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Detoxification of Copper and Chromium via Dark Hydrogen Fermentation of Potato Waste by *Clostridium butyricum* Strain 92

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Abstract: The accumulation of various types of waste containing both organic and inorganic metal-containing compounds is extremely hazardous for living organisms. The possibility of polymer degradation, biohydrogen synthesis, and metal detoxification via the dark fermentation of model potato waste was investigated. For this purpose, the strict anaerobic strain was isolated and identified as *Clostridium butyricum*. The high efficiency of dark hydrogen fermentation of potatoes with yield of hydrogen in 85.8 ± 15.3 L kg^{-1} VS_{potato} was observed. The copperand chromium salts solutions were added to the culture fluid to obtain the concentrations of 50, 100, and 200 mg L^{-1} Cu(II) and Cr(VI) in the active phase of growth (19 h of cultivation). Metals at a concentration of 200 mg L^{-1} inhibited the fermentation process the most. The hydrogen yield decreased in 7.2 and 3.6 times to 11.9 ± 2.1 and 23.8 ± 5.6 L kg^{-1} VSpotato in the presence of 200 mg L^{-1} Cu(II) and Cr(VI), respectively. The efficiencies of the chromium bioremoval in all variants of the experiment were 100%, and those of copper bioremoval were about 90%. A pure culture of strict anaerobes *Clostridium butyricum* strain 92 was used for the first time for the detoxification of metals. The presented results confirmed the possibility of this promising strain application for industrial H_2 production and the bioremediation of contaminated sites.

Keywords: anaerobic fermentation; hydrogen production; microbial degradation of plant polymers; potato waste; detoxification of metals; copper; chromium; strict anaerobes; *Clostridium butyricum*

1. Introduction

Currently, a significant environmental problem is the accumulation of waste of organic and inorganic origins in the environment [1,2]. Both of them pose a significant threat to living organisms, from bacteria [3] to humans [4]. The accumulation of carbohydrate-containing organic waste in landfills causes a high metabolic activity of microorganisms and, as a consequence, the emergence of toxic compounds (organic acids, alcohols, mercaptans, hydrogen sulfide, ammonia, etc.) and the contamination of the environment [5]. However, organic, easy biodegradable wastes are promising for the obtaining of energy sources,

especially biohydrogen [6]. Municipal and food wastes contain high concentrations of carbohydrates and can serve as substrates for anaerobic hydrogen-synthesizing bacteria [7]. As a result of such a biotechnological process, a double-positive effect is realized, i.e., the obtaining of environmentally friendly biohydrogen and degradation of carbohydrate-containing waste [8]. Potato waste is a good example of such economically valuable waste [9]. Potatoes contain from 11.8% to 14.3% of starch [10], which can be used as a viable alternative carbon source for biogas production. Potatoes as raw materials are processed in the food industry for the production of chips, starch, and alcohol. Starch, in turn, is used as a structural substance and filler for the manufacture of adhesives, plastics, paper products, pharmaceuticals, etc. Potato wastewater, potato pulp, peel, distillery wastewater, and pulp are the waste generated after potatoes processing [11]. The above wastes contain starch that can be hydrolyzed by microorganisms of the genus *Clostridium*. It is known that some *Clostridium* species (*C. acetobutylicum* [12], *C. thermosulfurogenes* [13], etc.) have enzymes (a-amylase, oligo-1,6-glucosidase, etc.), belonging to the family of glycoside hydrolases [14], and are involved in the hydrolysis of starch [15]. Hydrogen-synthesizing microorganisms produce H_2 and related products from simpler carbohydrates after hydrolysis according to the equations [16]:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2\,CH_3COOH + 2CO_2 + 4H_2,$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2.$$

This process is accompanied by the accumulation of reducing equivalents and the maximum decrease of the redox potential of the culture fluid ($2H^+ + 2e = H_2$, $E_o' = -414$ mV). Such an anaerobic process can be used for the detoxification of inorganic metal-containing wastes due to the highly reducing conditions created by hydrogen-synthesizing microorganisms. Inorganic wastes, such as oxidizing metal salts [17], are even more hazardous than organic ones. They have a toxic effect immediately, as they have oxidizing [18], bactericidal [19], carcinogenic [20], fungicidal [21], hepatotoxic [22], and others properties. The most common metals pollutants are chromium [23] and copper [24]. They are released into the environment from deposits and mining sites, as well as from industrial wastewater. A new and previously little-studied direction of metal detoxification is the reduction or immobilization of metals to insoluble and therefore non-toxic compounds with the participation of strict anaerobic microorganisms. At first glance, oxidizing metals that create high redox potential values in the media are incompatible with the existence of strict anaerobes that grow at low redox potential values ($E_h = -200 \div -400$ mV). However, spore-forming anaerobic bacteria (e.g., *Clostridium*) are able to autoregulated the redox potential in the absence of oxygen, followed by their active growth and development [25]. *Clostridium* strict anaerobic bacteria can be used for the purification of model metal containing sewage from toxic metals. The thermodynamic prediction allows one to theoretically substantiate the most effective mechanism of the microbial reduction of soluble toxic chromium(VI) and copper(II) ions to insoluble and non-toxic compounds such as insolvable $Cr(OH)_3 \cdot nH_2O$ and Cu_2O. The precipitation of soluble forms of Cu(II) by microbial exometabolites without changing the valence (redox state) is another way of copper bioremoval ($CuCO_3$(s), $Cu(OH)_2$(s), etc.). The standard redox potentials of chromium(VI) and copper(II) reduction are equal to +555 mV and +480 mV, respectively [26]:

$$CrO_4^{2-} + (n-1)H_2O + 5H^+ + 3e = Cr(OH)_3 \cdot nH_2O\downarrow \quad E_o' = +555\ \text{mV},$$

$$2Cu^{2+} + H_2O + 2e = Cu_2O\downarrow + 2H^+ \qquad E_{o(pH=4,6)} = +480\ \text{mV}.$$

We are considering a binary redox reaction of metal reduction by microorganisms. In this reaction, metabolically active microorganisms are the donor system, and high-potential compounds of chromium and copper are the acceptor system. It is obvious that the efficiency of metal reduction is proportional to the potential difference between the acceptor and donor systems. It follows that microorganisms with the lowest values of

the redox potential reduce chromium(VI) and copper(II) with the maximum efficiency. It is well-known that the lowest redox potential ($E_o' = -414$ mV) is created by hydrogen-forming anaerobic bacteria of the *Clostridium* genus: $2H^+ + 2e = H_2$, $E_o' = -414$ mV [27]. It is obvious that a large potential difference between the acceptor and donor systems ensures the fastest possible reduction of chromium and copper compounds. From this, it is obvious that precisely clostridia is the most effective to remove soluble chromium and copper compounds from solutions with the simultaneous degradation of waste and the obtaining of high energy environment friendly fuel biohydrogen.

