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Fermentation and Redox Potential

Chen-Guang Liu, Jin-Cheng Qin and Yen-Han Lin

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Abstract

Redox potential, known as oxidation–reduction or oxidoreduction potential (ORP), not only indicates the reduction and oxidation capacity of the environment but also reflects the metabolic activity of microorganisms. Redox potential can be monitored online and controlled in time for more efficient fermentation operation. This chapter reviews the enzymes that modulate intracellular redox potential, the genetically engineered strains that harbor specific redox potential–regulated genes, the approaches that were used to manipulate and control redox potential toward the production of desired metabolites, the role of redox potential in metabolic pathway, and the impact of redox potential on microbial physiology and metabolism. The application of redox potential–controlled ethanol fermentation and the development of three redox potential–controlled fermentation processes are illustrated. In the end, the future perspective of redox potential control is provided.

Keywords: redox potential, ORP, fermentation, bioprocess, ethanol

1. Introduction

The fermentation industry has a long history since human ancestor occasionally produced alcohol, yogurt, and pickled food. Most of these fermentation products are related to the pathways of glycolysis and TCA cycle, which required microaerobic or anaerobic conditions to avoid the desired products being oxidized by oxygen.

Precisely controlling microaerobic or anaerobic states is a challenge when using a general dissolved oxygen electrode because of the detection limit of the probe. Therefore, the measurement of redox potential (aka oxidoreduction potential, ORP) is considered as an ideal alternative approach because of its rapid response and high sensitivity to oxidation reaction.

What's more, redox potential also correlates to metabolic network, involving the genes, proteins, and metabolites. Since maintaining intracellular redox potential balance is a basic demand of cells, either intracellular or intercellular redox potential control could be the effective methods to redistribute metabolic flux toward targeted products. This idea has been applied to make a broad range of fermented products.

In this chapter, the basic principle of redox potential and its intracellular influence on genes, proteins, and metabolites are reviewed. Furthermore, redox potential control by metabolic modification and process engineering on the various metabolite fermentations are illustrated, specifically for ethanol production as an example.

2. Basic theory of redox potential

Chemically, the oxidation–reduction potential (aka ORP or redox potential) is defined as the tendency for a molecule to acquire electrons. It involves two components known as redox pair during the electron transfer process, of which the oxidizing one (Ox) attracts electrons and then becomes the reducing one (Red). This relationship is illustrated below:

$$Ox + ne^- = Red \ 1 \tag{1}$$

Electrons are exchanged during a redox reaction, in which a pair of oxidation reaction and reduction reaction must be involved. As an illustration, when oxidizing iodide by ferric iron to form iodine, the iodine ion loses two electrons to from iodine (known as oxidation), concurrently ferric ion receives the same amount electrons to form ferrous ion (known as reduction). As a result, a complete redox reaction is established.

Oxidation: $2I^- = I_2 + 2e^-$

Reduction: $2Fe^{3+} + 2e^- = 2Fe^{2+}$

Redox reaction: $2Fe^{3+} + 2I^- = 2Fe^{2+} + I_2$
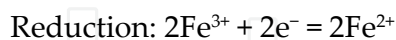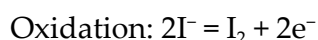
In an aqueous system, the redox potential is related to the capacity of releasing or accepting electrons from all redox reactions. Similar to pH where it indicates the availability of hydrogen ions, the overall redox potential portrays a relative state of gaining or losing electrons. However, the net changes of redox potential are caused by all oxidizing and reducing agents in the aqueous system, not just alkalis and acids that determine pH values.

In 1889, Walter Hermann Nernst (1864–1941; Nobel Prize: 1920) developed an equation to interpret the theory of galvanic cells by taking the changes of Gibbs free energy (ΔG) and the mass ratio into account. The Gibbs free energy is a thermodynamic potential, a reduction of G is a necessary condition for the spontaneity of processes at constant pressure and temperature. The chemical reaction can occur only if the ΔG is negative.

$$E_h = E^0 + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]} \quad 2$$ (2)

$$\Delta G = -nF\Delta E_h \quad 3$$ (3)

E^0 is the standard redox potential of a system obtained at standard state. Every chemical pair has its own intrinsic redox potential. The greater affinity for electrons, the higher standard redox potential could be. Generally, $NAD^+/NADH$, $NADP^+/NADPH$, GSSG/2GSH, ubiquinone (ox/red), and oxygen/water are some of the most common chemical pairs in cells, whose E^0 were −320, −315, −240, +100, and +820 mV, respectively.

R is the universal gas constant; T is the absolute temperature; F, Faraday constant (96,485 C/mol), is the number of coulombs per mole of electrons, and n is the number of transferred electrons. The equation implies the concentration of species and temperature plays the key roles for redox potential change.

