCl	4.44	–	–	–
NaCl	–	–	–	5.13
NH_4Cl	6.17	6.17	28.04	5.61
$CaCl_2 \cdot 2H_2O$	1.97	1.51	0.14	0.75
$MgCl_2 \cdot 6H_2O$	2.56	2.52	0.88	0.49
KH_2PO_4	2.43	2.43	2.21	3.01
$Na_2HPO_4 \cdot 2H_2O$	–	–	1.69	2.98
$NaHCO_3$	11.90	11.90	5.95	17.86
$Na_2S \cdot 9H_2O$	2.92	2.08	0.83	0.83
$NiCl_2$	0.002	0.002	0.002	0.002
Bioactivity results				
Hydrogen yield (%)	na	99	100	82
H_2 production rate[b] (μmol H_2/ml culture.h)	na	7.33	nd	26.6
Cell density (cells/ml)	na	$2.8\ 10^8$	nd	nd
Cell density (OD_{660})	na	nd	nd	0.11

na: not applicable; nd: not determined.
a Medium composition from the strain supplier (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).
b The production rate is obtained by multiplying the hydrogen yield and carbon monoxide consumption rate as reported by the authors.

optimal level may only limit the bioactivity, other nutrients or components in excess to the optimal level may inhibit the bioactivity or stimulate the production of non-desirable co-metabolites or secondary products [28]. It is assumed that it is what occurred actually during the growth of *C. hydrogenoformans* with the medium used. Considering the literature data referring to the growth defined media composition (Table 1) two anions, PO_4^{3-} and HCO_3^-, and two cations, Ca^{2+}, Mg^{2+}, were identified as the potential major factors of minerals' accumulation during the *C. hydrogenoformans* growth. NH_4Cl and Na_2S were only provided as the nitrogen source and the reducing agent, respectively. As well, $NiCl_2$ was added as a cofactor to stimulate the bioactivity rate [30]. Hence the objective of the present study was to determine their optimal concentrations via an experimental design according to the Taguchi's orthogonal method, using three levels for each factor, and considering the culture calcium and phosphorus (Ca + P) content, the H_2 yield and the SHPR as optimization criteria.

2. Materials and methods

2.1. Culture

C. hydrogenoformans DSM6008 was obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The strain was cultivated under strictly anaerobic conditions at 70 °C and 100 rpm in basal mineral medium buffered with bicarbonate-phosphate as described by DSMZ. The original medium formulation obtained from DSMZ contained (g/L): NH_4Cl (0.33), $MgCl_2 \cdot 6H_2O$ (0.52), $CaCl_2 \cdot 6H_2O$ (0.33), KCl (0.33), KH_2PO_4 (0.33), yeast extract (0.5), $Na_2S \cdot 9H_2O$ (0.3). This media was then supplemented with 10 ml/L trace metals solution and 10 ml/L of vitamins solution according to Stams [31]. The initial pH was always maintained between 6.8 and 7.0. The initial biomass was a culture enrichment prepared on the DSM medium and had an inorganic content of 88.5 ± 0.6 and 90 ± 3% dr. wt in the optimization and validation phases of the study, respectively.

2.2. Specific activity tests

The carboxydotrophic (CO-oxidizing) specific activity tests were performed in triplicate in 120 ml serum bottles. Both substrate (CO) depletion and catabolites (H_2, volatile fatty acids (VFAs) and alcohols) production rates were monitored. The biomass and its mineral content were also quantified at the end of each test. Briefly once inoculated with 60 ml of culture and filled with buffer solutions, bottles were capped, sealed and flushed with 100% CO as sole carbon source, and placed in a rotary shaker (New Brunswick, Edison, NJ) in a dark, thermostatically controlled environment (70 ± 1 °C) and gyrated at 100 rpm. Besides the components tested (PO_4^{3-}, HCO_3^-, Ca^{2+} and Mg^{2+}), the activity test media also contained: NH_4Cl 28 mM, $NiCl_2$ 0.002 mM, $Na_2S \cdot 9H_2O$ 0.83 mM, resazurin 0.05 mg/L, yeast extract 0.05 g/L, 10 ml of both trace metals and vitamins solutions prepared according to Stams [31]. All solutions were autoclaved, except the mother $CaCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 7H_2O$ and vitamins solutions, which were sterilized with 0.22-μm filters. The tests at neutral pH were pH-controlled by addition of 1 M HCl. The hydrogen yield (Y_{H_2}) was expressed as a percentage of the H_2 gas produced per CO consumed (mol/mol). The specific H_2 production rate (SHPR), expressed as mol H_2/g VSS·d, was obtained by reporting the rate of H_2 produced (mol/d) to the VSS-based biomass as estimated in the bottle.