Therefore, the aim of the study was to investigate the efficiency of the potato fermentation and detoxification of chromium and copper compounds via dark hydrogen fermentation by *Clostridium butyricum* strain 92.

2. Materials and Methods

2.1. Isolation and Identification of the Hydrogen-Synthesizing Strain

The strain of strict anaerobic hydrogen-synthesizing microorganisms was isolated from the culture fluid during the fermentation of multicomponent organic wastes containing potato peel as one of the main substrates. A granular microbial preparation (GMP1) was used as an inoculum for the isolation of hydrogen-synthesizing microorganisms [8]. The pure culture of strict anaerobes was isolated by the ten-fold dilution method [28], followed by inoculation by the Hangate roll tube method (rotation of the vials with agarizated media in an inert gas atmosphere) [29]. The volume of the vials was 120 mL. To create anaerobic conditions in the vials, they were purged with argon at a rate of 0.5 L min^{-1} for 3 min. Reduced Fe(II) citrate was added to the medium to the final concentration of 500 mg L^{-1} to ensure a low redox potential ($-220 \div -250$ mV). A sodium resazurinate (0.01%) indicator was used to detect anaerobic conditions in an agar medium. NA medium (HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India) with potato starch (10 g L^{-1}) was used for the isolation of pure culture of strict anaerobic microorganisms—polymer destructors (starch). The culture was transplanted three times on the reduced agarizated nutrient medium under strict anaerobic conditions to ensure the purity. The taxonomic position of the investigated microorganisms was determined by molecular biological methods. The microorganisms were cultured for 24 h in a Nutrient Broth liquid nutrient medium (HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India) for the obtaining of the microbial biomass. The isolation of bacterial DNA was performed from daily culture using the DNeasy UltraClean Microbial Kit (Qiagen) according to the manufacturer's instructions. The purity of the obtained DNA sample was determined spectrophotometrically using a NanoDrop OneC UV/Vis Spectrophotometer by absorption at wavelengths of 260, 230, and 280 nm, as well as using horizontal agarose gel electrophoresis. The visualization of electrophoresis was performed using a UV transilluminator MultiDoc-It Digital Imaging System (UVP). The obtained DNA was stored in TE buffer at −20 °C. Amplification of 16S rRNA gene sequences was performed by the classical method [30]. The amplification of the sequences was performed using oligonucleotide primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The BioRad thermal cycler was used for amplification. The polymerase chain reaction (PCR) reaction mixture consisted of 5.0 µL of PCR buffer (10X DreamTaq Green Buffer, Thermo Scientific™, Thermo Fisher Scientific Inc., Waltham, MA, USA), 5.0 µL of dNTP, 2 µL of primer mixture, 0.5 µL of Taq polymerase (DreamTaq DNA polymerase, Thermo Fisher Scientific Inc., Waltham, MA, USA), added immediately before the reaction, 1.0 µL of DNA sample, and 36.5 µL of deionized water. Thirty cycles of the amplification of the following temperature profile were performed: DNA denaturation (94 °C, 20 s), primer renaturation (56 °C, 15 s), and elongation (polymerization) (72 °C, 90 s). Nucleotide sequences of 16S rRNA genes were sequenced at Eurofins Genomics (Louisville, KY, USA). The obtained nucleotide sequences of 16S rRNA genes of microorganisms were used for the determination of closely related species and phylogenetic analysis. To do this, the obtained sequences of 16S rRNA genes of bacterial isolates were compared with those deposited in the GenBank database using the BLAST software

package. Related microorganisms were determined by calculating the pairwise similarity in percentage as the ratio of the number of matching/analyzed nucleotides of 16S rRNA genes of each strain with the compared bacteria. Nucleotide sequence correction, as well as the removal and replacement of degenerate nucleotides, were performed in the Bioedit program. A multiple alignment of the studied and closely related sequences was performed in ClustalX based on MEGA (Molecular Evolutionary Genetic Analysis). The phylogenetic position was determined by constructing trees (dendrograms), which showed the position of the studied strain among closely related and typical species (MEGAX program). The tree was constructed using the MEGAX program by the neighbor-joining method using 1000 bootstrap tests. Sequences of external groups of microorganisms (outgroups), which were more remotely related to the studied strains, were used for rooting the tree. The phylogenetic tree was built and edited in the MEGAX program.

2.2. Preparation of Chromium and Copper Solutions and Their Analytical Determination

To prepare 250 mL of a copper citrate solution, 29.4 g of $CuSO_4 \cdot 5H_2O$ (Sigma Aldrich, pure for analysis) was dissolved in 100 mL of distilled water. Then, 30 g of dry trisubstituted sodium citrate was added and stirred until completely dissolved. The solution had a pH value of 5.5, and therefore, it was neutralized with Na_2CO_3 to pH = 7.0. The resulting solution was added to a 250 mL Mora flask and made up to the mark with distilled water. The citrate chelation procedure was performed to stabilize the Cu^{2+} cation in a soluble form in the neutral or alkaline pH range. The final concentration of Cu(II) was 30.0 mg L^{-1}. The concentration of Cu(II) in the solution was determined by a qualitative reaction with a PAR (4-(2-Pyridylazo) resorcinol; Sigma-Aldrich), followed by titration with ethylenediaminetetraacetic acid (EDTA) [31].

To prepare 250 mL of a chromate solution, 28 g of K_2CrO_4 salt (Sigma Aldrich, pure for analysis) were dissolved in 200 mL of water. The solution was poured into a Mora flask after complete dissolution and made up to 250 mL. The final concentration of Cr(VI) was 30.0 mg L^{-1}. The concentration of Cr(VI) in the solution was determined by a colorimetric method using a qualitative reaction with 1,5-diphenylcarbazide (DFC) [32]. Metal solutions were sterilized by heating in a water bath in a hermetically sealed vial for 15 min at a temperature of 100 °C.

