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Review

Biohydrogen production from saline wastewater: An overview

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Abstract: The escalating global demand for sustainable energy has propelled the exploration of biohydrogen production with a promising avenue for simultaneously generating clean energy and managing waste effectively. This review mainly focuses on advances in sustainable biohydrogen production from saline wastewater, especially in a process that leverages the unique abilities of halotolerant and halophilic microorganisms adapted to high-salinity conditions. It provides an extensive understanding of various biohydrogen production methods, which are biophotolysis, photofermentation, dark fermentation, and microbial electrolysis. Additionally, this review elaborated on the enzymology of hydrogen production and the impact of salt stress, with a particular emphasis on the adaptive mechanisms of "salt-in" and "compatible solute" strategies. These adaptations are crucial for maintaining enzymatic activity and structural integrity under hypertonic conditions. Through a comprehensive examination of microbial pathways and strategies, this review aimed to furnish foundational insights that will drive future research and technological innovations in biohydrogen production.

Keywords: biohydrogen; metabolism; salt stress; microbial adaptation; fermentation; saline wastewater

1. Introduction

Given the current global energy crisis, finding sustainable energy sources is the most important issue. In recent decades, hydrogen (H_2) has garnered attention as a clean and sustainable energy carrier with considerable potential. As a high-density energy source, hydrogen boasts a specific energy content of 120−142 $MJ \cdot kg^{-1}$, surpassing those of other fuels, such as methane (50 $MJ \cdot kg^{-1}$) and ethanol (26.8 $MJ \cdot kg^{-1}$) [1]. Furthermore, hydrogen possesses an energy density 2.75 times higher than those of conventional hydrocarbons. Unlike fossil fuels, which contribute to air pollution and global warming through CO_2 emissions, the utilization of hydrogen in fuel cells or through combustion is a carbon-neutral process, with water (H_2O) as its sole byproduct. These compelling attributes position hydrogen-based technologies as promising alternatives to fossil fuel-based transportation systems in the foreseeable future.

Despite its potential, around 96% of hydrogen amount is still generated from fossil fuels via energy-intensive processes, such as pyrolysis, reforming, and biomass gasification, which are unsustainable in the long term [2]. This underscores the urgent

need to develop environmentally friendly, cost-effective, and sustainable hydrogen production technologies. Biological hydrogen production technology, leveraging biological processes, offers a clean, efficient, and renewable alternative. Among feedstock sources, utilizing various waste streams, including organic wastewater, for biological hydrogen production not only addresses waste management challenges but also demonstrates the principles of industrial symbiosis and the circular economy. Biological processes, particularly dark fermentation and photofermentation, are advantageous due to their lower operational costs, enabled by milder reaction conditions and the use of waste substrates. In contrast, conventional hydrogen production methods are not cost-efficient due to their high material costs and significant energy consumption [3].

Saline organic wastewater, which accounts for 5% of total industrial wastewater and is a byproduct of numerous industrial processes, presents a unique substrate for biohydrogen production [4]. However, its high salt content poses significant challenges, notably inhibiting the activity of biohydrogen producers [5]. Salt stress also imposes selective pressure that specifically favors the growth of halotolerant or halophilic microorganisms [6]. Recent advances have investigated the metabolic resilience and capabilities of these specialized microbes and demonstrated their ability to convert saline organic wastewater into biohydrogen effectively [5,7–12].

This review aimed to consolidate and elaborate on the progress in microbiology related to biohydrogen production from saline organic wastewater. It focused on the biological processes utilized for biohydrogen production under saline conditions, examined the influence of salinity on enzymology, and explored halotolerant biohydrogen producers, along with their adaptation mechanisms. By shedding light on the potential of hydrogen generation from saline wastewater, this review provides a foundational understanding for future research and applications in this critical and emerging field.

2. Biological processes for hydrogen production from saline organic wastewater

Biohydrogen production from saline organic wastewater falls into four categories—biophotolysis, photofermentation, dark fermentation, and microbial electrochemical technology—each with unique advantages and specific technical challenges that need to be overcome for practical applications. Biophotolysis harnesses solar energy to break down water molecules into oxygen and hydrogen, and it occurs in the cells of green algae or cyanobacteria [13]. Photo-fermentation utilizes light energy and organic compounds to produce biohydrogen, relying on nitrogenase enzymes under nitrogen-deficient conditions [14]. Dark fermentation employs bacteria to decompose carbohydrate-rich substrates into hydrogen and various byproducts [15]. Microbial electrochemical technology, typically implemented through microbial electrolysis cells, involves electroactive bacteria that metabolize organic compounds and convert electrons to the anode via extracellular electron transfer, with hydrogen evolution occurring at the cathode [16]. In this section, a detailed description is given of these four biohydrogen production technologies used in saline wastewater.

2.1. Biophotolysis

In the biophotolysis process, organisms such as green algae or cyanobacteria engage in photosynthesis similar to that of terrestrial plants (**Figure 1a**) [17]. These microorganisms harness solar energy to oxidize water in Photosystem II (PSII), producing oxygen and reduced ferredoxin [13,18]. This reduced ferredoxin subsequently facilitates the conversion of H^+ into H_2, mediated by enzymes, such as hydrogenases or nitrogenases, in Photosystem I (PSI) [13,19]. Biophotolysis can be divided into direct and indirect biophotolysis processes. Microalgal species and some specific unicellular cyanobacteria have been reported to use the direct biophotolysis pathway (Equation (1)). Indirect biophotolysis, which uses light to synthesize carbohydrates through a chemical reaction, can be performed by both microalgae and cyanobacteria (Equations (2) and (3)). The efficiencies of direct and indirect biophotolysis differ significantly due to the distinct mechanisms involved in each process. Direct biophotolysis, where water is directly split into hydrogen and oxygen by photosynthetic organisms, typically suffers from low efficiency due to the sensitivity of the oxygen-evolving complex and the inhibitory effect of oxygen on hydrogenase enzymes. This results in a low overall hydrogen production rate. But direct biophotolysis has been observed to achieve conversion efficiencies of solar radiation as high as 80% [20]. Indirect biophotolysis, on the other hand, involves two separate stages: the production of a carbon source via photosynthesis and its subsequent conversion to hydrogen in a dark, anaerobic environment. The efficiency of this process is relatively low compared with other hydrogen production methods due to energy losses during the two-step process [20].

