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Heavy metal effects on fermentative hydrogen production using natural mixed microflora

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ABSTRACT

The effects of ionic Cr, Cu and Zn on fermentative hydrogen production were examined using serum vial assays. The hydrogen-producing natural mixed microflora (dominated by the *Clostridium* species) was cultivated from sewage sludge with sucrose. The relative heavy metal toxicity to fermentative hydrogen production was Zn > Cu > Cr. The microflora hydrogen production activity was reduced by 50% for a biomass that came into contact with 4.5 mg Zn/L, 6.5 mg Cu/L and 60 mg Cr/L. However, low concentrations of 3 mg Cu/L and 15 mg Cr/L resulted in peak 10–20% in hydrogen production stimulation. The threshold concentrations were 4 mg Cu/L and 25 mg Cr/L, over which declining stimulation occurred. Heavy metals affected the hydrogen fermentation in hydrogen production potential, hydrogen production rate, lag-phase time and soluble microbial products; their influences were dependent on metal kind and concentration. Metabolic pathway shift occurred when the metal concentrations varied. Based on the maximum specific hydrogen production rate, the inhibition patterns of Cu and Zn dosages were kinetically competitive with inhibition coefficients of 2.9 and 4.5 mg/L, respectively. The metal-toxicity to the hydrogenesis, acidogenesis and methanogenesis in anaerobic digestion was compared. Strategies based on heavy metal concentration control in the substrate influent for optimal hydrogen production were proposed.

1. Introduction

The depletion and pollution problems from fossil fuels necessitate new energy resource development. Hydrogen is one of the ideal and clean energy sources for the future because of its high conversion, recyclability and nonpolluting nature. Hydrogen production using chemical or physical methods has been well developed. However, biological processes such as dark and photo-fermentation are environmentally friendly and less energy intensive compared to chemical processes [1]. Dark fermentation has been recently reported as the hydrogen production system with the greatest potential [2]. Biohydrogen production from biomass wastes can reduce both waste disposal problems and substrate cost and becomes attractive. In the case of converting biomass wastes into biohydrogen, natural mixed microfloras oriented from compost and sewage sludges have been shown as good hydrogen-producing microorganism sources [3,4].

Heavy metals affect biological processes. The acidogenesis and methanogenesis of an anaerobic process are readily affected by Cr, Cu and Zn [5–8]. Hydrogenesis is similar to the acidogenesis in biochemical characteristics but its efficiency under the influence of heavy metals has been little reported [9]. Understanding the influence of metal on hydrogenesis enhances the range of hydrogen fermentation application.

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In light of the above developments, this study is aimed at investigating the heavy metals' effect on biohydrogen production using anaerobic sewage sludge microflora at the presence of Cr, Cu and Zn ions. Specifically, the influences on hydrogen production activity, hydrogen yield (the ability converting substrate into hydrogen, hydrogen yield (HY) and volatile fatty acid (VFA) distribution was determined.

2. Materials and methods

2.1. Materials

The seed microflora was obtained by acclimating the wasted activated sludge of a municipal sewage treatment plant. The collected sludge was screened with a No. 8 mesh (diameter 2.35 mm) and then seeded into a 4 L completely stirred tank reactor (CSTR) fermenter. This seed microflora fermenter was operated at a temperature of $35 \pm 1\,^{\circ}C$, a pH of 6.8 ± 0.2, an operating hydraulic retention time of 12 h and a substrate sucrose concentration of 20 g COD/L. The substrate was fed in a continuous mode and contained bacterial nutrients ([10], mg/L): 5240 NH_4HCO_3, 125 K_2HPO_4, 100 $MgCl_2 \cdot 6H_2O$, 15 $MnSO_4 \cdot 6H_2O$, 25 $FeSO_4 \cdot 7H_2O$, 5 $CuSO_4 \cdot 5H_2O$, 0.125 $CoCl_2 \cdot 5H_2O$, 6720 $NaHCO_3$.

This seed fermenter was routinely (two to three times a week) monitored for oxidation–reduction potential (ORP), alkalinity, gas production, VFA and solids concentrations. The fermenter was operated for 180 days and had reached a steady-state condition, i.e. conditions under which the parameter values varied only slightly (less than 10% variation) for 90 days. Under the steady-state condition, the average operating parameters were: ORP −470 mV, alkalinity 4450 mg/L as $CaCO_3$, hydrogen content in biogas 40.6% (v/v), HY 2.3 mol H_2/mol sucrose, hydrogen production rate (HPR) 254 mmol H_2/L day, acetate (HAc) 2830 mg COD/L, propionate 830 mg COD/L, normal butyrate (HBu) 6300 mg COD/L, ethanol 2480 mg COD/L and volatile suspended solids (VSS, to express the biomass concentration) 4.0 g/L.