2.3. The Process of Fermentation and Metals Insertion

Clostridium butyricum strain 92 was cultivated in a mineral nutrient medium with the addition of sterile potato (0.5 × 0.5 cm) as the model substrate for a common potato waste and source of carbon and energy in a ratio of 3:1 (150 mL of the nutrient medium and 50 g of potatoes). The potato was sterilized, because it is (especially potato peels) a source of mixed facultative and strict anaerobic microorganisms. Mineral salts NH_4Cl (1 g L^{-1}), K_2HPO_4 (2 g L^{-1}), and K_2SO_4 (0.5 g L^{-1}) were used as sources of basic nutrients. The inoculum was also grown on a potato medium for 24 days. The inoculum (3 mL, 2% from the liquid phase) was added to each vial. A solution of copper(II) and chromium(VI) was added to the vial in the active phase of culture growth (19 h of fermentation) to final concentrations of 50, 100, and 200 mg L^{-1}. Thus, the effect of the concentration gradients of Cu(II) and Cr(VI) (50, 100, and 200 mg L^{-1}) on the growth of the culture in a mineral medium with potato was studied. The efficiency of metals bioremoval by the *Clostridium butyricum* strain 92 was determined by decreasing the concentration of soluble Cu(II) and Cr(VI) in the culture fluid. The initial gas phase in the vials was argon. The determination of gas composition was performed by the standard gas chromatography method [33]. The following metabolic parameters were determined as following [34]: pH, redox potential (Eh), volume, and composition of the gas phase (H_2, O_2, N_2, and CO_2), as well as the concentrations of total carbon and Cu(II) and Cr(VI) in the culture fluid. The concentration of dissolved organic carbon (DOC) was determined by a permanganate method [35]. A medium without metals was used as a control variant of the experiment. The coefficient of

potato degradation (reduction of dry weight after degradation) and the efficiency of the fermentation process (yields of H_2 and CO_2 from 1 L kg^{-1} VS_{potato}) were also determined.

2.4. Data Analysis

All experimental measurements were performed in triplicate. Mean values (AV) and standard deviations (SDs) were determined with a 95% confidence level. Each value is presented as the mean ± SD. The significance of the influence of metals on the effectiveness of dark hydrogen fermentation were determined by the one-way ANOVA test with the post-hoc test using the statistical platform of Microsoft Excel. The graphs were constructed using OriginLab software. The phylogenetic three was constructed using MEGAX program.

3. Results

3.1. Characteristic of the Strict Hydrogen-Synthesizing Anaerobic Strain

The strictly anaerobic hydrogen-synthesizing strain was isolated from the culture fluid containing a different species of spore-forming bacteria. The strain withstood heating at 90–100 °C for 20 min and grew only in low-potential conditions (−250 mV). The strain was also able to grow in the presence of dextrose, starch, and potatoes as the only sources of carbon and energy. The strain 92 was identified as *Clostridium butyricum* species. The phylogenetic dendrogram of the species affiliation of the strain is presented in Figure 1.

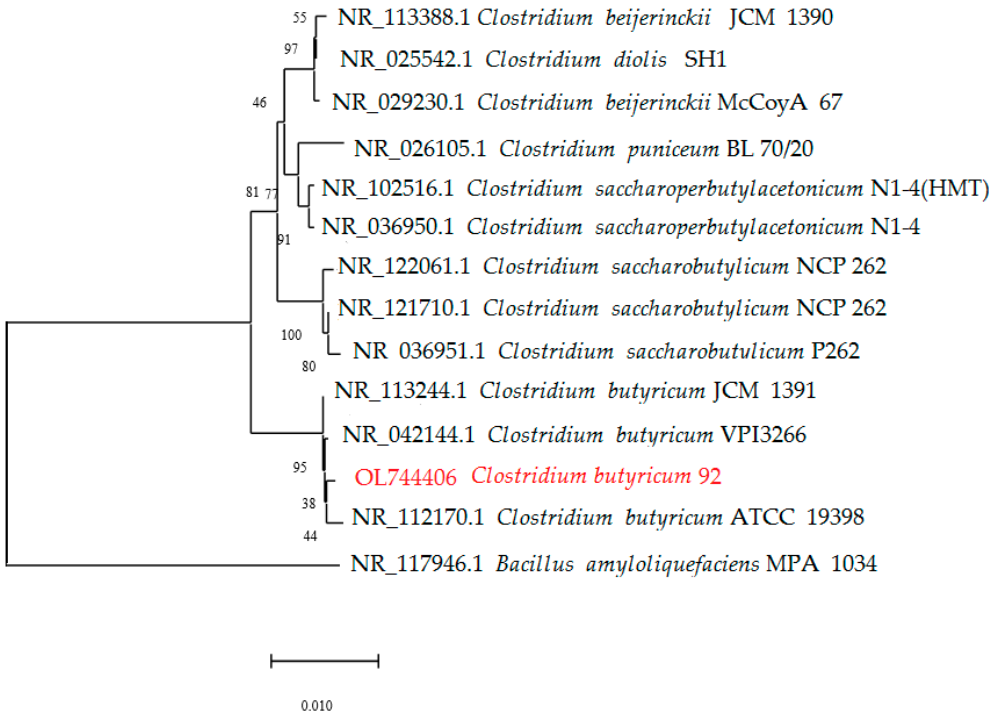

Figure 1. Phylogenetic tree, built using closely related species by the method of joining neighbors (using MEGAX software). The initial values (%) are set in the nodes obtained by repeating the analysis 1000 times. The size of the scale characterizes 0.01 nucleotide substitutions for each nucleotide position. The *Bacillus amyloliquefaciens* MPA 1034 demonstrated as an outgropup for rooting a phylogenetic tree.

According to the dendrogram (Figure 1), the strain of anaerobic microorganisms 92 was in a common cluster and was highly similar to strains *Clostridium butyricum* JCM 1391 (NR_113244.1), *Clostridium butyricum* VPI3266 (NR_042144.1) та *Clostridium butyricum* ATCC 19398 (NR_112170.1), the similarities to which were 100%, 99.81%, and 99.03%, respectively. The obtained results allowed carrying the studied isolate 92 to the *Clostridium butyricum* species. The GenBank accession number for strain nucleotide sequence is OL744406.

3.2. The Influence of Metals on the Gas Synthesis and the Efficiency of Potato Fermentation Process by Clostridium butyricum Strain 92

The main criteria for the efficiency of the anaerobic fermentation process were the concentration of biohydrogen in the gas phase, the volume of gas synthesized by microorganisms, as well as the decrease of the potato weight. All vials contained argon in the gas phase at the beginning of the experiment. The gas phase did not change and consisted only of argon throughout the experiment in the control vials without inoculum. The changes in the composition of the gas phase were not observed throughout the experiment. The values of pH and the redox potential, as well as the concentration of DOC, also did not change throughout the experiment in the control vials without inoculum. Microorganisms synthesized only H_2 and CO_2 under experimental conditions. Hydrogen synthesis took place from the first hours of cultivation and for 5 h the hydrogen concentration was 18.4 ± 3.9 vol.% under control conditions (Figure 2a,b). The H_2 concentration was 36.5 ± 1.8 vol.% for 24 h of cultivation. The maximum concentration of H_2 was 38.4 ± 1.9 vol.% for 48 h of fermentation and then began to decline, indicating a decrease in the growth activity of the *Clostridium butyricum* strain 92. The concentration of H_2 was 24.2 ± 1.2 vol.% for 168 h. The CO_2 concentration also increased during the whole fermentation process and was 62.5 ± 3.1 vol.% for 168 h of cultivation (Figure 2). No significant changes in the concentration of hydrogen or carbon dioxide were observed at the presence of 50 mg L^{-1} Cu(II). The resumption of hydrogen synthesis was observed after decreasing the concentration of soluble copper compounds in solutions to residual concentrations (see Section 4).