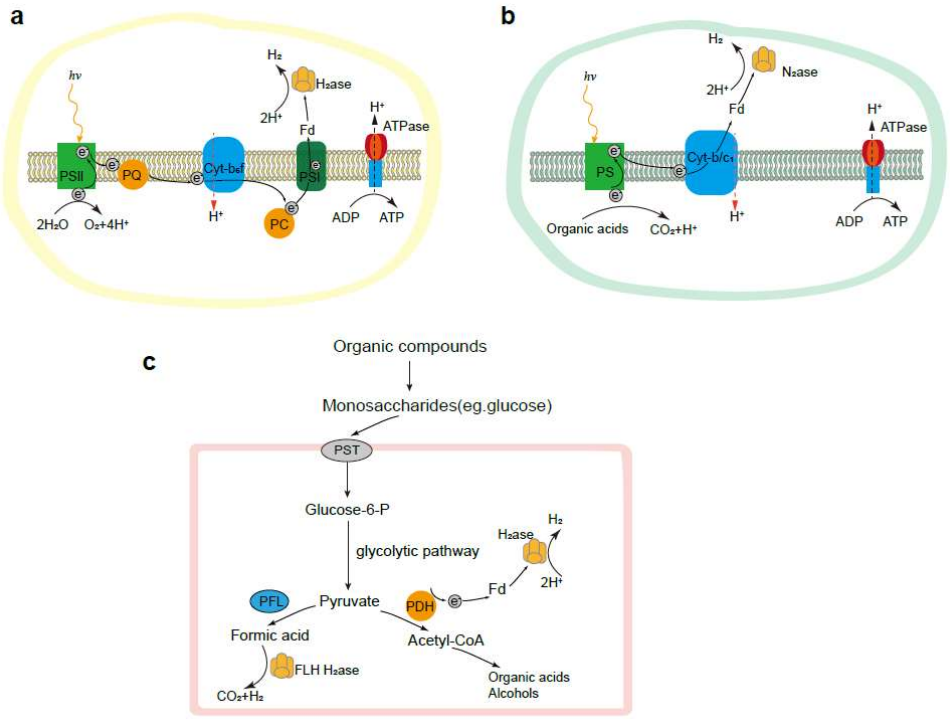

Figure 1. Metabolic pathways in basic biohydrogen-producing system: **(a)** biophotolysis, **(b)** photo-fermentative, and **(c)** dark fermentative.

$$2H_2O + \text{light energy} \rightarrow 2H_2 + O_2 \quad (1)$$

$$6H_2O + 6CO_2 + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2)$$

$$C_6H_{12}O_6 + 6H_2O + \text{light energy} \rightarrow 12H_2 + 6CO_2 \quad (3)$$

The effect of salinity on hydrogen production by biophotolysis has been studied in previous studies. Generally, an increase in salinity correlates with a decline in the hydrogen generation rate in biophotolysis, primarily due to the diversion of energy and reductants toward the extrusion of Na^+ or the prevention of Na^+ influx. In addition, salinity also exerts a significant impact on hydrogen production by affecting the biophotolysis system in organisms [21]. Salt stress could impede PSII-mediated oxygen evolution and result in NaCl accumulation in the cytoplasm, adversely affecting growth rates, photosynthetic activities, and electron transport [21]. A previous study demonstrated that restoration of PSII activity could be achieved using diphenylcarbazide, a synthetic electron donor to PSII in salt-exposed cyanobacterial thylakoids, indicating that the water-oxidizing complex is a critical target affected by salinity in *Synechococcus* cells [21]. Nonetheless, another study suggested that in cyanobacteria, the PSII center itself is the direct target of salinity. According to Allakhverdiev et al. [22], the combination of light and salinity inactivated PSII of *Synechocystis*, specifically inhibiting the synthesis of core protein in PSII, D1 protein [22]. Conversely, PSI electron transport activity was enhanced under salt stress, as evidenced by an increase in P700 and PSI reaction center quantities in response to high salt conditions [23]. Moreover, salt stress induces significant changes in the pigment content of a biophotolysis system. For instance, in *Synechocystis* sp. PCC 6803, medium salinity (0.3M NaCl) was found to increase the chlorophyll a (Chl a) content, whereas higher concentrations (0.6–1M NaCl) resulted in a significant decrease [24,25]. In contrast, it was observed that carotenoids, such as echinenone, oscillaxanthin, and myxoxanthophyll, tended to increase under a high-salinity condition (1.026 M NaCl), potentially diminishing the number of photons accessible for Chl a absorption and exacerbating irradiance as a secondary stress factor [24].

Biophotolysis is advantageous due to the abundance of its substrate (water) and the simplicity of its products (H_2 and CO_2) (Equations (1)–(3)). But mitigating the effects of salt stress is a key issue that needs to be solved in saline wastewater treatment by biophotolysis. Biophotolysis also encounters other challenges, such as limited light conversion efficiency, oxygen sensitivity of hydrogenase enzymes, and the requirement for a costly hydrogen-impermeable photobioreactor [14].

2.2. Photo-fermentation

Photo-fermentation involves certain photo-fermentative bacteria (e.g., purple non-sulfur bacteria) that engage in anaerobic photosynthesis. These bacteria utilize sunlight to produce adenosine triphosphate (ATP) and energetic electrons via reverse electron transport [26]. The produced electrons subsequently facilitate the reduction of ferredoxin, while ATP and the reduced ferredoxin facilitate the reduction of protons for hydrogen production via nitrogenase [14]. Unlike the biophotolysis process, photo-fermentative does not derive electrons from water but relies on organic compounds, typically volatile fatty acids, as substrates (Equation (4)).

$$CH_3COOH + 2H_2O + \text{light energy} \rightarrow 4H_2 + 2CO_2 \quad (4)$$

Most photo-fermentative bacteria typically thrive in freshwater habitats and are generally sensitive to salinity. Only a limited number of species have been identified for hydrogen production under salt stress, with few proving efficient in hydrogen accumulation [5]. These species can be classified as halotolerant photo-fermentative bacteria, which tolerate high salt concentrations but do not rely on NaCl for normal growth. Tsuzuki et al. [27] presented *Rhodobacter sphaeroides* that adapted to salt concentrations up to 0.4 M through the activation of salt stress response systems, including organic solute transport and synthesis, shortly after salt exposure [27]. *Rhodopseudomonas palustris* strain 42 OL has been shown to demonstrate the ability to produce H_2 under 1% salt stress, tolerating up to 4.7% of salts without the addition of compatible solutes [5]. The hydrogen production rates in the presence of salt were found to remain comparable to those obtained under unstressed conditions [5]. Also, Ike et al. demonstrated that *R. sphaeroides* could generate hydrogen under 3% salt stress [28].

Growth challenges and the decline in hydrogen generation caused by salinity in the photo-fermentative bacteria can be largely attributed to the effects of salinity on nitrogenase present in their cells [28]. In halotolerant *Rhodobacter capsulatus* strain E1F1, diazotrophic growth was found to be inhibited by salts through impacting nitrogenase activity [29]. This reduction in nitrogenase activity also correspondingly led to diminished hydrogen production capabilities at these salt concentrations. The presence of salt stress not only directly affected the ability to produce hydrogen through the enzyme but also influenced its synthesis within the cell. A negative effect on the expression level of nitrogenase was observed with increasing salt concentrations in *Rhodopseudomonas palustris* strain 42 OL, particularly noticeable between 0%–1% and between 2%–3% [5]. This suggests a regulatory mechanism controlling nitrogenase synthesis under salt stress. In *Rhodobacter capsulatus* strain E1F1, the nitrogenase was found to be reversibly inactivated and no longer synthesized under salt stress. Only the introduction of glycine betaine enabled the restoration of metabolic function at 1.2% of NaCl [28,29]. Furthermore, the synthesis of compatible solutes (e.g., trehalose and glycine betaine) also contributes to the reduction of hydrogen production. The carbon source is redirected toward the synthesis of other carbon compounds instead of being oxidized for H_2 production [5].