2.3. Analytical methods

Measurements of chemical oxygen demand (COD), suspended solids (SS) and suspended volatile solid (VSS) were made according to standard methods [32]. COD-based biomass was converted into VSS using a factor of 1.30 g COD/g VSS according to the average elemental formula $CH_{1.86}O_{0.6}N_{0.16}$ of anaerobic bacteria [33]. The cell's mineral content was estimated by difference between SS and VSS. Phosphorus was determined colorimetrically [34] and calcium and metals, by inductively coupled plasma [35], both on the centrifuged, rinsed and resuspended culture. Volatile fatty acids (VFAs, i.e. acetic, propionic and butyric acids) were measured on an Agilent 6890 gas chromatograph (Wilmington, DE) equipped with a flame ionization detector (FID) on 0.2 μl samples fortified 1:1 (vol./vol.) using an internal standard of iso-butyric acid in 6% formic acid, directly injected on a glass column of 1 m × 2 mm Carbopack C (60–80 mesh) coated with 0.3% Carbowax 20 M and 0.1% H_3PO_4. The column was held at 130 °C for 4 min and helium was the carrier gas at a rate of 20 ml/min. The injector and the detector were both maintained at 200 °C. For measurement of solvents (methanol, ethanol, acetone, 2-propanol, tert-butanol, n-propanol, sec-butanol, n-butanol) 100 μl of liquid were transferred in 20 ml headspace vial crimped with Teflon coated septum. The vial was heated at 80 °C for 2 min, then 1000 μl of headspace gas were injected on a DB-ACL2 capillary column of 30 m × 530 mm × 2 μm using a Combipal autosampler (CTC Analytics AG, Zwingen, Swizerland). The column was held at 40 °C for 10 min. Helium was the carrier gas at a head pressure of 5 psi. The injector and the detector were maintained at 200 °C and 250 °C, respectively. The gas composition (H_2, CO, CO_2) was measured by injecting 300 μl of gas (model 1750 gas-tight syringe, Hamilton, Reno, NV) taken from the bottle headspace after equilibrium at 70 °C into a HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a thermal conductive detector (TCD) and a 11 m × 3.2 mm 60/80 mesh Chromosorb 102 packed column (Supelco, Bellafonte, PA). The column temperature was held at 60 °C for 7 min and increased to 225 °C at a rate of 60 °C per min. Argon was used as the carrier gas. The injector and detector were maintained at 125 °C and 150 °C respectively.

2.4. Experimental design and statistical analysis

The experiments were designed according to the Taguchi's orthogonal method [24]. The optimization criterion was primarily the minimum mineral cell content but the maximum hydrogen production yield and the specific hydrogen production rate were also considered. The selected factors included PO_4^{3-}, HCO_3^-, Ca^{2+} and Mg^{2+} were examined using 3 levels for each factor in 9 experimental runs

[orthogonal layout $L_9(3^4)$]. The four variables and their levels (coded as 1, 2, 3) are given in Table 2. The values chosen for the each level were based on the concentration range found in the literature. The response of the culture mineral content, the H_2 yield and the SHPR to the 9 different combinations of the above four factors were evaluated using the range and variance analysis. Scores (response results) are summed for each factor and each level (k_1, k_2 and k_3, for levels 1, 2 and 3, respectively) as well as averaged (K_1, K_2 and K_3, for levels 1, 2 and 3, respectively). The range (*R*) among the score averages is the difference between the highest and the lowest score average (K), i.e. $K_{max}-K_{min}$, for each factor. The *R* value is used to rank the four factors with respect to their impact to the optimization objectives and to evaluate the importance of their contribution to the variation of each objective. The analysis of variance is also used to determine the significance level of factors' impact and rank them with respect to the variability magnitude [36]. The variance analysis is based on the sum of squares of deviations from the overall mean (SS), the degrees of freedom, df_F and df_{Error}, respectively the degree of freedom of factor (A, B, C, or D) and of error, the mean square (MS) i.e. SS/df_F, and F. The df_F is the number of observations minus one (i.e. 2 in this study, where scores are based on triplicates). The df_{Error} is the total number of observations minus one and minus the freedom degrees of each factor (i.e. $df_{Error} = n - 1 - df_A - df_B - df_C - df_D = 18$). The F distribution ratio is given by the MS/MS_{Error} ratio. The MS_{Error} estimates the experimental error. In this study, the minimum MS of the four factors is considered as the experimental error considering that the unbiased estimates of the sum of squares are much smaller than the variance of the other factors. A factor is considered as having an impact which is statistically significant at a probability of 99% and 95% if the F ratio calculated for the factor evaluated is greater than the critical value of the F distribution with 2 and 18 df for a *P*-value of 0.01 and 0.05, $F_{0.01}(2,18)$ and $F_{0.05}(2,18)$, i.e. 6 and 3.6, respectively.