More significant inhibition of hydrogen synthesis was observed in the presence of 100 and 200 mg L^{-1} Cu(II). Thus, the maximum concentration of H_2 was 34.2 ± 1.7 vol.% after the insertion of 100 mg L^{-1} Cu(II), which was 4.2 vol.% less than in the control variant. Subsequently, the H_2 concentration also decreased and at 168 h was only 21.4 ± 1.1 vol.% (Figure 2a). Even more inhibition of hydrogen synthesis was observed after the introduction of 200 mg L^{-1} Cu(II). Thus, the maximum concentration of H_2 was 32 ± 2.6 vol.% after the insertion of copper, which was 6.4 vol.% less than in the control conditions (Figure 2a). The increase in copper concentration correlated with the increase in CO_2 concentration in the gas phase of the vial. Thus, the CO_2 concentration was 62.5 ± 3.1 vol.% under control conditions for 168 days of cultivation, and 67.3 ± 3.4, 71.4 ± 3.6, and 72 ± 3.6 vol.% in the presence of 50, 100, and 200 mg L^{-1} Cu(II), respectively (Figure 2c).

The inhibition of H_2 synthesis was also observed in the presence of chromium compounds. Thus, the maximum concentration of H_2 in the control was 38.4 ± 1.9 vol.% for 48 h of fermentation and 34.2 ± 2.5, 33.2 ± 6.2, and 32.3 ± 3.1 vol.% in the presence of 50, 100, and 200 mg L^{-1} Cr(VI), respectively (Figure 2b). The concentration of CO_2 also increased slightly in the presence of metals compared to in the control (Figure 2d). Increasing the concentration of metals led to a decrease of the efficiency of the fermentation process, namely the reduction of the gas yield and K_d (Table 1).

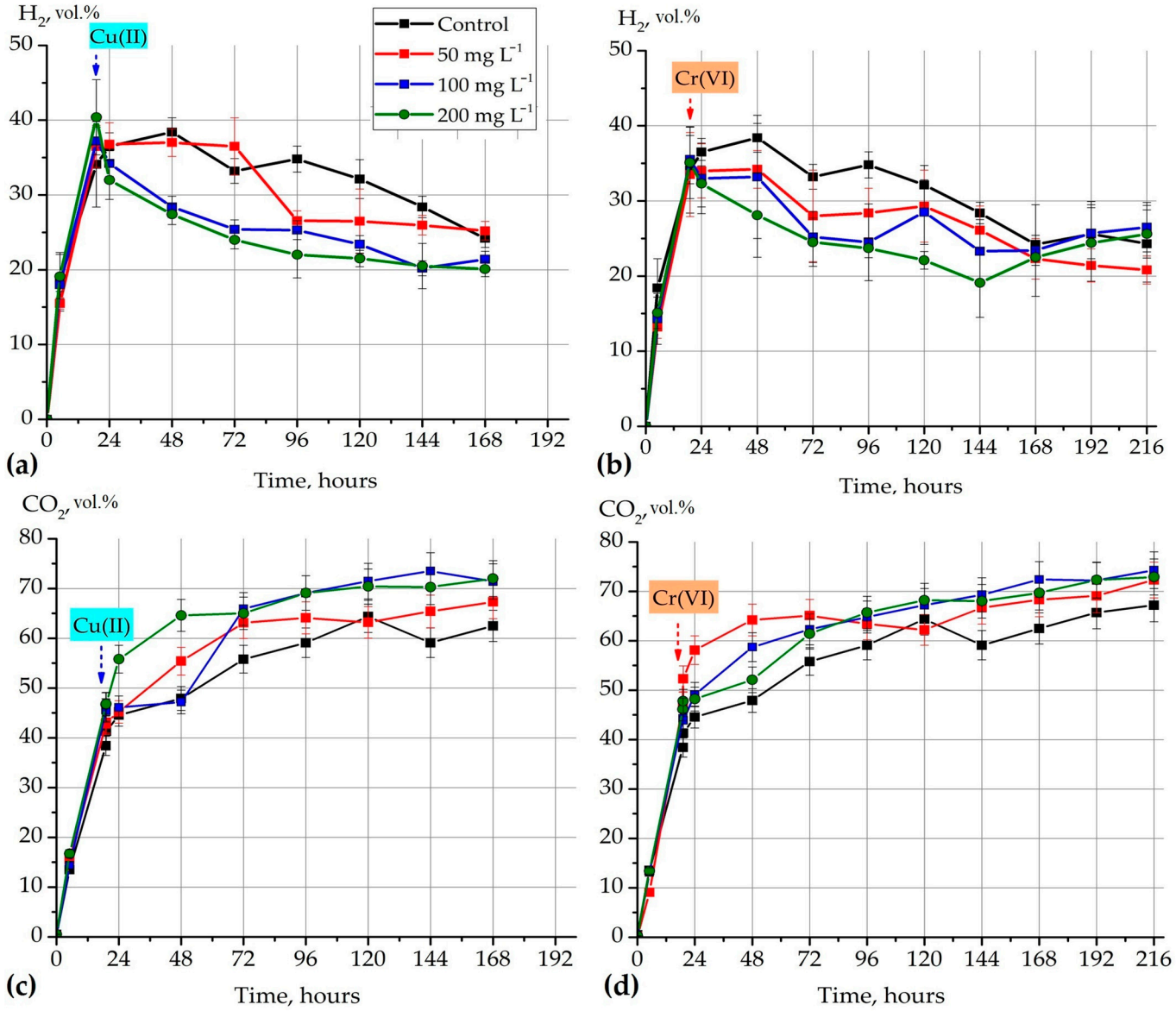

Figure 2. Dynamic of the concentrations of H_2 (**a**,**b**) and CO_2 (**c**,**d**) during the fermentation of potatoes by the *Clostridium butyricum* strain 92 under the influence of Cu(II) (**a**,**c**) and Cr(VI) (**b**,**d**). The black lines represent the control without metals; the red lines represent 50 mg L^{-1} Me; the blue lines represent 100 mg L^{-1} Me; the green lines represent 200 mg L^{-1} Me.

Table 1. The effectiveness of the fermentation process in the presence of Cu(II) and Cr(VI).