Photo-fermentation enables thorough conversion of organic waste materials into H_2, presenting potential applications in wastewater treatment. However, considering the sensitivity of most photo-fermentative bacteria to salinity, the focus of saline wastewater treatment through photo-fermentation is on discovering halotolerant photo-fermentative bacteria. Photo-fermentation also faces various challenges, such as limited light conversion efficiency, substantial energy requirements for nitrogenase activity, and the need for a hydrogen-impermeable photobioreactor [14].

2.3. Dark fermentation

Dark fermentation leverages a variety of anaerobic microorganisms to break down carbohydrate-rich substrates into hydrogen and byproducts, such as acids (e.g., lactic, acetate, and butyrate) and alcohols (e.g., ethanol and butanol) [30,31]. The process of dark fermentation initiates with the hydrolysis of complex polymers (e.g.,

cellulose) into monosugar (e.g., glucose), which is then converted into pyruvate via the glycolytic pathway, simultaneously generating adenosine triphosphate (ATP) [32]. Pyruvate then diverges into two distinct pathways to yield hydrogen (Equations (5)–(8)).

In facultative anaerobes, pyruvate is converted to formate via the pyruvate formate-lyase (PFL) pathway [32]. PFL catalyzes the conversion of pyruvate to formate and acetyl-CoA (Equation (5)). The formate is then further decomposed into H_2 and carbon dioxide (CO_2) through the actions of formate hydrogen lyase and various hydrogenases (Equation (6)) [32]. Conversely, in obligate anaerobes, pyruvate undergoes conversion via the pyruvate:ferredoxin oxidoreductase (PFOR) pathway (Equations (7) and (8)) [32]. In this pathway, pyruvate dehydrogenase catalyzes the release of electrons from pyruvate, resulting in the formation of acetyl-CoA. These electrons are conveyed to oxidized ferredoxin (Fd(ox)), converting it into reduced ferredoxin (Fd(red)). The reduced ferredoxin then drives the reduction of protons to produce hydrogen gas, facilitated by hydrogenase enzymes. Simultaneously, acetyl-CoA is further metabolized into acetate with the catalyzation of acetate kinase and alcohol dehydrogenase [32]. Through both the PFL and PFOR pathways, it is theoretically possible to obtain 1 mol of H_2 per mol pyruvate.

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{Acetyl-CoA} + \text{HCOOH} \quad (5)$$

$$\text{HCOOH} \rightarrow CO_2 + H_2 \quad (6)$$

$$\text{Pyruvate} + \text{CoA} + \text{Fd(ox)} \rightarrow \text{Acetyl-CoA} + \text{Fd(red)} + CO_2 \quad (7)$$

$$2H^+ + \text{Fd(red)} \rightarrow H_2 + \text{Fd(ox)} \quad (8)$$

Theoretically, 1 mol of glucose could yield up to 12 mol of H_2 [33]. But in an actual fermentation system, the complete transfer of energy from glucose to hydrogen is hindered by various factors, such as microbial growth and the formation of by-products. Thus, the actual hydrogen yield never reaches the theoretical maximum in any fermentative organisms. The maximum hydrogen yield for dark fermentation does not exceed 4 mol H_2/mol glucose, which is called the Thauer limit [34]. The yield of hydrogen production is also affected by the microbial composition in the system. Strict anaerobes, such as those in the genus *Clostridium*, generally achieve a higher hydrogen yield using the PFOR pathway compared with facultative anaerobes, which utilize the PFL pathway [35]. Research has indicated that the maximum hydrogen yield of *Clostridium* was 3.47 mol H_2/mol hexose, while genera *Enterobacter* and *Bacillus* typically reached up to 2.6 mol H_2/mol hexose [36].

The composition of volatile fatty acids (VFAs), formed as byproducts in the PFOR pathway, also significantly impacts hydrogen yield. The type of fermentation can be further inferred from the predominant VFA profiles and is classified into several categories: acetate-type, butyrate-type, ethanol-type, propionate-type, and mixed-type fermentations [19].

In acetate-type fermentation, glucose is initially metabolized to pyruvate via the glycolytic pathway. Pyruvate is subsequently converted into acetyl-CoA, hydrogen, and carbon dioxide by pyruvate dehydrogenase (**Figure 2a**). The maximum theoretical hydrogen yield for acetate-type fermentation is 4 mol H_2/mol glucose (Equation (9)) [15]. Nevertheless, the formation of acetate acid leads to an accumulation of H^+, which significantly decreases the pH and further inhibits the fermentation process. To

counteract this inhibition, butyric acid or ethanol is used to alleviate the accumulation of NADH and H^+.

A butyrate-type fermentation system is primarily predominated by *Clostridium* spp. with liquid metabolites of butyrate and acetate (**Figure 2b**). Pyruvate is transformed into acetyl-CoA, which is subsequently reduced by NADH to form butyryl-CoA. This conversion process results in a theoretical yield of 2 mol H_2/mol glucose, which is half that of acetate-type fermentation (Equations (9) and (10)) [37]. The ratio of acetate to butyrate is theoretically 2:1, reflecting the NADH consumption balance during fermentation (Equation (11)). This is because for every 2 mol of acetate produced, 2 mol of NADH are generated (**Figure 2a**), while the formation of 1 mol of butyrate consumes just 2 mol of NADH through a reductive reaction [37].

In ethanol-type fermentation, the byproducts of fermentation are dominated by ethanol and acetate (**Figure 2c**). The generation of ethanol also serves to stabilize the levels of NADH and H^+. The theoretical yield is 2 mol of H_2 per mol of glucose in ethanol-type fermentation (Equation (12)) [19].

