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## EFFECT OF Fe (III) IONS ON HYDROGENASE ACTIVITY AND GROWTH OF THE *CUPRIAVIDUS NECATOR* H16 STRAIN IN DIVERSE GROWTH MEDIA

*Cupriavidus necator* H16 possesses oxygen-tolerant [NiFe] hydrogenases, which can oxidize H<sub>2</sub> in the presence of oxygen (O<sub>2</sub>), easily adapts between autotrophic and heterotrophic lifestyles, and has significant biotechnological potential. **Aim.** To investigate the growth characteristics of *C. necator* H16 using different nutrient media and heavy metal, particularly iron ions, for determination of optimal conditions maximizing Hyd activity. **Methods.** *C. necator* H16 was cultivated heterotrophically using Nutrient Broth (NB) and Fructose-Nitrogen (FN) minimal growth media, pH 7.0. The effects of iron (Fe(III)) ions on the activity of the bacterial H<sub>2</sub>-oxidizing Hyds (hydrogenases) and its growth properties were investigated. Bacterial growth was monitored by determining the optical density, OD<sub>570</sub>, under 570 nm. The total H<sub>2</sub>-oxidizing Hyd activity of whole cells of *C. necator* H16 was measured over a 72-hour growth period. **Results.** In the control samples without adding Fe ions, the activity was observed in the range of 0.5–0.2 U mg<sup>-1</sup> cell dry weight (CDW) in both NB and FN media for up to 48 hours of bacterial growth. However, when 54 μM Fe ions were added to the media, the H<sub>2</sub>-oxidizing Hyd activity increased approximately 2–3 fold after 24 and 48 hours in both FN and NB media, compared to the control samples. Additionally, in the samples supplemented with Fe ions, a shift in the ORP from positive to negative values was observed, primarily starting from the exponential growth phase. After 24 hours, the NB medium showed stimulated biomass formation and Hyd activity compared to the FN medium. **Conclusions.** These findings suggest that certain conditions, such as the presence of higher amounts of iron ions and specific growth media, can enhance the Hyd activity during bacterial heterotrophic growth.

**Keywords:** *cupriavidus necator*, iron ions, bacterial growth, hydrogenase enzymes, oxidation reduction potential, heavy metal influence.

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*Cupriavidus necator* H16, formerly known as *Ralstonia eutropha*, *Alcaligenes eutrophus*, and *Wautersia eutropha*, can easily adapt between the autotrophic and heterotrophic lifestyles and has significant biotechnological potential. It possesses four different O<sub>2</sub>-tolerant [NiFe]-hydrogenases (Hyds) (Shafaat et al., 2013; Lenz et al., 2018) and holds promise for future H<sub>2</sub>-based biotechnology applications, including the production of commercially, environmentally, and medically valuable compounds (Davis et al., 2023; Fan et al., 2022; Jang et al., 2024). Hyds are considered promising candidates as anodic biocatalysts in enzymatic biofuel cells (EFC) or microbial fuel cells (MFC) (Wait et al., 2010; Greening et al., 2023). They exhibit long-lasting catalytic activity and utilize water-derived protons to synthesize hydrogen at turnover frequencies of up to 10<sup>4</sup> s<sup>-1</sup> (Shafaat et al., 2013). EFCs and MFCs play a crucial role in the physiological coupling of H<sub>2</sub> oxidation and O<sub>2</sub> reduction. They can be effectively used not only for electricity generation but also for addressing various environmental problems such as pathogen detection, soil corrosion, and COD/BOD measurement (Simoska et al., 2021).

Many microbes possess Hyd enzymes that either produce or utilize hydrogen gas (H<sub>2</sub>) as part of their metabolic processes. Some of these microbes can reduce toxic metals to less harmful forms through various mechanisms, including Hyd activity (Lloyd & Lovley, 2001; Lloyd et al., 2003). For example, the nickel-iron Hyd of *Desulfovibrio fructosovorans* has been attributed to the reduction of the problematic radionuclide <sup>99</sup>Tc(VII) to insoluble <sup>99</sup>Tc(IV) (Woolfolk & Whiteley, 1962). Additionally, Hyds have been involved in the reduction and recovery of precious metals from waste materials at the expense of H<sub>2</sub> (Yanke et al., 1995; De Luca et al., 2001; Lloyd et al., 1998). Studies using mutants of *D. fructosovorans* deficient in one or more of the three major Hyds have conclusively identified the participation of Hyds in these metal reductase activities (Yong et al., 2002). The genus *Alcaligenes*, also known as