Treatments (mg L^{-1})	H_2max (vol.%) *	H_2 Yield (L kg^{-1} VS_{potato})	CO_2 Yield (L kg^{-1} VS_{potato})	K_d (times)
Control	38.4 ± 1.9	85.8 ± 15.3 ^{a}	123.8 ± 14.5 ^{a}	75 ± 5.7 ^{a}
50 Cu(II)	37.0 ± 1.9	77.1 ± 16.1 ^{ab}	112.8 ± 17.1 ^{a}	71 ± 10.2 ^{a}
50 Cr(VI)	34.2 ± 2.5	79.2 ± 5.6 ^{ad}	127 ± 21.5 ^{a}	77.4 ± 12.1 ^{a}
100 Cu(II)	34.2 ± 1.7	12.5 ± 3.5 ^{c}	34 ± 15.1 ^{b}	32.1 ± 5.6 ^{b}
100 Cr(VI)	33.2 ± 6.2	45.3 ± 3.9 ^{be}	108 ± 9.8 ^{a}	62.7 ± 7.2 ^{a}
200 Cu(II)	32 ± 2.6	11.9 ± 2.1 ^{c}	32 ± 4.1 ^{b}	8.4 ± 3.2 ^{c}
200 Cr(VI)	32.3 ± 3.1	23.8 ± 5.9 ^{cf}	6.1 ± 2.2 ^{c}	25.6 ± 4.1 ^{b}

Within each row, the means (±SD, n = 3) with different letters (a–f) show the difference between the treatments based on the post-hoc test at $p \leq 0.05$, * Maximum H_2 concentration after metals insertion.

As can be seen from Table 1, metals affected not only the concentration of hydrogen, but also the cumulative H_2 production (Figure 3).

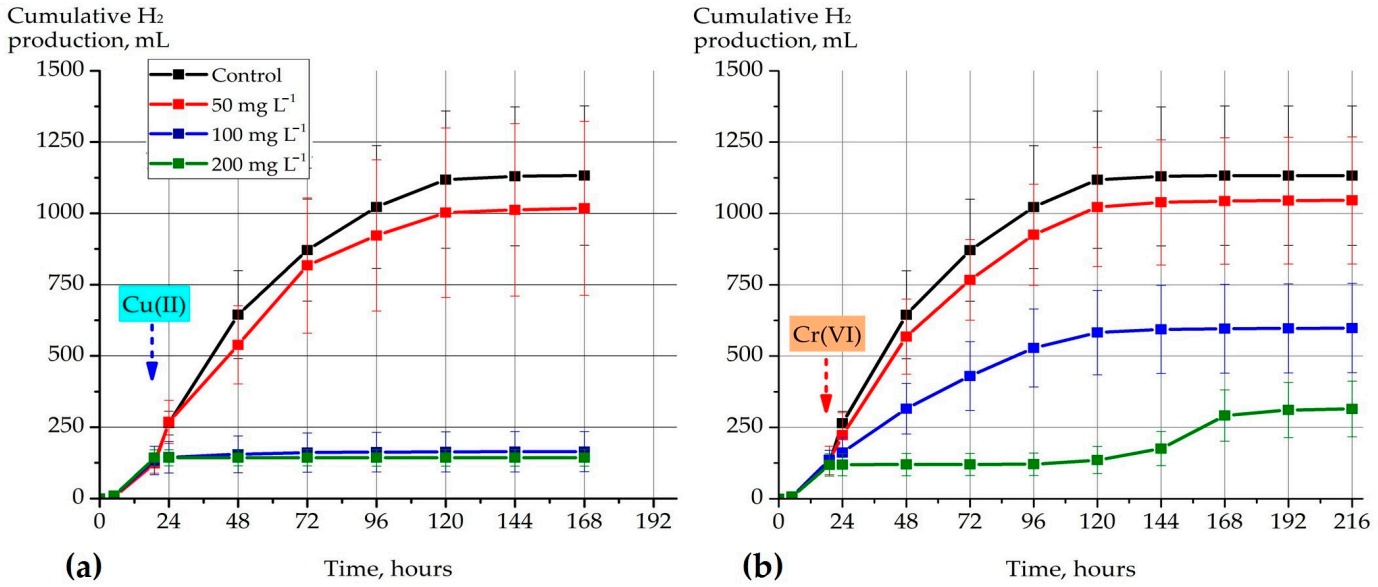

Figure 3. The influences of Cu(II) (**a**) and Cr(VI) (**b**) on cumulative biohydrogen production by *Clostridium butyricum* strain 92. The black lines represent the control without metals; the red lines represent 50 mg L^{-1} Me; the blue lines represent 100 mg L^{-1} Me; the green lines represent 200 mg L^{-1} Me.

Obviously, there was no statistically significant difference between the synthesis of hydrogen under control conditions in the presence of both 50 mg L^{-1} Cu(II) and Cr(VI). In this case, no inhibition of biohydrogen yield was observed (Figure 3a,b). Copper at concentrations of both 100 and 200 mg L^{-1} significantly inhibited the synthesis of hydrogen. It was identified 1132.5 ± 201.5 mL of H_2 were synthesized under control conditions (168 h). Under the influence of 200 mg L^{-1} was synthesized 156.7 ± 28.15 mL H_2 (Figure 3). Less inhibition was observed, and hydrogen synthesis continued under the influences of 100 and 200 mg L^{-1} Cr(VI) (Figure 3b), leading to 598 ± 50.8 mL H_2 and 314.8 ± 97.6 mL H_2 synthesized for 216 h, respectively (Figure 3b). Thus, copper at concentrations of 100 and 200 mg L^{-1} more inhibited the synthesis of hydrogen.

3.3. The Influence of Metals on Metabolic Parameters of the Potato Fermentation Process

The dynamics of pH, Eh, and the DOC were studied during the fermentation of potato by *Clostridium butyricum* 92 strain both in control conditions without metals and in the presence of Cu(II) and Cr(VI) (Figure 4). Therefore, the measurements of metabolic parameters were presented in control conditions during this time. The pH decreased from 7.32 ± 0.4 (beginning of the cultivation) to 5.1 ± 0.25 (168 h) under control conditions (Figure 4a,b).

The redox potential also decreased intensively and, at two days (48 h), was −281 ± 23 mV (Figure 4b,c). The initial concentration of DOC under control conditions was 2681 ± 334 mg L^{-1} and, within three days (72 h), decreased to 920 ± 246 mg L^{-1}.

The intensive degradation of potatoes was observed after the 72 h of cultivation, as evidenced by the increase in the concentration of soluble organic compounds to 1250 ± 162.5 and 1550 ± 177.5 mg L^{-1} for 96 and 120 h of cultivation, respectively. It should be noted that the rate of degradation of the substrate and its accumulation may correlate in the active phase of growth. Therefore, an increase in the concentration of DOC on the third day (72 h) may indicate a slowdown in the growth of anaerobic bacteria.