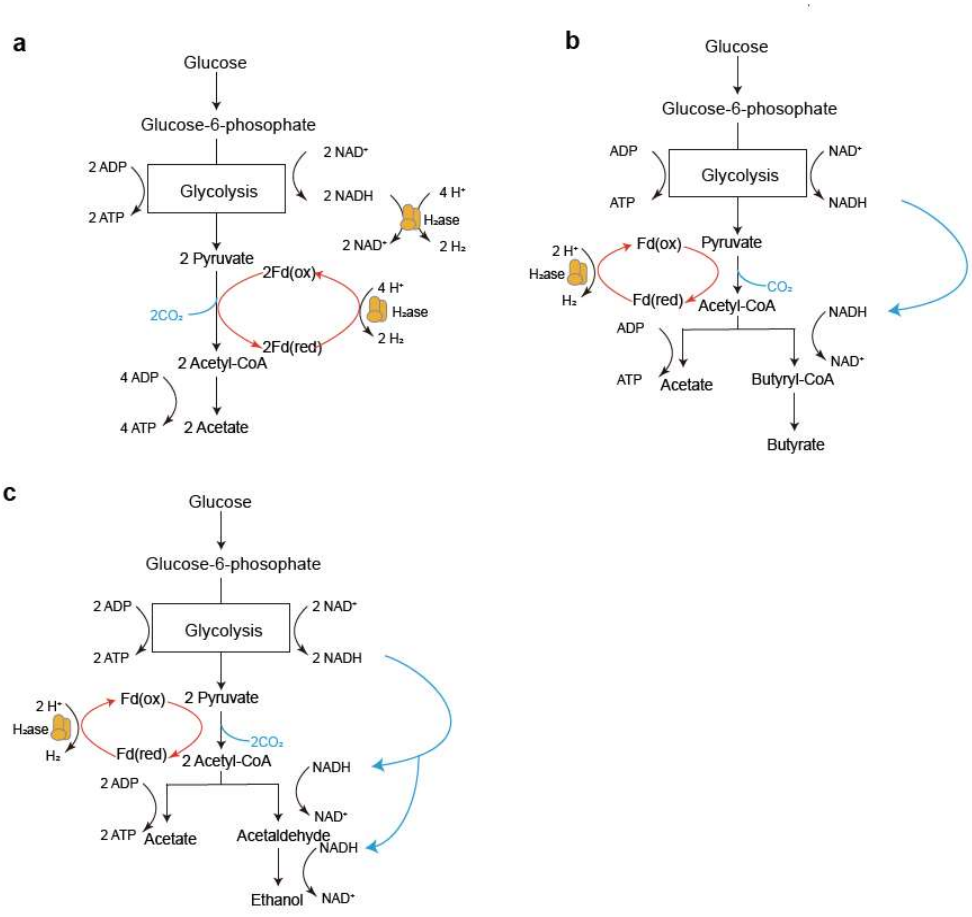

Figure 2. Fermentation types of biohydrogen production in dark fermentative process according to dominant VFA: **(a)** acetate-type fermentation, **(b)** butyrate-type fermentation, and **(c)** ethanol-type fermentation.

In propionate-type fermentation, the primary metabolites are propionate and acetate. The generation of propionic acid is directly associated with the concurrent production of excess NADH and H^+, which further leads to the formation of hydrogen

[38]. However, the limited metabolic pathways capable of producing hydrogen in this type of fermentation result in a relatively low hydrogen yield [38]. Theoretically, the degradation of 1 mol of glucose in this pathway results in the generation of 1 mol of acetate and propionate, alongside only 1 mol of H_2.

In mixed-type fermentation, no single liquid metabolite dominates the system, representing the concurrence of different fermentation types. This type often occurs during the initial stages of the fermentation process before a dominant fermentation type establishes itself [19]. This phase is characterized by the presence of a diverse array of microbial species, each contributing to a complex mixture of metabolic activities.

In addition to the above-mentioned pathways for hydrogen production, research has revealed that certain syntrophic acetogenic bacteria possess the ability to convert metabolites, such as ethanol, butyrate, and propionate, into H_2 and acetate [39]. However, these bacteria are characterized by notably slow growth rates, which presents significant challenges in establishing their dominance in microbial communities and consequently hinders the efficiency of metabolic conversions [39]. This limitation is especially problematic in continuous-flow systems, which do not support the slow growth kinetics of these bacteria, thus impeding the practical application of their hydrogen-producing capabilities [19].

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (9)$$

$$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2 \quad (10)$$

$$2C_6H_{12}O_6 + 2H_2O \rightarrow CH_3(CH_2)_2COOH + 2CH_3COOH + 6H_2 + 4CO_2 \quad (11)$$

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2H_2 \quad (12)$$

Salinity acts as a significant stressor in dark fermentation for hydrogen generation. A low salt concentration benefits the growth of bacteria, as Na^+ ions are essential for microbial metabolism and for the activity of Na^+-dependent membrane-bound ATP synthase, which is involved in ATP formation. For example, Bose et al. [40] observed an improvement in VFA generation when the salt concentration was maintained below 0.5g/L NaCl [40]. In contrast, high salinity negatively impacts bacterial growth due to osmotic stress [41]. A previous study reported that salinity could lead to shifts in fermentation pathways and caused the accumulation of butyric acid at NaCl concentration of 40 g/L [42]. Another study showed the alteration of microbial community composition under salt stress, shifting the dominant bacteria from *Bacteroidetes* to *Firmicutes* [43]. This change adversely affected proteolysis and glycolysis processes during dark fermentation [43]. Moreover, similar to photosynthetic and photo-fermentative microorganisms, for dark fermentative bacteria, elevated salinity triggers energetically costly stress responses [41]. These responses are necessary to balance the osmotic pressure within their cytoplasm, but diminish the energy conserved for metabolism and reduce the activity of intracellular enzymes [41]. The threshold of salt concentration to inhibit biohydrogen production has been intensively investigated, which showed that a low salinity level (approximately 0.6%) had a stimulatory effect on the biohydrogen production process, whereas higher levels led to significant inhibition [41].

Dark fermentation not only facilitates energy generation but also contributes to wastewater management [15]. Actual saline wastewater typically comprises complex

components, predominantly encompassing polysaccharides, proteins, and lipids, which act as suitable substrates for dark fermentation. Therefore, the treatment of saline wastewater by dark fermentation is attractive. But the major technical bottleneck in hydrogen production through dark fermentation under salt stress is the inhibition of microbial metabolic activity due to osmotic pressure and ionic imbalances. These stressors compromise the efficiency of hydrogen-producing bacteria, significantly reducing hydrogen yield. To overcome this limitation, recent approaches focused on developing and utilizing halotolerant microbial strains capable of sustaining metabolic activity under high-salinity conditions [44–46]. Additionally, engineering microbial communities can enhance the resilience and hydrogen-production capacity of these systems, pushing the boundaries of biohydrogen technology under saline conditions [34].

2.4. Biohydrogen production by microbial electrolysis cells (MECs)

As an emerging technology, MECs also produce H_2 using saline organic wastewater as a substrate and offer the benefit of pollutant removal. MECs are categorized into non-biocathode MECs and biocathode MECs based on the presence of microorganisms at the cathode [47].

MECs typically consist of an anode, a cathode, and a membrane (if present) that divides the two electrode chambers (**Figure 3**). MECs require a lower additional voltage of 0.4−0.8 V to meet operational demands, compared with 1.8−3.5 V for water electrolysis [16]. In the anode, active microorganisms oxidize organic substrates from the wastewater, such as glucose, into CO_2 while generating electrons and H^+ (Equation (13)) [16]. The electrons produced are then transferred to the cathode through an external circuit, and H^+ ions migrate directly to the cathode. Then H^+ ions react with electrons to form H_2 (Equations (14) and (15)) [16].

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e^- \quad (13)$$

$$8H^+ + 8e \rightarrow 4H_2 \quad (14)$$

$$CH_3COO + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2 \quad (15)$$

MECs can theoretically achieve up to 4 mol of H_2 per mol of acetate and 12 mol of H_2 per mol of glucose. Compared with dark fermentation, MECs can produce three times more H_2 from 1 mol of glucose (Equation (16)).

$$\text{MEC: } C_6H_{12}O_6 + 12H_2O \rightarrow 6CO_2 + 12H_2 \quad (16)$$

In biocathode MECs, microorganisms catalyze the reaction of H^+ and electrons to generate H_2 by using electrons from the anode. The mode of electron transfer from the cathode to the microorganism is classified as direct interspecies electron transfer (DIET) and mediated interspecies electron transfer (MIET). DIET processes are through electrically conductive pili and conductive materials (**Figure 3b**). MIET processes are through soluble electron carriers [48].