For instance, the reaction of NADH oxidized by oxygen is the final step of electron transport chain during aerobic respiration in mitochondrion. Usually, the reaction involves two redox pairs, just like oxygen/water (+820 mV) and $NAD^+/NADH$ (320 mV), thus $\Delta E_h = 820$ mV − (−320 mV) = 1140 mV, $\Delta G = -125$ kJ/mol, which indicates that this process occurs spontaneously due to the negative value of ΔG.

Although the Nernst equation has been broadly used in biological systems because of the involvement of electron transfer chain, one fact should be noticed that the redox potential measured by a platinum electrode is not a thermodynamically calculated value. It measures the redox state in an aqueous system as voltages. Although a living biosystem centers on cell growth and metabolism, it is an open system where the intracellular equilibrium state is not always established. Nevertheless, the significance of redox potential on functioning biological systems was predicted nearly one century ago by two prestigious British scientists at the University of Cambridge [1]. Many scientists, since then, have successfully explored various correlations between extracellular redox potential measured by an electrode and intracellular biological properties.

3. Extracellular redox potential

The extracellular redox potential is different from intracellular redox state due to cytomembrane separation and cell redox homeostasis. Environmental factors are critical to indirectly shift the cellular redox potential. Based on Nernst Equation, the redox potential is simply determined by the ratio of oxidative state to reductive state at a fixed temperature, which is always a constant parameter in most biological processes. **Figure 1** illustrates three general approaches to control extracellular redox potential in biological devices.
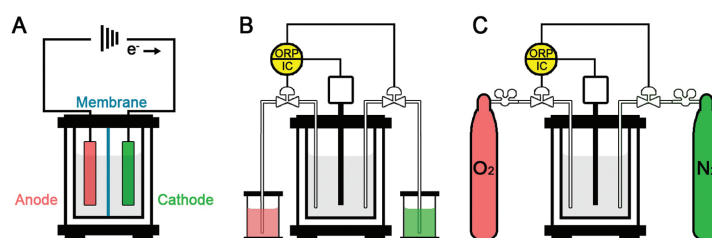
Figure 1. Approaches to control extracellular redox potential. (A) energy input, (B) redox reagents, and (C) gas sparging.

3.1. Control extracellular redox potential by energy input

Bioelectrical reactors (BERs), equipped with anodic and cathodic electrodes, were developed to regulate extracellular redox state in the medium through an external power source. It was used to replace chemical electron donor and acceptor in biosystem. BERs control redox potential at a certain level as easy as tuning a radio. It has been applied to microorganism cultivation and metabolites production [2]. Nevertheless, BERs have been implemented in a laboratory setting or for the production of high-value products in order to compensate for its complicated equipment requirement and extra electrical energy consumption.

3.2. Control extracellular redox potential by redox reagents

Numerous chemicals with higher or lower standard redox potential than common metabolic components are supplemented into fermentation broth in order to alter environmental redox potential. Some commonly used reductants and oxidants to control extracellular redox potential include $FeCl_3$, Na_2S, potassium ferricyanide, dithiothreitol, cysteine, methyl viologen, neutral red, H_2O_2, and even directly NADH and NAD^+ as additives. Unlike BERs requiring the design of a specific reactor, supplementing redox reagents can be employed in any type of bioreactor. However, the disadvantages are obvious: (a) extra chemicals added in media potentially interfere with intended bioprocessing and (b) some chemicals are too costly for industrial fermentation.

Those problems could be solved using substrates with different reducing degree. Girbal and Soucaille [3] used mixed substrates (glucose, glycerol, and pyruvate) to interfere with the intracellular NADH/NAD^+ ratio in *Clostridium acetobutylicum*. Snoep et al. [4] chose some energy source substrates, such as mannitol, glucose, and pyruvate, to govern cellular redox potential in *Enterococcus faecalis*.

3.3. Control extracellular redox potential by gas sparging

Oxygen and nitrogen are commonly used in aerobic and anaerobic fermentation, respectively. Thus, sparging pure or mixed gases into fermentation broth is one of the desired approaches to avoid unwanted reactions caused by redox salts. Generally speaking, oxygen elevates redox potential and hydrogen depresses it, whereas nitrogen and helium as inert gases remove dissolved oxygen or hydrogen from the medium. Furthermore, by adjusting the ratio of mixed

gases, a different redox potential level can be maintained. Carbon monoxide and SO_2 were also utilized to reduce the redox potential sometimes [5]. However, aerating a fermenter during fermentation is considered cost-effective only when air is used. As a mix of nitrogen, hydrogen and helium were applied to regulate redox potential in the above settings, these methods become too luxurious for industrial applications.

3.4. Extracellular redox potential and dissolved oxygen

Controlling the level of dissolved oxygen in a fermenter is essential for microorganisms to propagate under optimum physiological condition, not only because oxygen is involved in maintaining cell membrane integrity and function by synthesizing unsaturated fatty acid and sterol, but also for keeping metabolic flux channeling toward the production of desired products.