3. Results and discussion

As mentioned above, cultures of *C. hydrogenoformans* grown on the defined medium as formulated by the strain supplier (DSMZ) showed a large content of inorganic material (up to 93.6% dr. wt). This was confirmed by the rather elevated levels of calcium and phosphorus, 39.0 and 19.2% dr. wt, respectively, which corresponded to 42 and 21% of the cell mineral content (ash), respectively. This Ca/P ratio is close to that of a few known calcium phosphates such as hydroxyapatite and octacalcium phosphate [37,38]. Whereas a *Serratia* species has been shown to form crystals of calcium phosphate, identified as hydroxyapatite [39], such an attribute was never reported for *C. hydrogenoformans*. Therefore, should this excessive calcium phosphate production be avoided, the medium contents in PO_4^{3-}, HCO_3^-, Ca^{2+} and Mg^{2+} were considered as potentially the major factors of the mineral accumulation. The results of the $L_9(3^4)$ orthogonal design are analyzed considering as optimization criteria: the cell Ca + P accumulation (to minimize) as well as the H_2 yield and the SHPR (both to maximize) (Table 3). The parameters k1, k2, k3 and K1, K2, K3 are the summed and average scores (Ca + P %, Y_{H_2}, SHPR) of levels 1, 2 and 3, respectively, for each factor. The range of the scores (*R*) of levels 1, 2 and 3 is the maximal difference between the averages of the scores (K).

Table 2 – Factors and levels of the $L_9(3^4)$ orthogonal design.

Factor	A PO_4^{3-} (mmol/L)	B HCO_3^- (mmol/L)	C Ca^{2+} (mmol/L)	D Mg^{2+} (mmol/L)
Level 1	5.0	20.0	2.0	2.5
Level 2	2.5	10.0	0.7	1.0
Level 3	1.0	5.0	0.1	0.5

3.1. Optimal medium content for minimum cell content in calcium and phosphorus

Within the nine conditions experimented, the Ca + P content of the culture ranged from 4 to 38.9% dr. wt. The lowest experimental Ca + P content of cells (4%) was obtained at the seventh experimental run where: $PO_4^{3-} = 1.0$ mM, $HCO_3^- = 20.0$ mM, $Ca^{2+} = 0.1$ mM, $Mg^{2+} = 1.0$ mM. The optimal concentration for each factor for the minimization of the cell Ca + P content is determined by the minimal value of K, which occurred at levels 3 for PO_4^{3-} ($K_3 = 8.74$), 1 for HCO_3^- ($K_1 = 14.75$), 3 for Ca^{2+} ($K_3 = 16.91$) and 1 for Mg^{2+} ($K_1 = 17.52$) (Table 3). Accordingly, the optimal medium composition would be as following (mM): PO_4^{3-} 1.0, HCO_3^- 20.0, Ca^{2+} 0.1, Mg^{2+} 2.5. This is close to the results of the seventh run, and as expected, correspond to the lowest level of the concentration range tested for PO_4^{3-} and Ca^{2+}. Based on the magnitude of R, PO_4^{3-} (with $R = 19.15$) appeared as the factor whose variation has the greatest impact on the variation of the Ca + P content of cells. Furthermore based on the variance analysis (Table 4), the phosphate effect on the Ca + P content of cells was significant, as indicated by the F test. The F ratio is equal to 7.9, which is higher than the critical value of the F distribution with 2 factors' degrees of freedom (df), 18 error df, and a probability level (*P*-value) of 0.01 i.e. $F_{0.01}(2, 18) = 6$. Therefore phosphate concentration clearly is the most important factor that rules the mineral accumulation by cells, explaining more than 40% of the Ca + P content variation.

3.2. Optimal medium content for maximum H_2 yield

Within the nine conditions experimented, the H_2 yield reached a peak (92.7 ± 5.6%) at the sixth experimental run where: $PO_4^{3-} = 2.5$ mM, $HCO_3^- = 5.0$ mM, $Ca^{2+} = 2.0$ mM, $Mg^{2+} = 1.0$ mM (Table 3). The lowest H_2 yield was observed when the PO_4^{3-} and HCO_3^- concentrations increased to 5 mM and 10 mM, respectively. Small amounts of VFAs and alcohols were also observed in the culture liquid at the end of the test. The highest metabolite amount observed in the liquid corresponded to 16% of the substrate consumed, 97% of which was acetate. As above, the optimal medium composition was determined from the K values, as 2.5 mM for PO_4^{3-}, 5.0 mM for HCO_3^-, 0.1 mM for Ca^{2+} and 0.5 mM for Mg^{2+} (Table 3). Based on the magnitude of *R* the factors could also be ranked as following: PO_4^{3-} ($R = 9.64$) > HCO_3^- ($R = 6.42$) ≈ Ca^{2+} ($R = 6.40$) > Mg^{2+} ($R = 2.84$). Phosphate had the most important effect and explained with bicarbonate 60% of

Table 3 – Orthogonal design $L_9(3^4)$ and results.

Run	Factor				Ca + P content (% dr. wt)	H_2 yield (%)	SHPR (mol/gVSS·d)
	A	B	C	D			
1	1	1	1	1	22.3	79.1 ± 8.1	0.59 ± 0.20
2	1	2	2	2	38.9	71.8 ± 7.8	0.36 ± 0.16
3	1	3	3	3	22.5	87.5 ± 4.0	0.84 ± 0.21
4	2	1	2	3	17.9	85.0 ± 0.2	0.47 ± 0.10
5	2	2	3	1	24.2	89.7 ± 10.3	0.60 ± 0.12
6	2	3	1	2	34.6	92.7 ± 5.6	0.40 ± 0.01
7	3	1	3	2	4.0	82.1 ± 5.0	0.52 ± 0.09
8	3	2	1	3	16.2	82.6 ± 1.4	0.42 ± 0.00
9	3	3	2	1	6.0	83.2 ± 0.7	0.35 ± 0.03