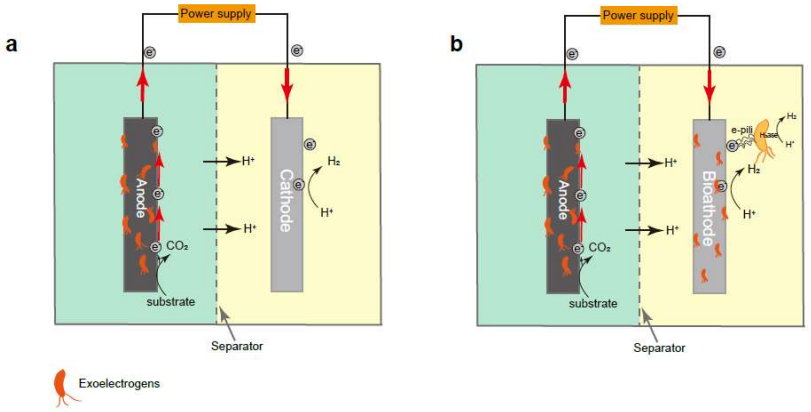

Figure 3. Schematic diagram for hydrogen production: **(a)** non-biocathode MEC and **(b)** biocathode MEC.

High salinity negatively affects hydrogen production in processes driven solely by microorganisms, as previously discussed. The scenario differs significantly in bioelectrochemical systems, which combine electrochemistry with microbial activity. The impact of salinity on hydrogen production in these systems manifests in two primary ways. Salt ions from saline water enhance electrical conductivity in MECs. The conductivity of saline wastewater can reach 9 S/m at a NaCl concentration of 35 g/L compared with just 1 S/m in domestic wastewater [49]. The slight increase in conductivity facilitates the electron transfer process essential for microbial reactions that produce hydrogen, thereby improving the efficiency of hydrogen generation [49]. Conversely, a high salt concentration can negatively affect electroactive bacteria (EAB). When electrolyte salinity surpasses the tolerance threshold of EAB, their ability to produce current is compromised, leading to increased internal resistance due to activation losses [50]. Therefore, an optimal salinity level is crucial to balance ohmic loss and bacterial activity for maximal MEC performance.

Salinity also significantly affects the microbial community structure of EAB, which are a key component of MECs [51]. The enrichment of moderate halophilic biofilms composing anode-respiring bacteria presents a viable solution for efficient hydrogen production from saline wastewater [49]. In previous studies, such moderate halophilic biofilms in a 4 L two-chamber MEC achieved a high current output of 10.6 ± 0.2 A/m^2 at the anode and a hydrogen production rate of 201.1 ± 7.5 L·H_2/m^2 cathode per day, or 0.9 ± 0.0 m^3·H_2/m^3 MEC per day, at 35 g/L of NaCl [49]. Microbiological analyses of these biofilms indicated significant selective colonization by *Deltaproteobacteria* at the anode [49]. Na^+ stress drives microbial community evolution and increases the activity of EAB, which acts as a "bacterial screening" mechanism for the creation of salt-tolerant "electrochemical hydrogen-producing" bacterial communities [49]. Another research also introduced an innovative Na^+-promoted MEC system for the treatment of saline wastewater. The selective Na^+ enhanced the electron transfer pattern and modified microbial metabolisms, boosting organic pollutant degradation to 91.34% and improving the hydrogen production rate by 1.57–1.7 times [52].

Overall, salinity does not always have a negative impact on hydrogen production in MECs. The establishment of biofilms comprising a high-salinity-tolerant microbial

community, as well as improved conductivity to impair electric resistance, makes MECs particularly advantageous in utilizing saline wastewater to produce highly demanded hydrogen.

3. Enzymology of hydrogen production and salt stress effect

Enzymes, serving as macromolecular biological catalysts, play a pivotal role in accelerating the rate of biochemical reactions [53]. Specific enzymes, especially hydrogenases, are crucial for hydrogen production. These enzymes harness energy directly from light or indirectly through the consumption of carbon compounds produced via photosynthesis, catalyzing the conversion of protons into molecular hydrogen (Equation (8)) [53]. Hydrogenases, which account for the majority of biological hydrogen production, are very important. The process of proton reduction is reversible: some hydrogenases catalyze the oxidation of H_2 back into H^+, utilizing various electron donors [54]. A comprehensive understanding of hydrogenase functionality is crucial for elucidating the mechanism of hydrogen production, regulating the metabolism of bacteria, and enhancing hydrogen output [55]. Therefore, this section focuses on hydrogenases and the salt stress effect on hydrogenases.

3.1. Hydrogenases

Hydrogenases are widespread in bacteria, archaea, and some eukarya and are categorized into three categories based on the metal of their active site: [Fe]-, [FeFe]- and [NiFe]-hydrogenases (**Figure 4**) [15].

[NiFe]-hydrogenases are one of the most extensively researched classes of hydrogenases [56]. These enzymes catalyze the reversible oxidation and formation of H_2 and serve as essential metabolic components in various bacterial and archaeal species. The production of [NiFe]-hydrogenases involves a complex process that includes the synthesis and insertion of the NiFe catalytic center with the participation of six hyp genes [19].

At the heart of these enzymes is an elaborate catalytic center featuring Ni and Fe, along with carbon monoxide (CO) ligands and cyanide [56]. Within this center, two sites are accessible for substrate binding: E2 acts as a bridging site between Ni and Fe, while E1 is located at the Ni-terminal. [NiFe]-hydrogenases also incorporate a small subunit with two domains, I_S and II_S, and three iron-sulfur (FeS) clusters [56]. The I_S domain binds [Fe_4S_4], while the II_S domain associates with the other two FeS clusters: mesial [Fe_3S_4] and distal [Fe_4S_4] [19]. Throughout the enzymatic reaction, the efficient transfer of electrons and H^+ between the surface and catalytic center is crucial (**Figure 4a**).

[FeFe]-hydrogenases can be divided into two main families based on their structural and functional characteristics [19]. Cytoplasmic, soluble, monomeric [FeFe]-hydrogenases are typically present in strict anaerobes, such as *Clostridium pasteurianum* and *Megasphaera elsdenii* [57]. They are oxygen-sensitive and play dual roles in both hydrogen generation and consumption. In *Clostridium pasteurianum,* the CpI hydrogenase accepts low-potential electrons via ferredoxin, which are generated from the degradation of organic matter, and uses protons to form hydrogen [57]. Periplasmic, heterodimeric [FeFe]-hydrogenases are present in various

species, such as *Desulfovibrio* spp., and primarily facilitate the oxidation of hydrogen [58]. The electrons generated from this process are used to reduce power or reduce sulfate to sulfide [58].