The metallic ions investigated were Cr^{6+}, Cu^{2+} and Zn^{2+}. The metallic ion concentrations tested were 15–120 mg Cr/L, 2–16 mg Cu/L and 5–40 mg Zn/L, which were added as aqueous $K_2Cr_2O_7$, $CuSO_4 \cdot H_2O$ and $ZnSO_4 \cdot H_2O$. In the nutrients there was a Cu^{2+} concentration of 1.7 mg/L. According to the present paper, the metallic concentrations were the dosed values.

2.2. Experimental methods

Hydrogen production experiments were conducted in serum vials with a working volume of 125 mL. The vials were initially gassed with argon gas followed by seed microflora (30 mL) and substrate (30 mL) addition. The vials were placed in a reciprocal water-bath shaker (reciprocation: 3.0 cm × 150 strokes/min) with the temperature controlled at $35 \pm 1\,^{\circ}C$. The total gas, VFAs and their compositions were determined at the 72 h to measure the hydrogen production and VFA distribution. The gas volumes were corrected to normal conditions: 0 °C, 1 atm. Each experimental condition was carried out in triplicate.

2.3. Monitoring and analysis

The parameters used in measuring the effects of the metallic ions were hydrogen gas production and its rate. Inhibition was quantified by determining the dose of the metallic ions that caused a 50% reduction for these parameters over a fixed period of time compared with that of a fed control. In general, assays were run for a 72 h period because in the metal fed control 95% of the sucrose substrate was degraded at this time interval.

Gas composition was analyzed with a gas chromatograph having a thermal conductivity detector. VFA and ethanol were analyzed with a Shimadzu (Japan) GC-14A gas chromatograph that was equipped with a flame ionization detector. Other analytical details for the VFA, ethanol and biogas assays were the same as those in our previous study [11,12]. Alkalinity and volatile suspended solids (VSS, to express the biomass concentrations) were measured according to the procedures in Standard Methods [13].

3. Results and discussion

Dark organic fermentation produces hydrogen and solvents such as ethanol and organic acids. Ethanol and VFAs are defined as soluble microbial products (SMP). Hydrogen production was employed to monitor the inhibition in this study. However, SMP production was also monitored to elucidate the heavy metals' effect on hydrogenesis. Hydrogen production activity (A_h) and SMP production activity (A_s) were defined to indicate the extent of hydrogen and SMP production inhibition, respectively. They are defined as the fractions of hydrogen and SMP produced in 72 h by metal-dosed seed microflora against the control.

$$A_h(\%) = H_m/H_c \times 100\%, \quad (1)$$

$$A_s(\%) = S_m/S_c \times 100\%, \quad (2)$$

where H_m and S_m denote the amounts of hydrogen and SMP produced in 72 h, respectively using metal dosed seed microflora. H_c and S_c denote the amounts of hydrogen and SMP produced at 72 h by the control. The experimental hydrogen production data discussed in the following are presented as mean values. The coefficients of deviation were 1.2–7.8% for all determinations.

The metal toxicity effect depends on the pH. However, the pH in the serum vials ranged from 6.3 to 6.6 during incubation for all tested metals. Since the dominant microbial species, *Clostridium*, functions within a pH range of 6.0–6.7, pH did not affect adversely affect the microbial activity.

3.1. Effects on production of hydrogen

Fig. 1 gives the hydrogen production time course for seed microflora dosed with Cr as an example showing heavy metal effect. The accumulative hydrogen amount was obtained from the total biogas production and hydrogen gas content. Differences in inhibition by various Cr concentrations were observed. For Cu, a similar hydrogen production variation trend was found after dosing with metal. However, differences

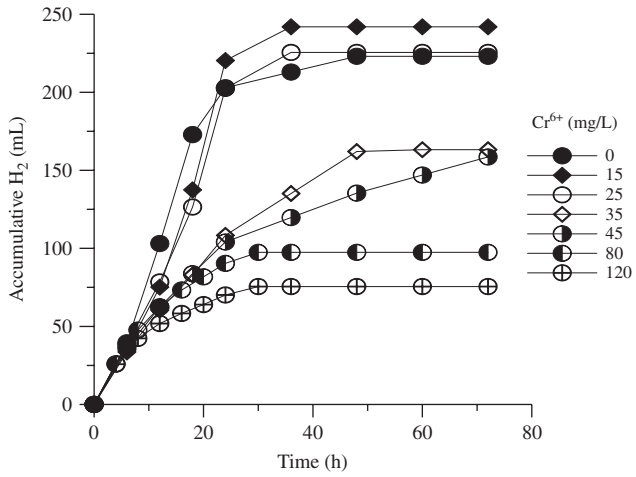

Fig. 1 – The time course of hydrogen production after dosing chromium (Cr^{6+}).