*Ralstonia*, has been found in sediments or soils with high concentrations of heavy metals such as zinc, cobalt, copper, and cadmium. Samples from such environments may contain up to 5–10% of these metals while still harboring viable bacterial populations (Yong et al., 2003). Most *C. necator* strains share common characteristics, including the growth on various organic substrates except sugars, oxidative metabolism, facultative chemolithotrophy, and the presence of megaplasmids carrying genes for heavy metal resistance (Mikheenko et al., 2008). *Cupriavidus metallidurans* strain CH34 is a well-studied model organism for heavy metal resistance. It can tolerate millimolar concentrations of heavy metals and has been utilized for bioremediation, recovery, and reduction of heavy metals (Mikheenko et al., 2008; Mahvi & Diels, 2004; Collard et al., 1993). *Cupriavidus necator* H16 and N9A strains, along with derivatives of strain CH34 lacking one or more of its natural metal resistance plasmids, have been used as recipients in various studies. The metal resistance plasmids pTOM8 and pTOM9, found in strain 31A, convey resistance features that are expressed except in *C. necator* H16 (Mergeay et al., 1985). *C. necator* H16 possesses four different O<sub>2</sub>-tolerant [NiFe]-hydrogenases: the membrane-bound Hyd (MBH), the soluble NAD<sup>+</sup>-reducing Hyd (SH), the actinobacterial-like Hyd (AH), and the regulatory Hyd (RH) (Janssen et al., 2010). The specific requirement for nickel was first demonstrated in autotrophically growing cells of *C. necator* H1 and H16 and subsequently confirmed in these strains by Repaske (1976) and Gruzinskii et al. (1977) (Lenz et al., 2015; Bartha & Ordal, 1965). Nickel and iron are essential components of the active sites of these Hyds. Iron metabolism not only influences Hyd activity by determining the availability of cofactors but also affects the transcription of the Hyd structural operons (Repaske, R. & Repaske, A., 1976). Furthermore, a new lipopeptide siderophore called cupriachelin has been isolated from *C. necator* H16 and structurally characterized. The bacteri-

um reduces Fe(III) using cupriachelin, leading to the release of ferrous iron (Goris et al., 2011).

*C. necator* H16 is a chemolithoautotrophic  $\beta$ -proteobacterium that can grow under both autotrophic and heterotrophic conditions using various organic substrates. However, the optimal conditions and mechanisms for Hyd enzyme synthesis, as well as its relation to metal availability during heterotrophic growth, have not been fully identified yet. It has been established that energy limitation, such as starvation and low  $O_2$  levels, promotes the catabolic de-repression of Hyd gene expression (Gruzinskii et al., 1997; Poladyan et al., 2019).

Therefore, our research aims to investigate the growth characteristics of *C. necator* H16 using different nutrient media and heavy metal conditions, particularly iron ions, to determine the optimal conditions for maximizing Hyd activity. ORP is an important physicochemical parameter that influences microbial growth and is also connected to the activity of Hyd enzymes, therefore, the changes in ORP during bacterial growth were investigated as well. Additionally, we aim to explore the link between  $H_2$ -oxidizing and metal-reducing activities for further applications in biotechnology.

**Materials and Methods. Media and inoculum preparation for the experiments in liquid medium.** *C. necator* H16 (DSM 428) was kindly provided by Dr. Oliver Lenz (Technical University of Berlin, Germany). *C. necator* was grown under heterotrophic conditions, using FN (Fructose-Nitrogen) minimal mineral (Lenz et al., 2018; Poladyan et al., 2019) and NB (Nutrient broth) solutions. The FN solution contains 10  $\times$  H16 buffer (100mL), 850mL  $H_2O$ , as well as 20% w/v of 10mL  $NH_4Cl$ , 20% w/v of 1mL  $MgSO_4 \times 7H_2O$ , 1% w/v of 1mL  $CaCl_2 \times 2H_2O$ , 0.001% w/v of 1 mL  $NiCl_2$ , 0.5% w/v of 1 mL  $FeCl_3 \times 6H_2O$ , and 40% w/v of 10 mL fructose. The 10  $\times$  H16 buffer was composed of 15g  $KH_2PO_4$ , 90g  $Na_2HPO_4 \times 12 H_2O$ , and 1 L  $H_2O$ , pH 7.0. The NB solution contains 5.0 g  $L^{-1}$  peptone, 5.0 g  $L^{-1}$  NaCl, 1.50 g  $L^{-1}$  beef

extract, and 1.50 g  $L^{-1}$  yeast extract, and 1 L  $H_2O$ , pH 7.0. Bacterial aerobic growth was performed on a shaker at 130 rpm at 33 °C. Aerobic conditions for cultivation experiments were attained by using 250 mL baffled flasks with 100 mL FN and NB media. 1.5% of a bacterial pre-culture (starter) was used to inoculate cultures, and cultivation was accomplished at 33 °C for 72 h. For inoculation (starter culture), bacteria were grown under the conditions described above, 37 °C (Poladyan et al., 2019).