[FeFe]-hydrogenases exhibit several fundamental similarities with [NiFe]-hydrogenases at their active sites [53]. Similar to [NiFe]-hydrogenases, the active site of [FeFe]-hydrogenases contains cyanide and CO ligands that coordinate the Fe in the active site [53]. These enzymes also utilize FeS clusters to facilitate the electron transfer chain from the active site to electron donors located on the surface of the hydrogenase. [FeFe]-hydrogenases possess the same gas transfer channels that facilitate the diffusion of gases, including H_2 and inhibitors (e.g., O_2) to and from the concealed active site (**Figure 4b**) [53].

[Fe]-hydrogenases, also referred to as H_2-forming methylene-tetrahydromethanopterin dehydrogenases (Hmd) or iron-sulfur-cluster-free hydrogenases, are distinct from other hydrogenases due to the lack of FeS clusters [54]. The enzymes feature three clusters: proximal, medial, and distal clusters. The active site of [Fe]-hydrogenases is buried and comprises an iron center coordinated to a cysteine S atom, two cis-CO ligands, a bidentate pyridone molecule, and an unidentified ligand [54]. Functionally, [Fe]-hydrogenases are involved in the consumption and production of hydrogen and catalyze the reduction of methenyl-tetrahydromethanopterin (methenyl-H_4MPT^+) with hydrogen to form methylene-H_4MPT (**Figure 4c**) [59].

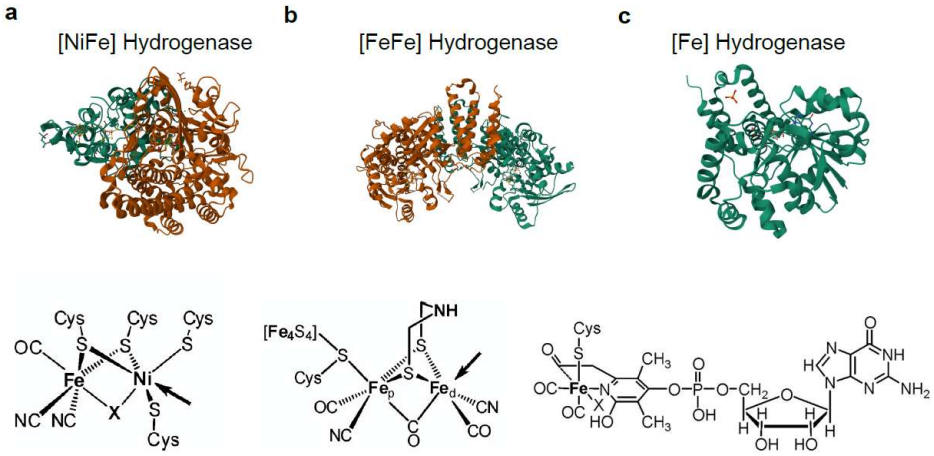

Figure 4. Stereoview schematic representations of three-dimensional structures of (a) [NiFe]-hydrogenase from *Nitratidesulfovibrio vulgaris* [60], (b) [FeFe]-hydrogenase from *Clostridium beijerinckii* [61], and (c) [FeFe]-hydrogenase from *Methanocaldococcus jannaschii* [54]. At the bottom, the active sites of the three types of hydrogenases are given [53].

3.2. Salt stress effect on hydrogenases

Like many enzymes, hydrogenases are sensitive to ionic imbalances caused by the high concentration of salts, such as NaCl. Salt stress significantly impacts the structural integrity of hydrogenases [62]. High salinity can lead to partial unfolding of the enzyme structure and decrease the enzymatic activity. This is because the osmotic

pressure exerted by a high salt concentration disrupts hydrogen bonding and electrostatic interactions within the enzymes, altering their tertiary and quaternary structures [62]. Such structural changes can deactivate the active sites of hydrogenases, thereby impairing their ability to catalyze hydrogen production [63]. A high salt concentration also influences the solubility of hydrogenases [64]. Proteins typically exhibit greater solubility in dilute salt solutions because ions interact with opposite charges in the protein structure, enhancing the hydration of a protein's surface [64]. As intracellular salt concentration rises, water's surface tension increases, creating competition between protein molecules and ions for hydration. This competition can lead the salts to strip the essential layer of water from the hydrophobic regions of the protein's surface, leading to protein denaturation when they are no longer sufficiently hydrated [64]. In particular, non-halophilic proteins are less capable of competing with salts and thus lose their structure and functionality at relatively lower ionic concentrations [64].

Halophilic and halotolerant microorganisms often exhibit modifications to the amino acid composition of hydrogenases, such as an increase in acidic residues that sequester positive ions [65]. This adaptation stabilizes the enzyme structure against the destabilizing effects of salts. Unlike their non-halophilic counterparts, which precipitate in high-salt environments, halophilic hydrogenases remain highly soluble. The "solvation-stabilization model" provides the most fitting explanation for the alteration of solvent properties observed in halophilic enzymes [62]. This model suggests that solubility and stability are inherently linked, supported by the established correlation between the increased acidic amino acid content and improved solubility in halophilic proteins [62]. Additionally, these microorganisms may produce specialized chaperone proteins, which help maintain the proper folding of hydrogenases under stress conditions, ensuring sustained enzymatic activity [66].

For instance, a halophilic hydrogenase in the sulfate-reducing bacterium *Desulfonatronum thiodismutans* was identified [63]. The hydrogenase of this organism exhibited high tolerance to Na^+ ions and stayed active in NaCl concentrations up to 4.3 M and Na^+ concentrations up to 1.2 M [63]. This attribute makes them particularly valuable for research in biochemistry and biotechnology.

The influence of salt stress on the enzymology of hydrogenases has significant implications for the scalability of biohydrogen production from saline wastewater. By understanding and mitigating the adverse effects of salt on these enzymes, it is possible to optimize the metabolic pathways responsible for hydrogen production.

4. Halotolerant biohydrogen producers

Among the diversity of microorganisms capable of biohydrogen production, certain strains have demonstrated notable resilience and productivity in saline environments. This section highlights some of the prominent halotolerant biohydrogen producers, as summarized in **Table 1**.

Table 1. Some typical salt tolerant hydrogen producers and their hydrogen yield.