Analysis of the Ca + P content

Factor	k_1	k_2	k_3	K_1	K_2	K_3	R (K_{max}–K_{min})
A	83.67	76.73	26.21	27.89	25.58	8.74	19.15
B	44.24	79.32	63.04	14.75	26.44	21.01	11.69
C	73.04	62.85	50.72	24.35	20.95	16.91	7.44
D	52.57	77.49	56.54	17.52	25.83	18.85	8.31

Analysis of the H_2 yield

Factor	k_1	k_2	k_3	K_1	K_2	K_3	R (K_{max}–K_{min})
A	238	267	248	79	89	83	9.64
B	246	244	263	82	81	88	6.42
C	254	240	259	85	80	86	6.40
D	252	247	255	84	82	85	2.84

Analysis of the specific hydrogen production rate (SPHR)

Factor	k_1	k_2	k_3	K_1	K_2	K_3	R (K_{max}–K_{min})
A	1.79	1.45	1.29	0.60	0.48	0.43	0.17
B	1.58	1.38	1.58	0.53	0.46	0.53	0.07
C	1.40	1.18	1.95	0.47	0.39	0.65	0.26
D	1.54	1.27	1.73	0.51	0.42	0.58	0.15

k_1, k_2 and k_3 are the sum scores of Level 1, Level 2 and Level 3 for each factor.
K_1, K_2, and K_3 are the average scores of Level 1, Level 2 and Level 3 for each factor.
R is the range among the average scores for each factor, estimated by the difference between the highest and the lowest score average (K), i.e. K_{max}–K_{min}.
Meaning of other acronyms given in the text.

the H_2 yield variability. The effect of those two major independent variables on the H_2 yield is shown in the response surface plot of Fig. 1. It is noteworthy that it was the middle phosphate concentration (level 2) which concurrently resulted in the highest H_2 yields. The analysis of variance (Table 4) showed that the PO_4^{3-} factor had the highest significant impact on the H_2 yield at a 99% confidence level (*P*-value of 0.01) whereas HCO_3^- and Ca_2^+ had a significant impact only at a 95% confidence level (*P*-value of 0.05) and Mg^{2+} had no influence (F ratio = 1.0).

3.3. *Optimal medium content for maximum specific hydrogen production rate*

The SHPR results obtained from the nine experimental runs of the $L_9(3^4)$ orthogonal design ranged between a maximum of 0.84 and a minimum of 0.35 mol H_2/g VSS·d. As above, the optimal medium composition was determined from the K values, as following: PO_4^{3-} 5.0 mM, HCO_3^- 5.0 mM, Ca^{2+} 0.1 mM, Mg^{2+} 0.5 mM (Table 3). Based on the magnitude of R the factors could also be ranked as following: Ca^{2+} (R = 0.256)> PO_4^{3-} (R = 0.167) > Mg^{2+} (R = 0.153) > HCO_3^- (R = 0.068). Accordingly Ca^{2+} had greater impact than the other factors, explaining alone more than 40% of the SHPR variability. In addition, the variance analysis (Table 4) indicated a high significance of the role of Ca^{2+} on SHPR, while the PO_4^{3-} and Mg^{2+} factors, whose F ratios were

Table 4 – Analysis of variance.

Source of variance	SS	MS	F	Significance level
Ca + P content challenge				
A	218.6	109.3	7.9	**
B	68.5	34.6	2.5	
C	27.7	13.9	1.0	
D	39.8	19.9	1.4	
Hydrogen yield challenge				
A	48.2	24.1	11.7	**
B	24.5	12.2	6.0	*
C	22.4	11.2	5.3	*
D	4.0	2.0	1.0	
Specific hydrogen production rate challenge				
A	0.015	0.007	4.8	*
B	0.003	0.002	1.0	
C	0.035	0.018	11.5	**
D	0.012	0.006	3.9	*

SS, sum of squares of deviations from the overall mean.
MS, mean square i.e. SS/df_F.
df_F, degree of freedom of factor (A, B, C, or D) i.e., 2 in this study.
F, F distribution ratio, i.e. MS/MS_{Error}.
MS_{Error}, experimental error.
Explanation of the significance level and of other acronyms are given in the text (section 2.4).