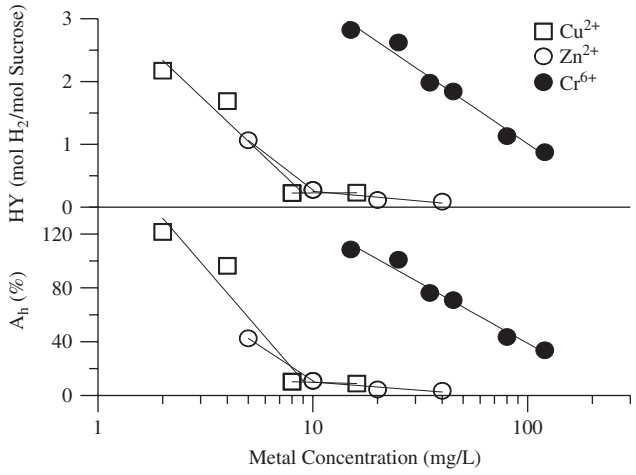

Fig. 2 – Relationships between hydrogen production activity (A_h), hydrogen yield (HY) and metal concentration.

in inhibition by concentration amount were observed. For Zn, all metal-dosed hydrogen volumes were less than those produced by the control. Heavy metals affected hydrogen production at various degrees.

Fig. 2 shows the relationships between hydrogen production activities (A_h), HY and metal concentrations. Both A_h and HY were metal and metal concentration dependent. A_h values exceeding 100% of 120% and 110% occurred at concentrations of 2 mg Cu/L and 15 mg Cr/L, respectively. Note that the influent nutrients contained 1.27 mg Cu/L. Therefore, if the concentration from the nutrients was included the Cu concentration causing 120% A_h value was approx. 3 mg/L. As shown by the activity exceeding 100%, production was stimulated. Low Cr and Cu concentrations slightly stimulated fermentative hydrogen production of the mixed microflora. Declining stimulation occurred when the Cr and Cu concentrations reached 25 and 4 mg/L (if the Cu concentration from the nutrients was included the value was approx. 5 mg/L), respectively. Metal dosages over these values resulted in A_h values lower than 100% which showing metal toxicity to hydrogen production. These metal concentrations were threshold concentrations [14]. For Zn dosage experiments, A_h values were always smaller than 100% showing no stimulation in hydrogen production; no threshold concentration was obtained.

An observation on the variation trends of A_h-metal concentration and HY-metal concentration curves in Fig. 2 also indicates the influence differences between these test metals. For these metals, both the A_h and HY indicator curves exhibit a similar variation trend. The A_h and HY-metal concentration curves for Cr were different from those of Cu and Zn. Moreover, both Cu and Zn revealed two stages of influence curves with an inflection point occurring at 10 mg/L metal concentration. When these two metals were at concentrations lower or higher than this value, steep and plateau curves are observed, respectively. Steep curves indicate that the activity markedly decreased with increasing metal concentration. For the plateau curve, the A_h value was only about 5% which showed the complete loss of hydrogen production activity. A linear curve was obtained for Cr at the tested concentrations showing that hydrogen production activity decreased with increased metal concentrations.

3.2. *Effects on production of liquid products*

Hydrogen formation is accompanied with VFAs or solvent production during an anaerobic digestion process. Therefore, the VFA concentration distributions and their fractions are useful indicators for monitoring hydrogen production. Fig. 3 gives the fraction variations for SMP at 72 h for microflora dosed with metals. Metals affected SMP production in metal kind and concentration dependent ways. HBu was the major fermentation VFA product at the tested Cu concentrations,