Bacterial growth was followed using a Cary 60 UV—vis spectrophotometer (Agilent, USA). The specific growth rate,  $\mu$ , was calculated as  $OD's \ln 2 / \text{doubling time}$  (logarithmic growth phase) and expressed as  $d^{-1}$  (Gabrielyan et al., 2010). The bacterial cell dry weight (CDW),  $g L^{-1}$ , was determined and applied to evaluate the yield of bacteria. The medium's pH was measured with a Hanna Basic pH/ORP Benchtop Meter HI2211-02 (Hanna, USA) meter: medium pH was poised with solutions of HCl (0.1 N) and NaOH (0.1 M).

**Determination of the maximum tolerable concentration (MTC).** The MTC of *C. necator* H16 grown on media with heavy metals was determined by gradually increasing the concentration of the heavy metal: 1  $\mu L / 20 mL$  (0.5 mM) each time on the nutrient agar plate until the strains failed to give colonies on the plate (Hassen et al., 1988). The starting concentration was 1  $\mu L / 20 mL$  (0.5 mM) in solid agar plates. Bacterial precultures were cultivated in 5 mL LB broth at pH 7.0, under aerobic conditions, and incubated at 33 °C for 24 h, a period that corresponds to the exponential phase. The research conducted by Hassen et al. (1988) demonstrates that the maximum tolerable concentrations (MTC) of heavy metals in liquid media are 10 to 1,000 times lower than those found in solid media. Hence, in our study, we selected initial concentrations of heavy metals in the liquid media that were 100 times lower than the levels observed in solid media, falling within the range of 10 to 1,000 times lower.

The following salts of analytical purity were used as sources of the heavy metals: cadmium sulfate octahydrate ( $\text{CdSO}_4 \times 8\text{H}_2\text{O}$ ), pentahydrate copper sulfate ( $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ ), cobaltous sulfate hexahydrate ( $\text{CoSO}_4 \times 6\text{H}_2\text{O}$ ), nickel chloride hexahydrate ( $\text{NiCl}_2 \times 6\text{H}_2\text{O}$ ), iron chloride hexahydrate ( $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ ), and heptahydrate zinc sulfate ( $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ ). Solutions with a concentration of 1 M of the mentioned heavy metals, besides iron, served as a stock solution, which was sterilized via microporous filtrations. As a stock solution of iron, 0.5% w/v of 1 mL  $\text{FeCl}_3 \times 6\text{H}_2\text{O}$  served (see the composition of the FN media mentioned above). MTCs were determined at 33 °C for 7 days.

**Oxidation and reduction potential measurement.** The medium's oxidation and reduction potential (ORP) was measured by using a Hanna Basic pH/ORP Benchtop Meter HI2211-02 (Hanna, USA) meter. The electrode was checked in a solution with a composition of 0.049 M  $\text{K}_3[\text{Fe}(\text{CN})_6]$  and 0.05M  $\text{K}_4[\text{Fe}(\text{CN})_6] \times 3\text{H}_2\text{O}$ , pH 6.86: the readings for the redox electrode at 25 °C was  $+ 250 \pm 5$  Mv (Poladyan et al, 2019).

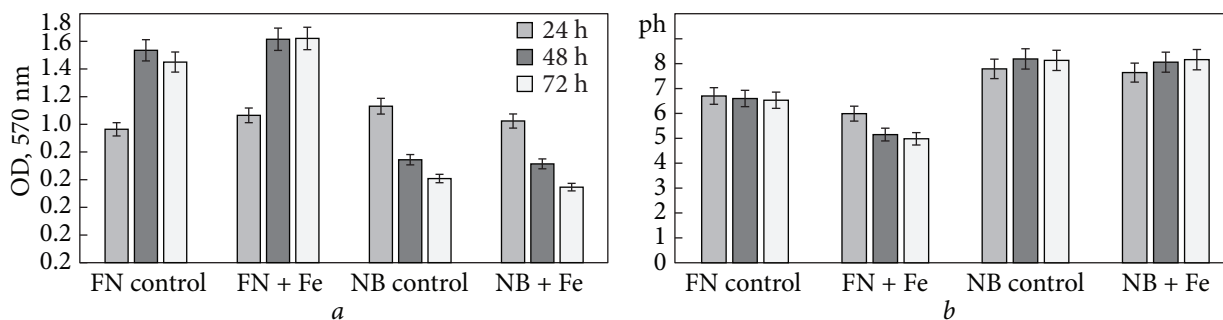
**Monitoring of the total Hyd activity.** Bacterial growth was monitored by determining the optical density,  $\text{OD}_{570}$ , under 570 nm. Cells were harvested after 72 h by centrifugation at 6500 rpm, 20 °C, and 20 min centrifugation. Collected bacteria were washed with 50 mM potassium phosphate buffer, pH 7.0.