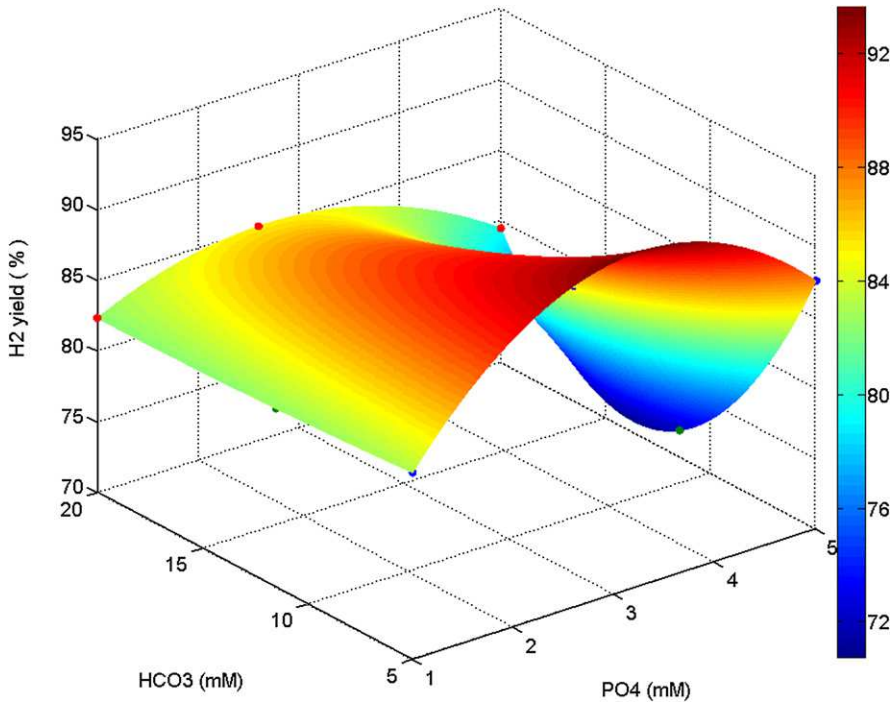

Fig. 1 – Effect of PO_4^{3-} and HCO_3^- concentrations on the hydrogen yield. HCO_3 (y-axis) and PO4 (x-axis) stand for HCO_3^- and PO_4^{3-} concentrations, respectively.

larger than the critical value of $F_{0.05}(2,18)$, had also a significant effect although at a lesser degree. The effect of the two major independent variables, Ca^{2+} and PO_4^{3-}, on the SHPR is shown in the response surface plot of Fig. 2. Most favorable Ca^{2+} and Mg^{2+} concentration values for the SHPR are 0.1 and 0.5 mM, respectively (Table 3), while higher concentration of those cations might repress the H_2 production rate, as shown in Fig. 3.

3.4. *pH effect on cell mineral content and biological activity*

An important pH variation was observed during the tests, as the medium pH increased in some runs up to 8.9. Interestingly, the culture was able to grow and produce H_2 at reasonable yield for a broader pH range than previously reported by Svetlichnyi

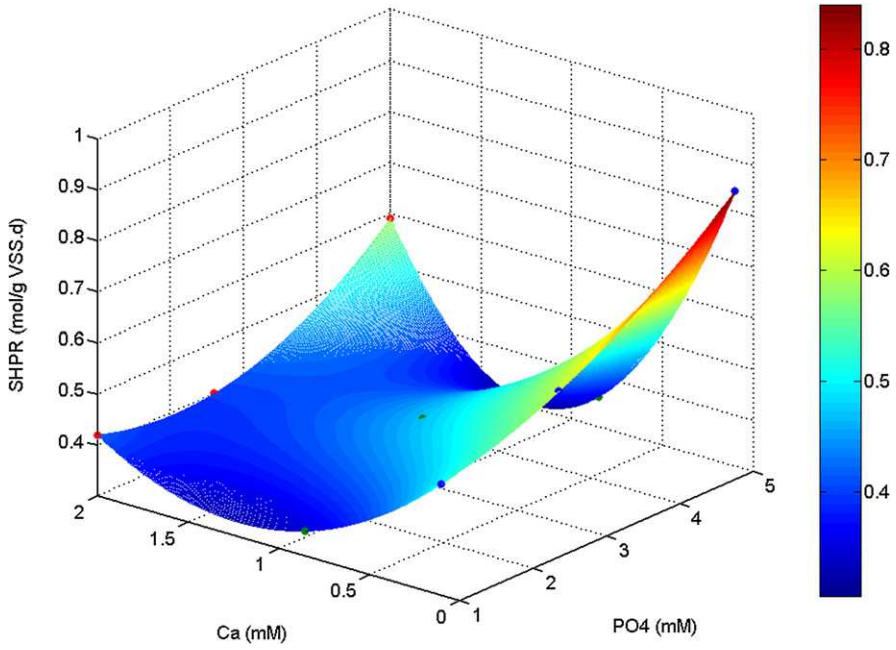

Fig. 2 – Effect of Ca^{2+} and PO_4^{3-} concentrations on the specific hydrogen production rate (SHPR). Ca (y-axis) and PO_4 (x-axis) stand for Ca^{2+} and PO_4^{3-} concentrations, respectively.