A number of bioreactions toward the syntheses of intended metabolites requires maintaining dissolved oxygen at a proper level. For most microaerobic and anaerobic fermentations, conventional oxygen probe has trouble in distinguishing trace level dissolved oxygen from background noise, and its response time is not sufficient for the purpose of regulating dissolved oxygen level. Even for aerobic fermentation, redox potential still offers much more details about gaseous conditions than that collected from dissolved oxygen measurement [6]. The standard redox potential for the O_2/H_2O pair has the highest value among typical metabolites related to microbial metabolism during fermentation. If electrons were transferred to acceptors, oxygen must be the preferable choice even though its concentration is lower than other metabolites. Therefore, redox potential is much more sensitive in monitoring the presence of a trace amount of dissolved oxygen under microaerobic and anaerobic conditions.

4. Intracellular redox potential

Currently, advanced technologies, such as a nanosensor that can embed into individual cells, have been developed to measure intracellular redox potential directly for in-depth understanding on intracellular redox balance and its impact on cell physiology and metabolism. However, the indirect approaches, such as the measurement of NAD(P)H pools, NAD(P)$^+$/NAD(P)H, GSH/GSSG, and the total oxidization power, are still commonly adopted to monitor the distribution of intracellular redox potential.

4.1. Universal redox pairs in a cell

A conjugate pair that constitutes a complete redox reaction is the fundamental of metabolic network in a cell. Many metabolic functions are realized through keeping intracellular redox balance with the main redox pairs, such as glutathione (GSH)/glutathione disulfide (GSSG), thioredoxin (TrxSS/Trx(SH)$_2$), nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphatase (NADP). These redox systems, such as NADP$^+$/NADPH, GSSG/2GSH, and TrxSS/Trx(SH)$_2$, are not isolated systems. Both the Trx and GSH systems use

NADPH as a source of reducing equivalents; thus, they are thermodynamically connected to each other. The role of NAD(P)$^+$/NAD(P)H in redox reaction is illustrated in **Figure 2**.
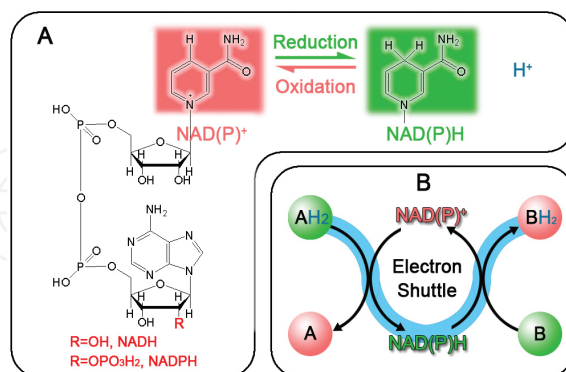

Figure 2. The structure (A) and function (B) of NAD(P)H.

Both glutathione (GSH) and thioredoxin are important reducing agents in all organisms, involved in cell oxidative stress response where they play an antioxidant role. Glutathione is a tripeptide (glutamine, cysteine, and glycine) that prevents damage to cellular components caused by reactive oxygen species such as free radicals and peroxides, lipid peroxides, and heavy metals. Thioredoxin is another class of small redox proteins with thiol system in the cell, which appears in many crucial biological processes, including redox signaling.

The coenzymes are essential electron carriers in cellular redox reactions with the oxidized form NAD(P)$^+$ and the reduced form NAD(P)H. The reduction reaction requires an input of energy and the oxidation reaction is exergonic. During carbohydrate metabolism, NADH plays as a notable reducing substance in catabolism, whereas NADPH, the other reducing component connected to anabolism, favors formation of amino acids, fatty acids, and nucleic acids. There are 129 enzymes that need NAD$^+$ as cofactor in order to serve 931 redox reaction and 108 enzymes that require the involvement of NADP$^+$ as cofactor in order to catalyze 1099 redox reaction (KEGG, 2016-3).

4.2. Redox effect across the membrane

Cytosol is isolated from the extracellular environment by a selectively permeable cytomembrane, which not only prevents the main redox pair escaping from the plasma freely but also conditionally allows the external redox chemicals to enter into the cytoplasm. As shown in **Figure 3**, chemicals with different reduction degrees, such as dithiothreitol (DDT), diamine, hydrogen peroxide, and oxygen, can unrestrictedly cross the membrane bilayer, causing the changes to the intracellular redox potential. However, most of these chemicals are prohibited to across the membrane. In another scenario, membrane proteins, such as oxidoreductase, involved in electron transport will respond and change the extracellular redox potential. For example, ferric reductase assists ferrous iron transport across the cell membrane [7]. Hydrogenase facilitates electron flow through the membrane with the conversion of NADH and NAD$^+$ [8]. A low redox potential level results in the changes of thiol and di-