The whole cells'  $\text{H}_2$ -oxidizing total activity was monitored by methylene blue (MB) reduction, 570 nm, 33 °C, using a spectrophotometer, Cary 60 UV—vis, Agilent Technologies, USA. 15 to 20 mL of bacterial whole cells were supplemented into a 1.9 mL reaction mixture of 2.5 mL of 50mM potassium phosphate buffer, pH 7.0, and  $\text{H}_2$ -saturated. MB was provided as an acceptor of artificial electrons (Lenz et al., 2018) One unit (U) of Hyd enzyme activity corresponds to  $1\mu\text{M}$   $\text{H}_2$  oxidized per min and 1mg protein (Lenz et al., 2018) or 1g CDW.

**Data evaluation and reagents used in the research.** Microsoft Excel 2016 was used for data processing. The data show values determined from 3 independent measurements; the standard average of the mean data with standard errors was determined using the corresponding Microsoft Excel 2016 function, and Student's criteria (P) were considered to approve the difference in average data among different series of measurements. The difference was valid when  $P < 0.05$ .

Cadmium sulfate octahydrate, pentahydrate copper sulfate, cobaltous sulfate hexahydrate, nickel chloride hexahydrate, iron chloride hexahydrate, heptahydrate zinc sulfate (Sigma-Aldrich (St. Louis, MO, USA)), and all other reagents used in the study were of analytical grade.

**Results. Determination of maximum tolerable concentrations of heavy metals against *C. necator* H16.** *C. necator* H16 (DSM 428) was assessed for its metal resistance capability using nutrient agar media containing metal concentrations ranging from 0.1 to 3.5 mM for Cd, Zn, Co, Ni, Fe, and Cu ions. The results of the maximum tolerable concentrations (MTCs) test for each heavy metal with the *C. necator* H16 strain demonstrated varying levels of tolerance on solid agar plates. The highest resistance was observed against  $\text{Fe}^{3+}$  at a concentration of 3.0 mM (3000  $\mu\text{M}$ ). This was followed by  $\text{Ni}^{2+}$  and  $\text{Cu}^{2+}$  at 2.5 mM, and  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Zn}^{2+}$  at 2.0 mM.  $\text{Fe}^{3+}$  and  $\text{Ni}^{2+}$  were chosen for further observation due to their higher resistance levels. According to Hassen et al. (1998), *C. necator* H16 was cultivated in liquid media (NB and FN) supplemented with  $\text{Fe}^{3+}$  and  $\text{Ni}^{2+}$  ions at concentrations 100 times lower than those used in solid media. This was done to assess the growth characteristics and Hyd activity of bacteria in the presence of those metal ions. The liquid media test proved to be sensitive, detecting toxicity at concentrations 10 to 1000 times lower than those observed in solid media. Despite its aforementioned limitations, the liquid media test provided a reliable means of evaluating metal toxicity in polluted environments, such as industrial



**Fig. 1.** *C. necator* H16 growth in FN and NB media in the presence of  $\text{Fe}^{3+}$ . Bacteria were grown aerobically at pH 7.0 for 72 h: (A) Growth kinetics measuring OD (570 nm) with the addition of  $\text{Fe}^{3+}$ ; and (B) pH is shown for media studied; The average data of three independent measurements are presented ( $p < 0.05$ )

effluents, incinerator residues, landfill municipal refuse, and sewage sludge leachates.