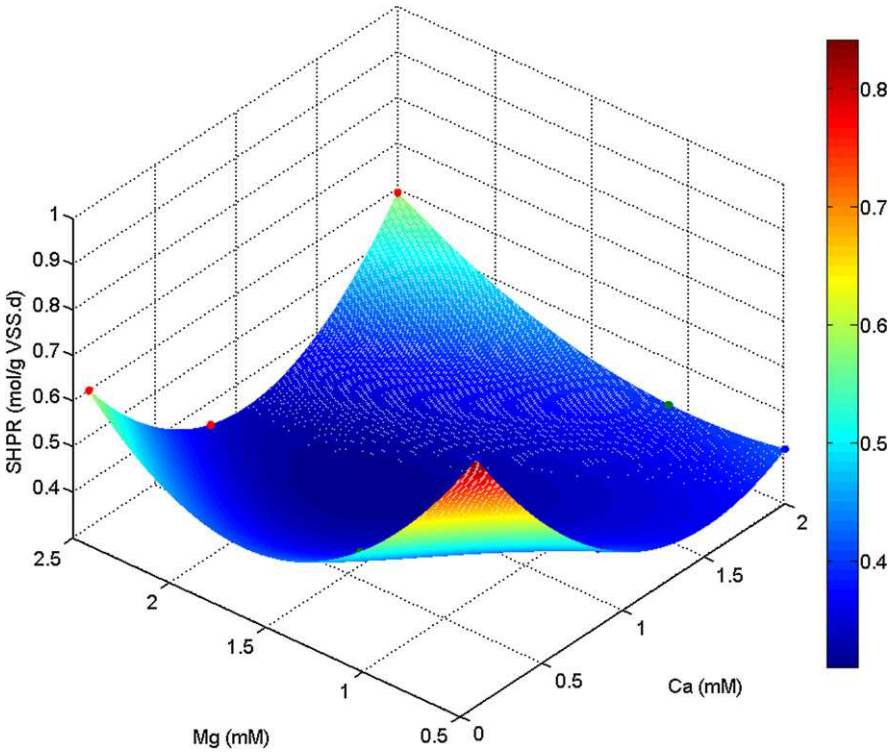

Fig. 3 – Effect of Ca^{2+} and Mg^{2+} concentrations on the specific hydrogen production rate (SHPR). Mg (y-axis) and Ca (x-axis) stand for Mg^{2+} and Ca^{2+} concentrations, respectively.

[14]. On the other hand, pH value did affect the SHPR. At a given medium composition (for instance $PO_4^{3-} = 1.0$ mM, $HCO_3^{-1} = 5.0$ mM, $Ca^{+2} = 0.7$ mM, $Mg^{+2} = 2.5$ mM as in the 9th experimental run), SHPR staid at 0.57 ± 0.05 mol/g VSS·d when the pH was kept neutral as compared to 0.35 ± 0.03 when pH rose to 7.8 (Table 3). This is consistent with literature, which reported optimal *C. hydrogenoformans* activity at neutral pH [14], while hydrogen dark fermentation also is often repressed at alkaline pH [6,40–42]. As well pH affected the cell mineral content. In Fig. 4, the mineral content response surface has been plotted for different pH and PO_4^{3-} concentrations, since the latter factor was the most effective on the calcium and

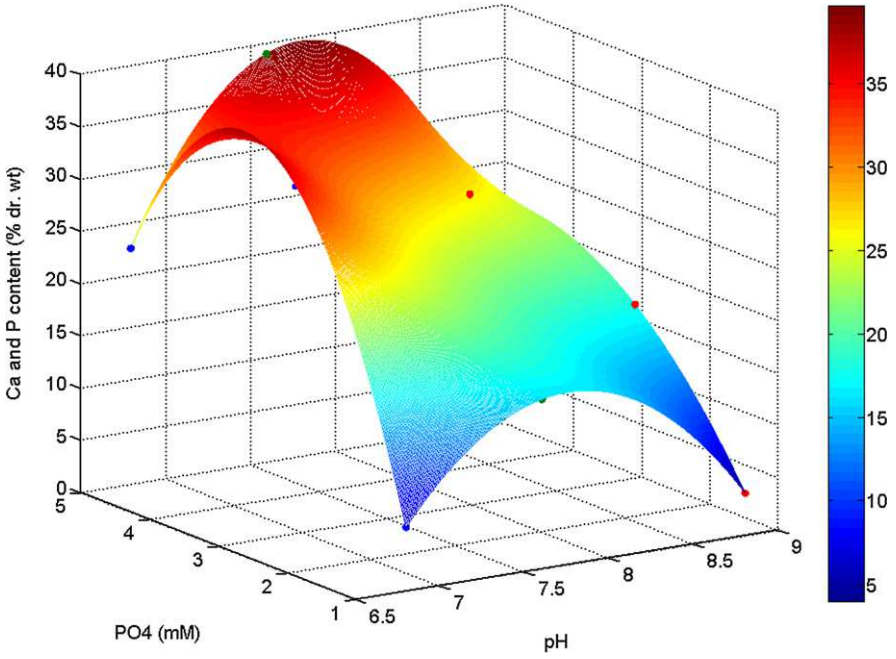

Fig. 4 – Effect of PO_4^{3+} concentration and pH on the cells content in calcium and phosphorus. PO_4 (y-axis) stands for PO_4^{3+} concentration.

phosphorus accumulation, as discussed in Section 3.1. A visual analysis of Fig. 4 reveals that a minimal Ca + P content (4 and 6%) could be obtained at highest PO_4^{3-} concentration and at neutral and highest pH values (7.5 and 8.9 respectively). This is consistent with literature which reported that solubility of a Ca- and P-rich chemical, such as hydroxyapatite, increases in presence of carbonate and phosphate with the increase of pH [38,43]. Since biological activity as well as solubility of the mineral content varied with the pH independently of the ions content of the culture medium, this variation interfered with the objective of the present study. In order to analyze the effect of the defined medium composition on mineral content only in biological optimal conditions a second series of tests was carried out at pH controlled in the neutral range (6.8–7.3).