Microorganism	Substrate	Type	Hydrogen yield	Salt	Ref.
Chlamydomonas reinhardtii	H_2O	Biophotolysis	-	0.05 M	[67,68]
Synechocystis sp. PCC 6803	H_2O	Biophotolysis	-	0.15 M	[13,69]
Anabaena sp. PCC 7120	H_2O	Biophotolysis	-	0.1 M	[18,70]
Mixed culture	Glucose	Dark fermentation	2.19 mol/mol glucose	2.6 M	[71]
Synechococcus elongatus PCC7942 PAMCOD	Sucrose	Biophotolysis	23.09 ± 9.1 nmol/mol Chl a×h	0.4 M	[72]
Synechocystis sp. PCC6714	Sucrose	Biophotolysis	22.86 ± 9.1 nmol/mol Chl a×h	0.4 M	[72]
Rhodovulum sulfidophilum P5	Acetate	Photo-fermentation	2.06 ± 0.08 mol/mol acetate	0.5 M	[9]
Rhodobium marinum	Lactic acid	Photo-fermentation	6 mol/mol starch-glucose	0.5 M	[28]
Rhodovulum sulfidophilum	Volatile fatty acids	Photo-fermentation	200 mL/L	0.5 M	[10]
Rhodopseudomonas palustris 42 OL	Volatile fatty acids	Photo-fermentation	10 mL/g biomass	0.4 M	[5]
Bacillus sp. B2	Glucose	Dark fermentation	1.65 ± 0.4 mol/mol glucose	0.5 M	[44]
Halanaerobium saccharolyticum sp. senegalensis	Glycerol	Dark fermentation	1.6 mol/mol glycerol	2.6 M	[45]
Halanaerobium saccharolyticum sp. saccharolyticum	Glycerol	Dark fermentation	0.6 mol/mol glycerol	2.6 M	[45]
Halanaerobium hydrogeniformans	Cellobiose	Dark fermentation	2.3 mol/mol cellobiose	1.2 M	[73]
Clostridium bifermentans 3AT-ma	Glucose	Dark fermentation	1.1 mol/mol glucose	0.3 M	[46]
Haloanaerobacter chitinovorans sp. nov.	Glucose	Dark fermentation	-	2 M	[12]
Halonanaerobacter salinarius sp. nov.	Glucose	Dark fermentation	0.48 mol/mol glucose	2.4–2.6 M	[11]
Vibrio tritonius AM2	Mannitol	Dark fermentation	1.7 mol/mol mannitol	0.4 M	[74]
Halanaerobium hydrogeniformans	Glucose	Dark fermentation	-	1.2 M	[75]
Co-culture	Glucose	Dark- and photo-fermentation	1694 ± 21 ml/L	0.5 M	[10]
Mixed culture	Glucose	Dark fermentation	0.9 ± 0.02 mol/mol glucose	1.3 M	[7]
Mixed culture	Wastewater	Dark fermentation	38.7 mL/gVSS	0.3 M	[8]
Mixed culture	Glucose	Dark fermentation	1.45 mol/mol glucose	4.5 M	[6]

Photosynthetic microorganisms, such as *Chlamydomonas reinhardtii* and *Synechocystis* sp., have demonstrated significant adaptability in utilizing water as a substrate to produce H_2 under varying salinity conditions. *Chlamydomonas reinhardtii* was found to operate effectively up to 0.05 M salt concentration, whereas

Synechocystis sp. *PCC 6803* extended this tolerance to 0.15 M. *Anabaena* sp. PCC 7120 further exemplified cyanobacterial robustness by functioning at 0.1M salt concentration [13,18,67–70]. This variation underscores the diverse osmoregulatory and metabolic capabilities inherent to different photosynthetic species.

Photo-fermentative microorganisms include purple sulfur bacterium (e.g., *Chromatium*), green sulfur bacterium (e.g., *Chlorobium*), purple nonsulfur bacterium (*Rhodobacter*), and gliding bacterium (e.g., *Chloroflexus*). These microbes convert organic substrates, such as VFA, into hydrogen under illumination. For example, in a study, *Rhodopseudomonas palustris* 42 OL was able to produce H_2 in a defined medium with up to 0.4 M salt concentration, achieving a hydrogen yield of 10 mL/g biomass [5]. *Rhodovulum sulfidophilum* and *Rhodobium marinum* also demonstrated high efficiency under 0.5 M salt stress with yields of 2.06 mol of H_2 per mol of acetate and 6 mol of H_2 per mol of starch-glucose [9,10,28].

It should be noted that microbes producing hydrogen through dark fermentation are more prevalent than their photosynthetic or photo-fermentative counterparts. Dark fermentative species can convert complex substrates (e.g., lignocellulosic biomass) into hydrogen without illumination. Among them, *Bacillus* sp. *B2* and *Halanaerobium saccharolyticum* are adept at converting glucose and glycerol into hydrogen [44,45]. *Bacillus* sp. *B2* was found to achieve a yield of 1.65 mol of H_2 per mol of glucose at 0.5 M salinity, demonstrating good salt tolerance and efficiency [44]. The *Halanaerobium* species showed remarkable salt tolerance, with *Halanaerobium saccharolyticum* subspecies *senegalensis* and *saccharolyticum* thriving in up to 2.6M salt concentration, producing 1.6 and 0.6 mol of H_2 per mol glycerol, respectively. *Haloanaerobacter* and *Halanaerobium* has also been identified to have remarkable salt tolerance and capability for hydrogen production [41]. Among the various identified bacteria capable of producing H_2, *Clostridia* are especially a promising candidate due to their relatively high yield of H_2 production. In a study, *Clostridium bifermentans* 3AT-ma produced 1.1 mol of H_2 per mol glucose under a 0.3 M salt condition [46]. The use of mixed and co-cultures represents another strategy to optimize hydrogen production. These systems often benefit from synergistic interactions among microbial species, which enhance the overall hydrogen yield. For instance, one co-culture of *Rhodovulum sulfidophilum* and dark fermentative microbe produced 1694 ml/L of H_2 at 0.5 M salinity, significantly surpassing the yields from photo or dark fermentation processes [10]. This shows the efficacy of integrating dark and photo-fermentative pathways. Research works on a mixed culture of extremely halotolerant hydrogen-producing bacteria demonstrated the bacteria's ability to tolerate a high concentration of Na^+ ion, while maintaining a high hydrogen production rate, illustrating the potential for integrating a diverse strategy to optimize hydrogen production from saline wastewater [6–8,71].

5. Salt adaptation mechanism on microorganisms involved in biohydrogen production

As discussed previously, high salinity poses significant challenges to microbial activity and survival due to osmotic stress, ionic toxicity, and disruption in metabolic processes. Halotolerant biohydrogen producers exhibit remarkable adaptability to

high-salt environments. This section explores cellular and molecular strategies that enable these microorganisms to thrive under such stress conditions, which are crucial for sustaining biohydrogen production from saline wastewater.

5.1. "Salt-in" strategy

Effective ion regulation is essential for halotolerant and halophilic microorganisms. The "salt-in" strategy involves achieving osmotic balance by the accumulation of a high level of ions within the cells [76]. These organisms are equipped with specialized ion pumps and transport systems designed to expel excess sodium ions, while retaining potassium ions [77]. This selective ion transport helps maintain intracellular ionic balance and stabilizes cellular components, including enzymes critical for hydrogen production [77]. For example, the halotolerant biohydrogen producer *Halanaerobium* primarily employs the "salt-in" strategy to manage osmotic stresses in saline environments [77]. Osmoadaptation in these microbes is typically regulated through the fine-tuning of intracellular potassium ions and osmolytes. Sodium/proton antiporters, such as NhaA, NhaD, NhaP, and Mrp, play a crucial role in this process, driven by the H^+ motive force to export Na^+ and import H^+, allowing these microbes to maintain a favorable internal environment despite external salinity pressures [78].