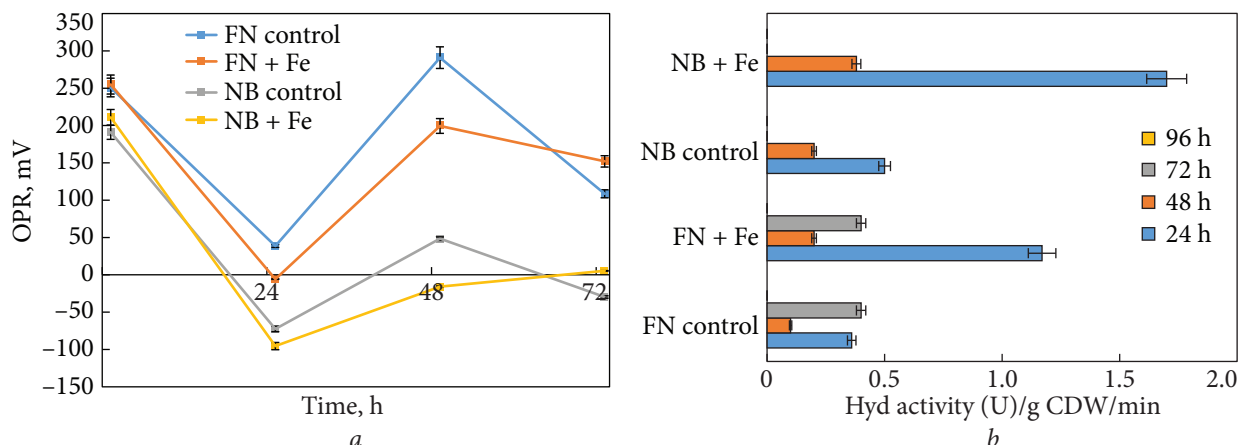
**Growth characteristics and Hyd activity of *C. necator* H16 in FN and NB media in the presence and absence of  $\text{Fe}^{3+}$  ions.** *C. necator* H16 was cultivated under heterotrophic conditions using FN minimal mineral and NB media, as described in the Materials and Methods section. Bacterial growth was conducted aerobically on a shaker at 130 rpm and 30 °C. Throughout the study, various growth properties including optical density (OD), specific growth rate ( $\mu$ ), pH, and oxidation-reduction potential (ORP) kinetics were monitored. Additionally, the total  $\text{H}_2$ -oxidizing Hyd activity of *C. necator* H16 bacteria was investigated in both FN and NB media in the presence and absence of Fe ions. The FN minimal and NB media without Fe ions served as control samples. To examine the impact of  $\text{Fe}^{3+}$  ions on the bacterial growth, 300  $\mu\text{L}$  of  $\text{FeCl}_3$  was added at a final concentration of 54  $\mu\text{M}$ , which was 100 times lower than the maximum tolerable concentration (MTC) determined in solid media. Fig. 1 demonstrates that after 24 hours, bacterial growth was observed in both FN and NB media. Furthermore, the highest optical density ( $\text{OD } 1.13 \pm 0.10$ ) was observed in the NB control media after 24 hours compared to FN. A similar biomass formation ( $\text{OD of } 1.06 \pm 0.03$ ) was observed in FN upon supplementation with Fe ions. Compared to FN, the specific growth

rate ( $\mu$ ) of bacteria in NB medium was increased ~ 1.4-fold. However, after 48 hours, the OD decreased by approximately 1.5 times in both NB and NB +  $\text{Fe}^{3+}$  media. In contrast, the OD remained unchanged in FN +  $\text{Fe}^{3+}$ -supplemented medium after 72 hours, while it continued to decline in both NB and NB +  $\text{Fe}^{3+}$  media.

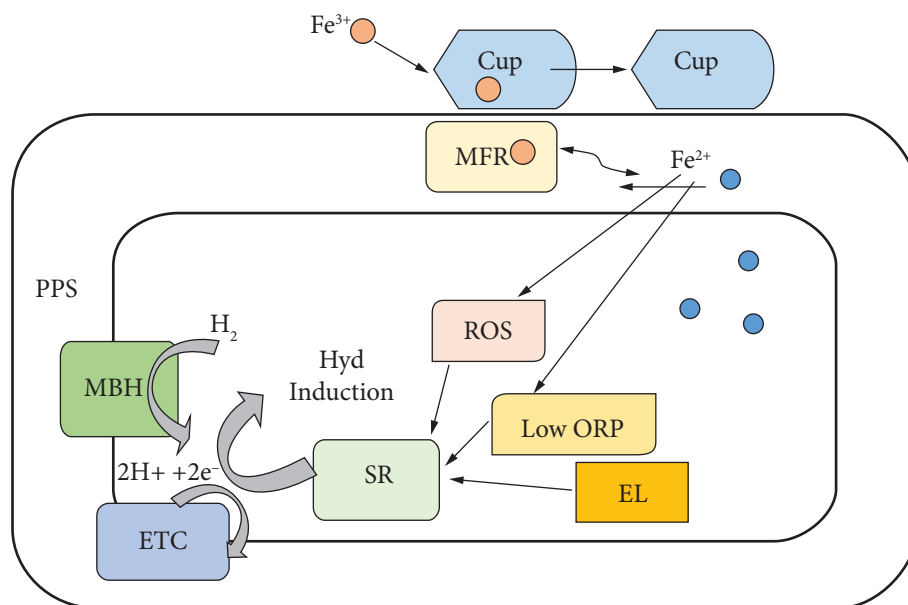
The pH kinetics during bacterial growth was monitored and shown in Figure 1B. Bacteria were initially grown at pH 7.0. After 24 hours, the pH decreased to  $6.7 \pm 0.13$  in the FN control and  $6.0 \pm 0.17$  in FN +  $\text{Fe}^{3+}$  medium, while it increased to  $7.8 \pm 0.25$  in NB and  $7.64 \pm 0.19$  in the NB +  $\text{Fe}^{3+}$  media. There was little difference observed after 48 and 72 hours.