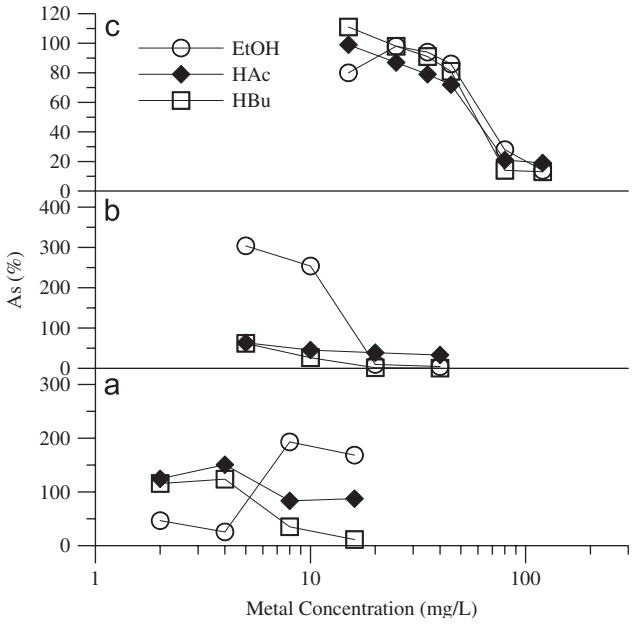

Fig. 3 – Relationship between soluble microbial product production activity (A_s) and metal concentration. (a) Cr^{6+}; (b) Zn^{2+}; (c) Cu^{6+}.

but its concentration fraction decreased with increasing Cu concentration. HAc was the secondary major liquid product and its concentration fraction increased with increasing Cu concentration. These examples show that an increase in metal concentrations resulted in a liquid product composition variation. This indicates that the fermentation metabolic pathway shifted when the metal concentrations varied [15].

The extents of metallic inhibition to SMP production evaluated by SMP production activity (A_s) are illustrated in Fig. 3. Ethanol was the most metal kind and concentration dependent component in production activity because it had obvious fluctuations in the variation curves. At lower Zn and higher Cu concentrations, high ethanol A_s values were determined. For HAc and HBu, their A_s values always decreased with increasing metal concentrations for the tested metals. Moreover, at the presence of Cr the A_s values of these SMP were below 120%, which was generally lower than these at the presence of Cu and Zn .

The ratio of HBu/HAc has been reported as a simple indicator showing the performance of a hydrogen fermenter. HBu/HAc ratio values of 3–4 are reported for efficient hydrogen production [11]. In this study HBu/HAc ratios ranged 0.5–5.5 and were quite metal kind and concentration dependent (Fig. 4). A similar wide range of HBu/HAc ratios were experienced in a study on hydrogen production at different iron concentrations [9]. The variation in the HBu/HAc ratio also indicates a shift in the metabolic pathway.

3.3. *Metal toxicity comparison*

The metal concentrations which caused inhibition of the selected activity levels were determined for the tested metals. The concentration at which a metal caused 50% inhibition (C_{50}, a 50% reduction in hydrogen production over 72 h with metal dosage) indicated the toxicity of the metals. This 50% inhibition term has been used to describe a reduction in VFA production by acidogenesis from dosed metals [6]. Table 1 summarizes the experimental results for 50% inhibition in hydrogen production activities (C_{50}). The metal concentrations causing a 50% reduction in activity ranged from a few to tens of mg/L for the hydrogenesis process. These results indicate that the hydrogenic microfloras had a varied tolerance to the tested metals. The C_{50} values were 4.5 mg Zn/L, 6.5 mg Cu/L (if the Cu concentration from the nutrients was included the value was approx. 7.8 mg/L) and 60 mg Cr/L. According to these results, the degree of hydrogen production inhibition was compared on the basis of the C_{50} results. The degree was considered to be the same when the concentration variation was calculated to be under ±10%. A comparison on the values of C_{50} indicates that the relative toxicities to hydrogen production were Zn > Cu > Cr. Zn was the most toxic metal tested. However, an inhibition study on acidogenesis of dairy wastewater by zinc and copper showed that Cu was 1.4–4.3 folds more toxic than Zn [8]. Another inhibition study on hydrogenesis of glucose by zinc and copper also showed that copper was more toxic than zinc [16]. These differences might relate to the substrate used and the toxic comparison basis being on acidogenesis/hydrogenesis (discussed below). That study used a synthetic dairy wastewater prepared from a full-cream powdered milk and was according to the overall production patterns of fatty acids and hydrogen as well as degradation patterns of carbohydrate and protein [8].