The uptake of K^+ ions in response to osmotic shock is managed by Trk and Ktr systems [79]. These systems are composed of transmembrane subunits, which facilitate K^+ permeation, and cytosolic regulatory subunits, which form a ring-like structure [79]. These TrkA and KtrA regulatory subunits—also known as potassium conductance regulators—can interact with cytosolic signaling molecules to regulate the gating of transmembrane pores TrkH and KtrB (**Figure 5**) [79].

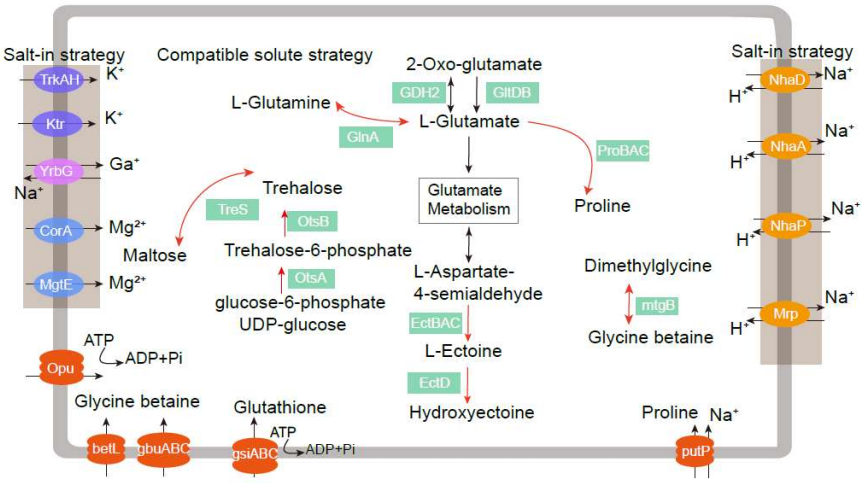

Figure 5. Salt adaptation mechanism of microorganisms involved in biohydrogen production.

5.2. "Compatible solute" strategy

To counteract a hypertonic external environment, many microorganisms also accumulate compatible solutes—organic compounds that do not interfere with cellular

functions and are essential for maintaining cellular turgor and enzyme functionality [77]. These compounds include a diverse range of organic molecules, such as sugars, amino acids, polyols, glycine betaines, ectoines, and N-acetylated diamino acids. Compatible solutes function as a potent water-structure stabilizer and are typically excluded from the hydration shells of enzymes, consequently stabilizing these shells and decreasing water activity coefficients [77]. This stabilization is crucial for preserving metabolic activity essential for hydrogen production.

Most halophilic or halotolerant cyanobacteria commonly employ this strategy of accumulating substances, such as glycine betaine, sucrose, or trehalose, to achieve osmotic balance [78]. However, microbes from the genus *Halanaerobium* do not typically follow this pattern. In *Halanaerobium* organisms, typical solutes (e.g., glycine betaine, glycerol, and amino acids) have not been detected [80]. Conversely, the use of L-glutamate betaine has been reported in other contexts [77]. It is important to note that the biosynthesis of compatible solutes is energetically more expensive compared with the "salt-in" strategy. Therefore, in the "salt-in strategy", microorganisms that adopt the "compatible solute" strategy will also accumulate solutes if available in the environment [78].

5.3. Protein and enzyme modification

Salt-adapted microorganisms often feature proteins and enzymes with altered amino acid compositions, enhancing stability and activity in high-salt conditions. The surface of proteins in halophiles is usually rich in negatively charged amino acids, while the interior is enriched in positively charged ones [81]. These highly acidic enzymes and proteins generally require molar salt concentrations to maintain activity and structural integrity [77]. This trait is characteristic of organisms employing the "salt-in" strategy.

For instance, the optimal salt concentration for the activity of hydrogenase ranges from 0.5 to 3 M [82]. This requirement stems from the weak interaction of salt with specific sites on the surface, where the high negative surface charge enhances solubility and flexibility, counteracting the aggregation and rigidity exhibited in non-halophilic proteins [82]. The charge on the surface is typically counterbalanced by firmly bound water dipoles, and acidic residues also play crucial roles in preventing protein aggregation. Further examination of the composition of bulk proteins of *Halanaerobium* in cell pellets confirmed the high proportion of negatively charged amino acids over basic amino acids [77].

The ability of microorganisms to withstand salinity is pivotal to leverage their potential in sustainable biohydrogen production from saline wastewater. Understanding the complex interplay of these adaptation mechanisms offers valuable strategies for enhancing biohydrogen production. By elucidating and harnessing these adaptive mechanisms, biotechnological applications can be developed to enhance the efficiency and viability of biohydrogen production in challenging salt stress environment. Exploring microbial communities in naturally saline or hypersaline environment could uncover novel insights into unexplored metabolic pathways and organisms with inherent high salt tolerance and efficient hydrogen-production capability. Continued research and development in this area are crucial for advancing

understanding and application of salt-stressed biological systems in renewable energy production.

6. Conclusion and perspectives

This review depicts substantial advances in the arena of sustainable hydrogen production with saline wastewater, highlighting the indispensable role of halotolerant and halophilic microorganisms in surmounting salinity challenges. We elaborated an understanding of various production methods of biohydrogen from saline wastewater, which are biophotolysis, photofermentation, dark fermentation, and microbial electrolysis. The adaptive mechanisms of microorganisms under salt stress, particularly those involving "salt-in" and "compatible solute" strategies, are pivotal in sustaining biohydrogen production. These adaptations aid in maintaining enzymatic functionality and structural integrity under hypertonic conditions.

Looking ahead, the integration of multidisciplinary approaches holds promise for addressing the remaining technical challenges and for scaling up biohydrogen production. Hydrogen generation is intricately linked to the metabolic activities of microbes. Identifying and characterizing novel halotolerant hydrogen-producing microorganisms, and understanding the mechanisms that contribute to their salt adaptation and efficiency, are pivotal steps toward enhancing H_2 production rate and yield with saline wastewater. Additionally, the transformation of organic materials into hydrogen involves multiple steps and the cooperation of diverse microbial species. This complexity is heightened when using real saline organic wastes as substrates, which may necessitate specific microbial consortia tailored to the substrate composition to minimize adaptation times and maximize hydrogen output. Further research should focus on the roles of microbes in the hydrogen production process and investigate ways to optimize microbial interactions manually for improved outcomes. Moreover, innovations in reactor design and system integration are critical for improving the operational viability and efficiency of biohydrogen production technologies. Collectively, a thorough understanding of the ecological impacts and economic feasibility of salt-stressed biohydrogen production systems is essential to ensure their success in industrial applications.

As the demand for sustainable energy solutions grows, biohydrogen production from saline organic wastewater presents a dual benefit of energy recovery and environmental protection, making it a compelling area for future research and development. However, to support the commercial-scale synthesis of hydrogen from saline organic wastewater, significant infrastructure developments are necessary. These include establishing large-scale bioreactors designed to handle diverse and variable wastewater, along with efficient waste-collection and pre-treatment facilities. Moreover, scaling up biohydrogen production will require advancements in process monitoring and control technologies to ensure a consistent yield and operational stability. Continued efforts in this field will be crucial for advancing our capabilities to produce clean energy, while managing waste effectively in an environmentally sustainable manner.

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