Fig. 2 A shows the changes in oxidation-reduction potential (ORP) during growth in FN, FN +  $\text{Fe}^{3+}$ , NB, and NB +  $\text{Fe}^{3+}$  media. After 24 hours, a decrease in ORP was observed in both FN and FN +  $\text{Fe}^{3+}$  media ( $40 \pm 10$  mV,  $-10 \pm 5$  mV, respectively) as well as in NB and NB +  $\text{Fe}^{3+}$  media ( $-75 \pm 10$  mV and  $-100 \pm 10$  mV, respectively). Notably, negative ORP values were observed in FN +  $\text{Fe}^{3+}$  and NB +  $\text{Fe}^{3+}$  media.

The total  $\text{H}_2$ -oxidizing activity of *C. necator* H16 whole cells was assessed using the methylene blue (MB) reduction assay. Approximately 15 to 20 mL of bacterial whole cells were added to a 1.9 mL reaction mixture, pH 7.0, and saturated with  $\text{H}_2$ . MB was used as an electron acceptor (Poladyan et al., 2019). The  $\text{H}_2$ -oxidizing activity of *C.*



**Fig. 2.**  $\text{Fe}^{3+}$  impact on the kinetics of ORP of *C. necator* H16 (A). Bacteria were grown aerobically, at pH 7.0, for 72 h,  $n=3$ ,  $p<0.05$ . (B)  $\text{H}_2$ -oxidizing total Hyd activity of whole cells of *C. necator* H16 is shown.  $\text{Fe}^{3+}$  ( $54\mu\text{M}$ ) was added to both FN and NB media. CDW is cell dry weight. For the other details, see the section «Materials and Methods» and the legend to Fig. 1 (72 h,  $n=3$ ,  $p<0.05$ )



**Fig. 3.** Hypothesis for possible mechanisms by which iron ions stimulate Hyd activity. Cup — cupriachelin, MFRb — membrane ferric reductase, ROS — reactive oxygen species, Low ORP — low oxidation-reduction potential, EL — energy limitations, SR — stress response, ETC — electron transport chain, MBH — a membrane-bound hydrogenase, PPS — periplasmic space

*necator* H16 whole cells was measured at different time points during bacterial growth (24 h, 48 h, and 72 h). Without the addition of Fe ions, the activity ranged from 0.5 to 0.2  $\text{U mg}^{-1}$  cell dry weight (CDW) in both NB and FN media for 24

h and 48 h of bacterial growth. However, the addition of Fe ions increased the  $\text{H}_2$ -oxidizing activity by approximately 2 to 3-fold after 24 h and 48 h in both FN and NB media. Interestingly, after 72 h (late stationary growth phase), no  $\text{H}_2$ -ox-

idizing activity was detected in the NB medium, while minimal activity ( $0.1\text{--}0.2\text{ U mg}^{-1}\text{ CDW}$ ) was observed in the FN medium.

**Discussion.** Bacterial hydrogenases (Hyds) have been utilized in the removal of heavy metals from solutions through their reduction to less soluble metal species (Yanke et al., 1995; De Luca et al., 2001; Lloyd et al., 1998; Yong et al., 2002). Thus, Hyds play a crucial role in biotechnological applications, including the Hyd-mediated bioreduction of heavy metals. *C. necator*, along with its  $\text{O}_2$ -tolerant Hyds, is a promising candidate for bioremediation purposes. Additionally, as mentioned earlier, the  $\text{O}_2$ -tolerant Hyds of *C. necator* can be employed as suitable biocatalysts in enzymatic fuel cells (Lenz et al., 2018). Therefore, optimizing the growth of *C. necator* and the production of Hyds in the presence of various heavy metals becomes an appealing strategy for biotechnology (Logan et al., 2006).