The reported 50% inhibition in the acidogenesis activities (VFA production from glucose by VFA producing-microorganisms [6]) and methanogenesis (methane production from VFA by VFA degrading-microorganisms [17]) process is also

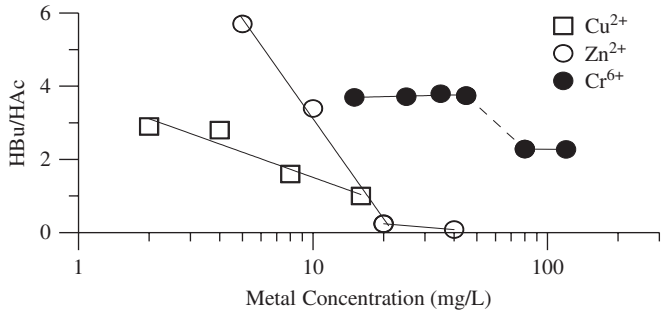

Fig. 4 – Relationship between butyrate/acetate (HBu/HAc) ratio and metal concentration.

Table 1 – Results for 50% inhibition of hydrogen production (hydrogenesis), acidogenesis and methanogenesis (mg/L)

Metal	Hydrogenesis		Acidogenesis			Methanogenesis
	[this study]	[16]	[6]	[7]	[16]	[17]
Cr	60	N.A.	17	N.A.	N.A.	14.7
Cu	6.5	350	0.9	2–8	364	12.5
Zn	4.5	>350	3.5	7–18	>364	16

included in Table 1 for comparison. An observation on the values of C_{50}, on the basis of metal concentration, for hydrogenesis, acidogenesis and methanogenesis showed that metal toxicity was process-dependent. The orders of toxicity-resistance were hydrogenesis > acidogenesis > methanogenesis, methanogenesis > hydrogenesis > acidogenesis and methanogenesis > hydrogenesis > acidogenesis to Cr, Cu and Zn, respectively. This resulted from the microbial species responsible for theses processes being different and is consistent with a speculation by Hickey et al. [5]. They speculated that some trophic group(s) or organisms within the anaerobic consortia of methanogenic digesters might be more severely inhibited by a pulse addition of heavy metals than the methanogenic populations. Note that although hydrogen production (hydrogenesis) is accompanied with VFA production (acidogenesis), their toxicity resistances were different. This is because the metabolic pathway is complicated and might shift under environmental change conditions [12,15]. A similar experience was reported in observing the metal effect differences on VFA degradation and methane production [17].

3.4. *Kinetic analysis*

The hydrogen production potential, maximum HPR and lag-phase time were elucidated using the modified Gompertz equation (Eq. (3)) that has been used to describe the progress of cumulative hydrogen production obtained from a batch experiment [3,12]. Table 2 summarizes the results that using the cumulative hydrogen production data obtained to fit this equation. The correlation coefficient values (R^2) ranged from 0.955 to 1.

$$H(t) = P \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\}, \quad (3)$$

where $H(t)$ is the cumulative hydrogen production (mL), P is the hydrogen production potential (mL), R_m is the maximum hydrogen production rate (mL/h), e is the 2.71828…, λ is the lag-phase time (h), t is the time (h).

Table 2 reveals that the peak hydrogen production potential (P) values occurred at 0, 2 and 15 mg/L for Zn, Cu and Cr, respectively. These values show inhibition (Zn) and stimulation (Cu and Cr) in hydrogen production for the tested metals. This is consistent with the results read from Fig. 2. However, the metals' R_m values peaked at the zero metal concentration. This shows that these metals did not enhance the maximum HPR. In other words, the maximum HPRs were inhibited in the presence of the tested metals. Low concentrations of Cu and Cr enhanced only hydrogen production at the maximum HPR. An observation on the lag-phase time (λ) values also gives that the tested metals affected hydrogen production in different ways. For Cu and Zn, λ values increased with increasing metal concentration and peaked at the highest tested concentrations. The presence of Cu and Zn delayed hydrogen production. For Cr, the highest test concentration (120 mg/L) resulted in the smallest λ value (0.8 h). Moreover, at 15 mg Cr/L, which having a peak stimulation in hydrogen production potential, peak λ value was also obtained. This indicates that low Cr concentration could stimulate the hydrogen production but its production was delayed simultaneously.

On the other hand, for Cr and Cu, when their dosages were over the threshold concentrations (5 mg Cu/L and 25 mg Cr/L), P and R_m reduced markedly. When the Cu dosage was increased from 4 to 8 mg/L, P and R_m decreased by 90% (from 140 to 14 mL and from 9.1 to 0.8 mL/h). When the Cr dosage was increased from 25 to 35 mg/L, P and R_m decreased by 27% and 47%, respectively (from 227 to 167 mL and from 9.1 to 4.8 mL/h).