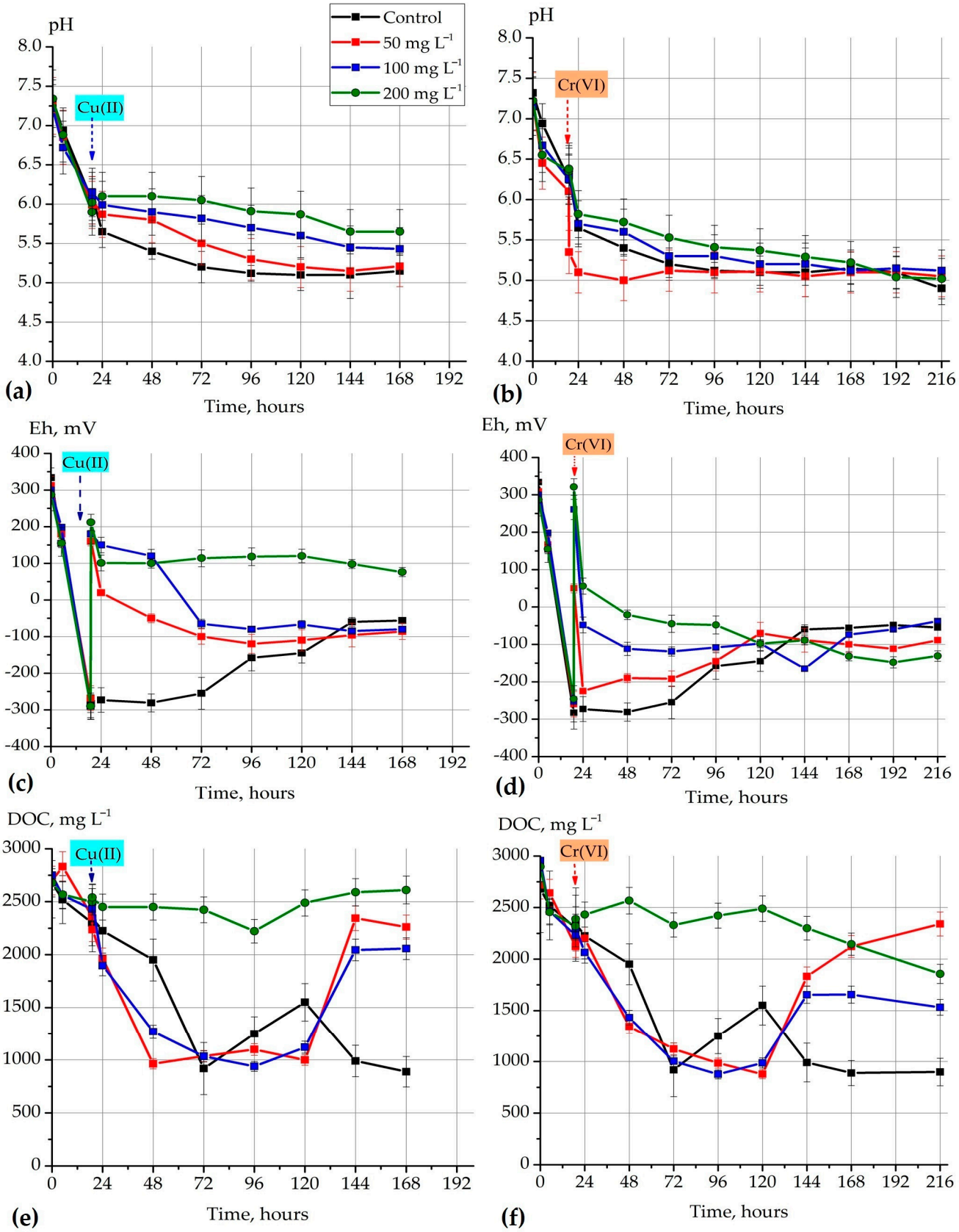

Figure 4. The influences of Cu(II) (**a**,**c**,**e**) and Cr(VI) (**b**,**d**,**f**) compounds in different concentrations on metabolic parameters (pH, Eh, and dissolved organic carbon (DOC)) of *Clostridium butyricum* 92 growth. The black lines represent the control without metals; the red lines represent 50 mg L^{-1} Me; the blue lines represent 100 mg L^{-1} Me; the green lines represent 200 mg L^{-1} Me.

The values of metabolic parameters of potato fermentation differed significantly in the presence of a gradient of metal concentration. Solutions of copper and chromium were inserted to the culture fluid in the active phase of fermentation (for 19 h). The Eh of the culture fluid was very low during this phase. The metals had the greatest effect on the value of the redox potential of the culture fluid. The Eh rapidly increased from -268 ± 34 to $+160 \pm 12$ mV immediately after the insertion of Cu(II) solution at a concentration of 50 mg L^{-1}, as well as from -290 ± 35 to $+212 \pm 22$ mV (Figure 4b) after the insertion of 200 mg L^{-1} Cu(II), and did not decrease significantly until the end of fermentation. A similar situation was observed after the insertion of a chromate solution. Thus, the E_h rapidly increased from -290 ± 34 to $+50 \pm 12$ mV after the insertion of 50 mg L^{-1} Cr(VI). However, the redox potential stabilized and again amounted to -225 mV after 5 h, which was quite an optimal value for the growth of *Clostridium butyricum* stain 92. However, a significant increase of the redox potential from -266 ± 35 mV (before insertion) to $+321 \pm 22$ mV (after insertion) at 200 mg L^{-1} Cr(VI) was observed, which slowed down the fermentation process and substrate consumption. Thus, in contrast to control conditions, the concentration of soluble organic compounds was not decreased in the presence of 200 mg L^{-1} Cr(VI). The DOC concentration did not change significantly for six days after chromium insertion, because the growth of microorganisms was inhibited by this concentration of chromium. No statistically significant changes in the DOC concentration were observed in the presence of 200 mg L^{-1} Cu(II). Thus, similar patterns of the influences of both chromium and copper on the degradation of the substrate were observed (Figure 4e,f). The patterns of change in pH during fermentation were similar in all variants of the experiment (Figure 4a,b). However, a less intense decrease in pH was observed in the presence of 200 mg L^{-1} Cu(II) and Cr(VI). For example, the pH decreased from 7.25 ± 0.4 to 5.2 ± 0.3 in the presence of 50 mg L^{-1} Cu(II) and from 7.34 ± 0.4 to 5.65 ± 0.3 for 168 h of cultivation in the presence of 200 mg L^{-1} Cu(II) (Figure 4a).