In our study, the *C. necator* H16 strain exhibited varying levels of tolerance, with the highest resistance observed against  $\text{Fe}^{3+}$  at a concentration of 3.0 mM. According to the maximal tolerable concentrations (MTCs,  $\text{Fe}^{3+}$  and  $\text{Ni}^{2+}$  were selected for further investigation due to their higher resistance. However, it is worth noting that the bacteria showed negligible growth in liquid media containing  $0.1\text{--}0.5\text{ mM Ni}^{2+}$ . This observation suggests that high  $\text{Ni}^{2+}$  concentrations may be more detrimental in liquid media compared to  $\text{Fe}^{3+}$  (Sai et al., 2000).

After 24 hours, growth was observed in both NB and FN media. Additionally, maximum growth was noticed in the NB control at 48 hours, and biomass (OD) increased by approximately 1.6 times in both the FN control and in the presence of  $\text{Fe}^{3+}$ , likely due to the utilization of fructose by *C. necator* H16 with the long-lasting stationary growth phase. In contrast to FN, bacteria exhibit faster and more stimulated growth in NB, with a shorter stationary growth phase.

The presence of ferric salts may influence pH during the process. The dissociation of fer-

ric chloride releases  $\text{H}^+$  ions into the FN +  $\text{Fe}^{3+}$  medium, leading to a decrease in pH compared to the control (Daoud & Karamanev, 2006). On the other hand, there is no significant difference in pH between NB and NB +  $\text{Fe}^{3+}$  media. The higher pH values observed in NB and NB +  $\text{Fe}^{3+}$  media can be attributed to the generation of  $\text{NH}_4^+$  from amino acid degradation (Mohiuddin & Khattar, 2023).

A decrease in ORP was observed in both FN and FN +  $\text{Fe}^{3+}$  media and NB and NB +  $\text{Fe}^{3+}$  media after 24 hours, which is consistent with previous studies (Lenz et al., 2018; Iskandaryan et al., 2023). Previous studies have demonstrated that the ORP of the medium determined Hyd catalytic as well as its activation properties (Petrov et al., 1989), and in most cases, Hyd activities are higher at low ORP values, taking into consideration few exceptions (Zorin et al., 1984; Zorin, 1986). It is noteworthy that more negative ORP values were observed in media supplemented with Fe ions. Previous studies have identified that the initial redox potential values depend on the concentration of  $\text{Fe}^{3+}$  ions (Sand & Gehrke, 2006; Cain & Smith, 2021), as this parameter is directly related to the  $\text{Fe}^{3+}/\text{Fe}^{2+}$  ion exchange ratio (Cain & Smith, 2021). Considering the activated oxidation processes, a correlation between the presence of  $\text{Fe}^{3+}$  ions and changes in ORP over time was assumed. As bacterial activity is the main controller of the  $\text{Fe}^{3+}/\text{Fe}^{2+}$  ratio for 48 hours and 72 hours, the ORP in the four samples fluctuated over time.

$\text{H}_2$ -oxidizing Hyd activity was stated in both FN and NB, as well as in the presence of  $\text{Fe}^{3+}$ . It is worth mentioning that, for the first time, we demonstrate that the addition of Fe ions at a concentration 3 times higher than  $18\text{ }\mu\text{M}$  (Lenz et al., 2018) increased  $\text{H}_2$ -oxidizing Hyd activity by approximately 2- to 3-fold after 24 hours and 48 hours in both FN and NB media.

The proposed effect of iron ions on the Hyd activity and growth of *C. necator* H16 is illustrated in Fig. 3.

*C. necator* H16 is known to secrete cupriachelin (siderophore) (Cup), which can acquire  $\text{Fe}^{3+}$  from the environment (Kreutzer et al., 2012). Although cupriachelin secretion is typically triggered by iron limitation, our observations indicate that this secretion can continue even in conditions of high iron abundance. This persistence may be due to the limited availability of free  $\text{Fe}^{3+}$  ions, specific intracellular iron-sensing thresholds, or as part of a stress response to oxidative conditions caused by excess iron. Additionally, the production of siderophores in the presence of excess iron helps sequester the surplus iron. This mechanism protects the cells from damage caused by reactive oxygen species and aids in maintaining cellular iron homeostasis (Andrews et al., 2003).