3.5. *Optimal medium formulation at neutral pH*

Next experiments were carried out using the same orthogonal layout $L_9(3^4)$ as previously (Table 5). The Ca + P content of the culture varied from 21 to 50.5%. A maximum SHPR of 0.84 ± 0.22 mol H_2/g VSS·d was observed at third experimental group, which also showed a relatively low Ca + P content (22.5%) and an Y_{H_2} of 87.5 ± 7% (i.e. not significantly different from the maximal one, 92.7 ± 5.6%). Comparing the observed results in Table 2 and Table 5, both hydrogen production rate and yield were improved (except in 2 cases out of 9 for SHPR) due to the control of pH in the neutral range. The results were analyzed considering the minimization of the Ca + P content of cells as the optimization criterion (Table 5). From variance analysis (not shown), only the F ratios of Ca, 4.2, was larger than the critical value of $F_{0.05}(2,18)$, indicating that only this factor had a significant impact on the mineral content. The magnitude of the variance, expressed in percentage, reflects the significance of the contribution of each factor, hence their ranking, toward this target (Table 6). Calcium was definitely the main factor for minimizing the mineral content, explaining 32% of the variance effect on the mineral content, as well as 43% and 28% of the variance effect on H_2 yield and SHPR, respectively (not shown). Accordingly, considering that minimum mineral content is the primary target, the optimal defined medium formulation should contain (mM): PO_4^{3-} 1, HCO_3^- 5, Ca^{2+} 0.1, Mg^{2+} 0.5. We presumed that this formulation was also appropriate for satisfactory H_2 yield and SHPR levels since the 3rd run performed with the HCO_3^-, Ca^{2+}, Mg^{2+} levels as in the optimal formulation resulted in highest SHPR and Y_{H_2}, as above-mentioned, and that the runs performed with 1 mM PO_4^{3-} (runs 7, 8 and 9 in Table 5, PO_4^{3-} at level 3) as in the optimal

Table 5 – Orthogonal design $L_9(3^4)$ and results for pH controlled in the neutral range.

Run	Factor				Ca + P content (% dr. wt)	H_2 Yield (%)	SHPR (mol/gVSS·d)
	A	B	C	D			
1	1	1	1	1	50.5	94.1 ± 6.8	0.70 ± 0.15
2	1	2	2	2	38.9	71.8 ± 7.8	0.36 ± 0.06
3	1	3	3	3	22.5	87.5 ± 7.4	0.84 ± 0.22
4	2	1	2	3	24.0	85.6 ± 9.6	0.58 ± 0.18
5	2	2	3	1	30.5	90.0 ± 1.7	0.53 ± 0.00
6	2	3	1	2	34.6	92.7 ± 5.6	0.39 ± 0.01
7	3	1	3	2	21.0	85.4 ± 6.3	0.72 ± 0.23
8	3	2	1	3	29.7	89.9 ± 6.2	0.51 ± 0.05
9	3	3	2	1	22.7	84.2 ± 13.5	0.57 ± 0.05

Analysis of the Ca + P content

Factor	k_1	k_2	k_3	K_1	K_2	K_3	R (K_{max}–K_{min})
A	111.81	89.01	73.37	37.27	29.67	24.46	12.82
B	95.49	99.01	79.69	31.83	33.00	26.56	6.44
C	114.67	85.56	73.95	38.22	28.52	24.65	13.57
D	103.57	94.52	76.10	34.52	34.51	25.37	9.16

Analysis of hydrogen yield

Factor	k_1	k_2	k_3	K_1	K_2	K_3	R (K_{max}–K_{min})
A	253	268	259	84	89	86	4.97
B	265	252	264	88	84	88	4.45
C	277	242	263	92	81	88	11.66
D	268	250	263	89	83	88	6.12

Analysis of specific hydrogen production rate (SHPR)

Factor	k_1	k_2	k_3	K_1	K_2	K_3	R (K_{max}–K_{min})
A	1.90	1.50	1.80	0.63	0.50	0.60	0.13
B	2.01	1.40	1.80	0.67	0.47	0.60	0.20
C	1.60	1.52	2.09	0.53	0.51	0.70	0.19
D	1.80	1.47	1.93	0.60	0.49	0.64	0.15

k_1, k_2 and k_3 are the sum scores of Level 1, Level 2 and Level 3 for each factor.
K_1, K_2, and K_3 are the average scores of Level 1, Level 2 and Level 3 for each factor.
R is the range among the average scores for each factor, estimated by the difference between the highest and the lowest score average (K), i.e. K_{max}–K_{min}.
Meaning of other acronyms given in the text.