3.4. Bioremoval of Metals from a Medium by Clostridium butyricum Strain 92

Metals were inserted into the potato medium in the active phase of microbial growth at 19 h of cultivation without metals. The efficiency of the bioremoval of metal compounds depended on the concentration of each metal. The bioremoval rate of copper and its efficiency at 50 mg L^{-1} Cu(II) were very high. The duration of bioremoval was only 5 h, and the concentration of Cu(II) decreased from 48.9 ± 4.4 to 3.5 ± 1.1 mg L^{-1} (Figure 5a). However, a complete bioremoval of Cu(II) could not be achieved. The bioremoval efficiency was 92.8%. A similar result was also observed with the removal of 50 mg L^{-1} Cr(VI). Chromium was completely removed by microorganisms during 4 h after its insertion (Figure 5b). The duration of the bioremoval of 100 mg L^{-1} Cr(VI) was 10 h, and 100% toxic Cr(VI) was removed. However, its concentration decreased from 98 ± 15.2 to 15.3 ± 4.1 mg L^{-1} already for 1 h of cultivation (Figure 5d). The duration of removal of 100 mg L^{-1} Cu(II) was 9 h, but the concentration of Cu(II) did not change after 9 h and stayed at 12.5 ± 1.6 mg L^{-1} (Figure 5c). The bioremoval efficiency was 87.25%. The greatest inhibition of the growth of *Clostridium butyricum* strain 92 was observed in the presence of 100 mg L^{-1} Cu(II). Bioremoval was very slow and lasted four days in the presence of 200 mg L^{-1} Cu(II). Under such conditions, only 93.8% of Cu(II) was also removed (the concentration decreased from 201 ± 20 to 12.5 ± 2.6 mg L^{-1}). As expected, 100% Cr(VI) was removed at 200 mg L^{-1} Cu(II).

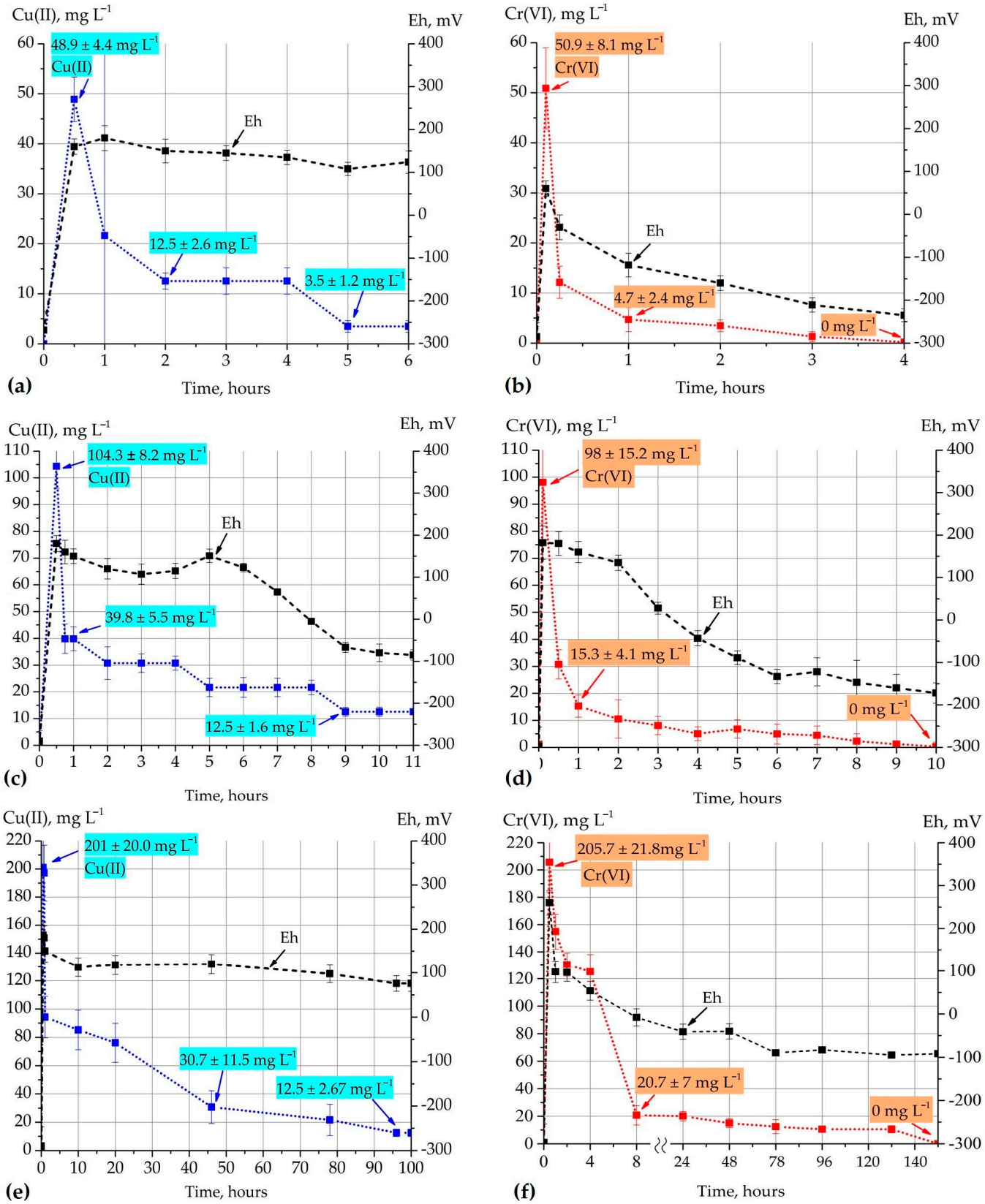

Figure 5. The dynamics of the bioremoval of Cu(II) (**a**,**c**,**e**) and Cr(VI) (**b**,**d**,**f**) by *Clostridium butyricum* strain 92. The black lines represent the value of the redox potential; the blue lines represent the Cu(II) concentration; the red lines represent the Cr(VI) concentration. Metals were inserted into the medium in the active phase of microbial growth at 19 h of strain cultivation.

The duration of bioremoval was seven days (144 h). However, during the first eight hours after application, 89.9% of Cr(VI) was removed (the concentration decreased from 205.7 ± 21.8 to 20.7 ± 7.0 mg L^{-1}; Figure 5e). The decrease of the chromium concentration correlated with a decrease of the Eh to permissible value for the growth of anaerobic microorganisms (−100 ÷ −200 mV). The redox potential did not decrease to negative values in the variants with Cu(II) during the bioremoval process (Figure 5).

The process of potato fermentation in the presence of metals is shown in Figure 6.