The coordinated  $\text{Fe}^{3+}$  is then liberated from cupriachelin via membrane-ferric reductases (MFR), resulting in the release of ferrous ( $\text{Fe}^{2+}$ ) iron (Ohyashiki et al., 2002). The Fe ions might also stimulate Hyd maturation and enzyme activity. However, an excess amount of intracellular reduced iron might potentially generate reactive oxygen species (ROS) through lipid peroxidation, forming trivalent iron-oxygen complexes (Cain & Smith, 2021; Ohyashiki et al., 2002), or by inducing low redox potential (ORP) (low ORP mimics energetic limitation), which can trigger a stress response leading to Hyd induction. These stress responses can stimulate the activity of membrane-bound hydrogenases (MBH). Thus, hydrogenase enzymes (Hyd) have various applications due to their ability to catalyze the oxidation of hydrogen gas ( $\text{H}_2$ ) at high turnover frequencies. They can be used in bioelectricity generation in fuel cells and can also mediate reductive reactions for metal detoxification. The results of this study indicate that the presence of high concentrations of iron ions in different growth media can significantly affect the Hyd activity of *C. necator* H16, most likely through the modulation of the medium's ORP.

These findings may have practical applications in  $\text{H}_2$ -based technologies, as they provide insights into promoting faster and stimulated biomass production and improving Hyd enzyme obtaining processes.

**Conclusions.** *C. necator* H16 produces highly active hydrogenase (Hyd) enzymes that catalyze the oxidation of hydrogen gas. In addition to their biotechnological applications in energy production and metal reduction, these enzymes may also act as sensitive indicators of heavy metal pollution. This study demonstrates that elevated concentrations of iron ions in various growth media significantly affect Hyd enzyme activity, likely by altering the oxidation-reduction potential (ORP) of the environment. Since heavy metals can disrupt redox balance and interfere with the metalloenzyme function, changes in Hyd activity may reflect alterations in environmental metal stress. These findings suggest that *C. necator* H16, through its hydrogenase response, could be developed as a biological sensor for detecting and monitoring heavy metal contamination in environmental settings. Optimizing growth conditions to enhance this sensitivity may facilitate its use in ecotoxicology and bioremediation monitoring.

**Author Contributions.** AP conceived and designed the study. SN performed the experiments. AM provided new reagents and analytical tools. SN wrote the manuscript. AP and AM edited the manuscript and gave recommendations for the experiment. All authors read and approved the manuscript.

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**Conflict of Interest.** The authors declare no competing interest.

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#### ВПЛИВ ІОНІВ Fe (III) НА АКТИВНІСТЬ ГІДРОГЕНАЗИ ТА РІСТ ШТАМУ *CUPRIAVIDUS NECATOR* H16 У РІЗНИХ КУЛЬТУРАЛЬНИХ СЕРЕДОВИЩАХ

*Cupriavidus necator* H16 має киснево-толерантні [NiFe] гідрогенази, які можуть окислювати H<sub>2</sub> в присутності кисню, легко пристосовуються до автотрофного та гетеротрофного способу життя і мають значний біотехнологічний потенціал. **Мета.** Дослідити характеристики росту *C. necator* H16, використовуючи різні поживні середовища та важкі метали, зокрема іони заліза, для визначення оптимальних умов для максимізації активності Нуд. **Методи.** Загальну Нуд активність цілих клітин *C. necator* H16 вимірювали впродовж 72-годинного періоду росту. *C. necator* H16 культивували гетеротрофно, використовуючи переважно поживний бульйон (NB) та мінімальне фруктозо-азотне (FN) середовище для росту, рН 7.0. Досліджували вплив іонів заліза Fe(III) на активність бактеріальних H<sub>2</sub>-окислюючих гідрогеназ (Нуд) та їхні властивості росту. **Результати.** У контрольних зразках без додавання іонів Fe активність спостерігали в діапазоні 0.5—0.2 U мг<sup>-1</sup> сухої маси клітин як у середовищі NB, так і в середовищі FN впродовж 48 годин росту бактерій. Однак при додаванні 54 мкМ іонів Fe до середовищ, активність Нудс збільшилася приблизно в 2—3 рази через 24 і 48 годин в обох середовищах порівняно з контрольними зразками. Крім того, у зразках, доповнених іонами Fe, спостерігали зміщення окислювально-відновного потенціалу середовища (ORP) від позитивних до негативних значень, починаючи з фази експоненціального зростання. Через 24 години середовище NB продемонструвало стимульоване утворення біомаси та вищу активність Нуд у порівнянні з середовищем FN. **Висновки.** Ці результати свідчать про те, що певні умови, такі як наявність більшої кількості іонів заліза та специфічні середовища для росту можуть посилювати активність Нуд під час гетеротрофного росту бактерій.

**Ключові слова:** *Cupriavidus necator*, іони заліза, ріст бактерій, ферменти гідрогенази, окисно-відновний потенціал, вплив важких металів.