Table 6 – Variance and ranking of each factor and optimal medium composition at controlled pH, with respect to the Ca + P content, the H_2 yield and the specific hydrogen production rate (SHPR). Explanations given in the text (section 3.5).

	Ca + P content (% dr. wt)		H_2 yield (% mol H_2/mol CO)		SHPR (mol H_2/g VSS·d)	
	Objective: min		Objective: max		Objective: max	
	Optimal conc. (mM)	Ranking (strength)	Optimal conc. (mM)	Ranking (strength)	Optimal conc. (mM)	Ranking (strength)
PO_4^{3-}	1	2 (31%)	2.5	3 (18%)	5	4 (20%)
HCO_3^-	5	4 (15%)	20	4 (16%)	20	1 (30%)
Ca^{2+}	0.1	1 (32%)	2	1 (43%)	0.1	2 (28%)
Mg^{2+}	0.5	3 (22%)	2.5	2 (23%)	0.5	3 (22%)

formulation, also produced SHPR and Y_{H_2} values in the upper range.

3.6. *Experimental validation*

In order to confirm the optimality of the defined medium as formulated in the above work, experiments were repeated under those conditions using different *C. hydrogenoformans* cultures. Batch fermentation tests were performed with the original DSM pure strain, and results were compared to those obtained with the culture previously enriched on the DSM medium (Fig. 5). Generally, no major inconvenient was observed in the conciliation of different optimization objectives. No significant difference was observed for the H_2 yield and SHPR parameters with the optimum medium formulation. However, the Ca + P content of the culture drastically decreased from 58.2 to 15.5% after the DSM medium was changed out by the optimized medium. Moreover, mineral content accumulation was further limited to 12.9 ± 4.1% (and to a calcium and phosphorus content 9.51% dr. wt) when original culture was directly grown on the optimized medium. Considering that standard inorganic content of bacterial cells

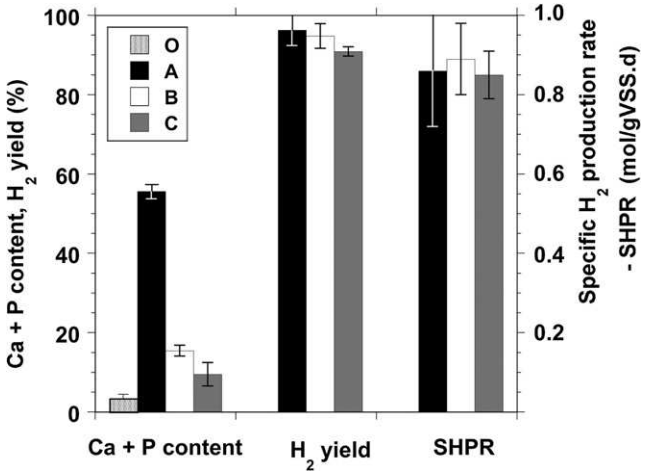

Fig. 5 – Impact of different conditions on the culture content in calcium and phosphorus.
O: Original culture (DSM6008)
A: Culture A (DSM6008 in DSM medium)
B: Culture B (culture A in optimized medium)
C: Culture C (DSM6008 in optimized medium).

generally varies from 7 to 12% [44], it is clear that the present result are fully satisfactory with respect to the optimization target. At the same time, an acceptable H_2 yield of 90.9 ± 1.2% and a reasonable SHPR of 0.85 ± 0.06 mol H_2/g VSS·d were also preserved with the optimized medium formulation.

4. Conclusions

The orthogonal experiment design was employed for process optimization of fermentative hydrogen production using carbon monoxide as substrate. The effect of PO_4^{3-}, HCO_3^-, Ca^{2+}, Mg^{2+} concentrations in culture medium on the calcium and phosphorus content of cells, hydrogen yield and SHPR were investigated in batch test. Experimental results showed that PO_4^{3-}, HCO_3^-, Ca^{2+}, Mg^{2+} factors all had an individual significant influence on H_2 yield and activity rate. Considering minimizing the cell mineral content as the primary target, the optimal defined medium formulation should contain (mM): PO_4^{3-} 1.0, HCO_3^- 5.0, Ca^{2+} 0.1, Mg^{2+} 0.5 at a neutral pH. Neither calcium or magnesium at the above low concentration resulted in notable limitations on the H_2 yield, or the bioactivity rate of *C. hydrogenoformans*, except in alkaline conditions for the latter. As well the validation results proved that the orthogonal experimental design was effective in optimizing an important number of factor combinations with a minimal number of experimental runs. As a result of this optimization strategy, the medium formulation was able to keep down the cell mineral content at only 13% dr. wt and the calcium plus phosphorus content at ca. 9% (as compared to ca. 94 and 56%, respectively, with the original medium) while allowing for H_2 production yield and rate of ca. 91% (mol/mol) and 0.9 ± 0.1 mol/g VSS·d, respectively. Therefore the optimized medium formulation preserved the culture from mineral accumulation or when cells had a high mineral content at inoculation, allowed for a significant reduction of it. In conclusion the optimization test results can be considered to be reliable for the control of the mineral content in *C. hydrogenoformans* cultures, without significant interference with their biological activity.

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