The inhibition mechanisms of the hydrogenesis from the tested metals are discussed as follows. The assumptions made were (1) the biochemical kinetics followed the Michaelis–Menten expression, (2) quasi-steady state approximation was valid and (3) the effects of substrate loadings were not taken into consideration [18]. The inhibition coefficients were determined using an initial rate method with a Lineweaver–Burk plot on the substrate utilization rate and concentration data. Based on the specific R_m (the hydrogen production ability of the biomass) data only the inhibitions by Cu and Zn could be kinetically analyzed by competitive model with inhibition coefficients of 2.9 and 4.5 mg/L, respectively. The reason why the Cr inhibition could not be analyzed using the initial rate method could be because the initial reaction between the hydrogenic microflora and Cr were not reversible and could not reach a quasi-steady state and so conflicted with the assumptions. Cr inhibition to methanogenesis is also not able to be kinetically analyzed using the same assumptions [15].

3.5. *Significance of the experimental results*

Cr and Cu were found to be stimulative to hydrogen production at concentrations of 15 and 3 mg/L, respectively. They enhanced the HY by 10–20%. However, dosages over the threshold concentrations (25 mg Cr/L and 5 mg Cu/L) resulted

Table 2 – Modified Gompertz equation parameters

	Metal dosage (mg/L)	P (mL)	R_m (mL/h)	λ (h)	R^2
Cr	0–120	74(120)[a]–243(15)	4.2(45)–13(0)	0.8(120)–4.3(15)	0.9768–0.9981
Cu	0–16	12.8(16)–177(2)	0.8(8)–19.2(0)	3.0(0)–33.7(16)	0.9912–0.9998
Zn	0–40	7.5(40)–220(0)	1.0(10)–11.8(0)	4.0(0)–31.2(40)	0.9546–0.9923

[a] Values in parentheses are the metal concentrations that resulted in the parameter values.

in decreasing HY. Cr has not been reported as a nutrient supplement but Cu has been used at small concentrations of 0.13–1.3 mg/L in some hydrogen production studies [10,19–21]. Zn was found to be quite inhibitive and no stimulation to hydrogen production was found at the tested concentrations. However, Zn has been reported as an important nutrient factor enhancing hydrogen production from sucrose using anaerobic sewage sludge microflora as used in this study [22]. This difference in the role of Zn on hydrogen production might arise from the difference in test concentrations used in these studies. In our previous study it was found that Zn was the most neglected important tracemetal in nutrient supplements for anaerobic hydrogen production [22]. We found that small amounts of Zn (0.12 mg/L) combined with proper concentrations of Mg, Na and Fe enhanced the HY by nearly 30%. In this study, the tested Zn concentrations were 5, 10, 20 and 40 mg/L, which values were higher than the reported value necessary for enhancing hydrogen production. Mizuno et al. used Zn at small concentration of 0.24 mg/L as a nutrient supplement in hydrogen production [23,24]. Based on the above developments, hydrogen production might be promoted with proper low dose of chromium, copper or zinc ion concentrations in a hydrogen fermentor. However, too high in dose concentrations should be avoided to prevent inhibition problems. These heavy metals relate to functions in reactions and transformations of dehydrogenase, dismutase, hydrogenase and methyltransferase [25].

4. Conclusions

Cr, Cu and Zn significantly affect hydrogen-producing microflora enriched from sewage sludge with Zn and Cr being the most and least toxic metals, respectively. These heavy metals affect the fermentative hydrogen production in metal kind and concentration dependent ways. The metal influence causes shifts in the metabolic pathway. The microflora's hydrogen production activity could be reduced by 50% for a biomass in contact with 4.5 mg Zn/L, 6.5 mg Cu/L and 60 mg Cr/L. However, low concentrations of 2 mg Cu/L and 15 mg Cr/L resulted in peak hydrogen production by 20% and 10%, respectively. In the presence of heavy metals at various concentrations, the effect on hydrogen production potential was exhibited in different degrees. The metal-toxicity to the hydrogenesis, acidogenesis and methanogenesis in anaerobic digestion was different and dependent on metal kind. The inhibition patterns of Cu and Zn dosages were kinetically competitive with inhibition coefficients of 2.9 and 4.5 mg/L, respectively.

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