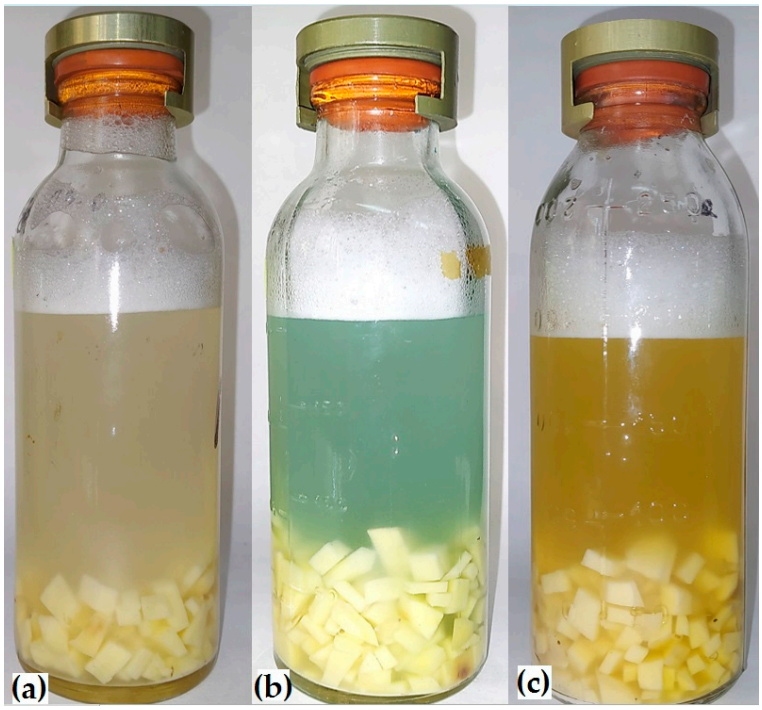

Figure 6. Dark hydrogen fermentation of potato in the absence of metals (**a**) and in the presence of 200 mg L^{-1} Cu(II) (**b**) and 200 mg L^{-1} Cr(VI) (**c**) on the first day (24 h) of the growth of *Clostridium butyricum* strain 92.

Thus, an unadapted strict anaerobic strain was able to efficiently remove Cu(II) and Cr(VI) compounds from the culture fluid via the dark hydrogen fermentation of the potato.

The chemical detoxification of metals was completely excluded, because in the control versions without inoculum, the concentration of metals remained at a constant level from the moment of metals insertion until the end of the experiment. The metal concentration remained at baseline (about 50, 100, and 200 mg L^{-1}) throughout the experiments in the absence of inoculum. Physicochemical changes in the state of copper and chromium compounds in the solution were not observed.

4. Discussion

Strict anaerobic bacteria are very promising for the biogas production and fermentation of different types of organic waste such as food [36], fruits [37], date seeds [38], and rice straw [39]. Bacteria of the *Clostridium* species are one of the most common representatives used for the efficient synthesis of biohydrogen [40]. Herein, we also showed a high efficiency of potato fermentation by *Clostridium butyricum* strain 92. The H_2 yield was as high as −91.4 ± 9.4 L kg^{-1} VS_{potato}. The presented studies also showed a high yield of hydrogen. For example, the hydrogen yield was 61.0 L kg^{-1} VS_{potato} achieved via the hydrogen fermentation of the food waste of a western-style restaurant [41]. An example of super-high hydrogen productivity is the production of 226 L kg^{-1} by *Clostridium thermocellum* from sugarcane bagass (SCB) [42]. However, this substrate is high-energy and easier to ferment than polymeric compounds.

In addition, microorganisms are used to detoxify toxic metals in wastewater [43,44]. However, an incomplete removal of metals from solutions is usually achieved. For example, the *Cupriavidus gilardii* strain CR3 was able to remove only 27% of Cu(II) from the contaminated waste water [45]. Strain *Bacillus cereus* RC-1 was able to remove 16.7%, 38.3%, 81.4%, and 40.3% Cu(II), Zn^{2+}, Cd^{2+}, and Pb^{2+}, respectively, with an initial concentration of only 10 mg L^{-1} at pH 7.0 [46]. Thus, most of the presented data relate to the extraction of metals by aerobic or facultative anaerobic microorganisms.

For the first time, we used a pure culture of strict anaerobe to extract copper(II) and chromium(VI) from solutions. We have previously shown that anaerobic hydrogen- [26,31] and methane-synthesizing microbial communities [34] are able to remove copper from solutions. In addition, the possibility of hexavalent chromium reduction in an anaerobic hydrogen-synthesizing bioreactor was shown [47]. The only one research on the use of a mixed culture (*Thiobacillus* sp. and *Clostridium* sp.) to remove Cu(II) was found [48]. That is why we first investigated the patterns of copper and chromium compounds bioremoval by *Clostridium butyricum* strain 92 of strict anaerobes. In addition, we had a clear theoretical approach and assumed that the bioremoval of copper and chromium by obligate anaerobes would be very effective. The removal of metals can be carried out simultaneously by several mechanisms. First, the reduction of Cu^{2+} to Cu_2O (CrO_4^{2-} to $Cr(OH)_3$) may be due to the significant difference of the redox potentials between the acceptor and donor systems. Really, the acceptor systems (the metals) have very high values of potentials (i.e., E'_0 = +486 mV of Cu^{2+} and E'_0 = +555 mV of CrO_4^{2-}), and strict anaerobes create very low redox potentials, such as −200 ÷ −300 mV [26,49]. Second, the accumulation of metals inside a microbial cells also takes place due to the stereochemical analogy with macroelements [50]. The third mechanism by which copper can be removed is the precipitation of copper carbonate $CuCO_3$ by the CO_3^{2-} anion [31]. Several mechanisms of metals bioremoval can be implemented simultaneously during the growth of the strict anaerobes in the presence of oxidizing metal. We showed a 100% bioremoval efficiency for 50, 100, and 200 mg L^{-1} Cr(VI) and a more than 90% bioremoval efficiency for 100 and 200 mg L^{-1} Cu(II) as a result of potato fermentation by strict anaerobe *Clostridium butyricum* strain 92.

5. Conclusions

A technologically promising strictly anaerobic strain was isolated, capable of the degradation of organic waste, biohydrogen production, and the detoxification of metal-containing waste. A pure culture of strict anaerobes *Clostridium butyricum* strain 92 was used for the first time for the detoxification of copper and chromium. The presented results confirmed the possibility of this strain application for the bioremediation of contaminated by dangerous organic compounds sites and industry H_2 production. The presented results change the idea of the current approach for the microbial detoxification of metals and concentrate on the study of anaerobic metabolism of fermentative bacteria for this purpose.

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