sulfide balance on membrane proteins, making the membrane more permeable to protons [9]. A thiol-rich membrane protein transduces external GSH reducing power across the erythrocyte membrane, which can be explained as a thiol/disulfide exchange mechanism [10].
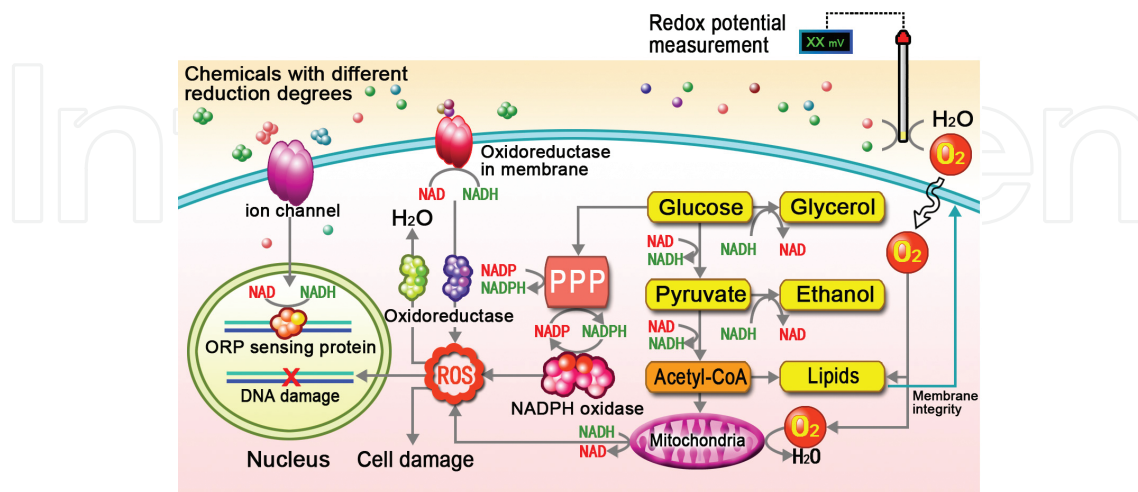

Figure 3. Intracellular redox response to extracellular redox potential and effects of redox potential on cellular metabolism and stress response.

4.3. Effects of redox potential on a cell

The influences of redox potential on enzymes activity have also been reported. Almost all enzymes related to oxidation–reduction reaction are redox potential sensitive, such as alcohol dehydrogenase, D-glyceraldehyde-3-phosphate dehydrogenase, quinone reductase (involved in quinone detoxification), NADH diphosphatase (involved in peroxisomal function), ubiquinone oxidoreductase (catalyzing the oxidation of NADH in the respiratory chain or in cytoplasm), mitochondrial NADH kinase (response to oxidative stress), and so on. The above-mentioned proteins have been investigated in *Saccharomyces cerevisiae* in the past decades. Numerous proteins contain sulfhydryl groups (PSH) due to their cysteine content. In fact, the concentration of PSH groups in cells and tissues is much greater than that of GSH. These groups can be present as thiols (-SH), disulfides (PS-SP), or mixed disulfides; Hsp33 as a possible chaperone and cysteine protease in heat shock protein families is regulated by redox potential, whose conformation changes from reduced state to oxidized state with the exposure of hydrophobic surface [11]. Being a key regulator of glutathione and, in turn, of redox potential, the identification of GSTp as, a JNK regulator, provides an important link between cellular redox potential and the regulation of stress kinase activities [12].

Gene expression is controlled by redox states as well. It has been reported that overexpressing genes related to redox process in *Escherichia coli* resulted in the decrease of NADH/NAD$^+$ ratio, which improve the cell growth profiles, because sufficient NAD$^+$ is required to oxidize carbohydrate substrate during cell growth [13]. *GPD2* encodes NAD-dependent glycerol 3-phosphate dehydrogenase, the key enzyme of glycerol synthesis, and is essential for cell survival under osmotic and low redox potential conditions. Unlike its homologous gene *GPD1*

controlled by high osmolality glycerol response pathway, *GPD2* is regulated under anoxic conditions or, more accurately, oxygen-independent reducing environment [14]. *YAP1*, a transcription factor for sensing the high redox state (e.g. H_2O_2), usually exists in the cytoplasm but is transferred into nucleus to activate the transcription of antioxidant genes *SOD1*, *TWF*, *TRX2*, *GLR1*, and *GSH1*, when Yap1p C-terminal region with three conserved cysteine residues is oxidized in response to oxidative stress [15]. A redox sensing protein (RSP) binds transcriptional regulation regions located upstream from *adh*A, *adh*B, and *adh*E as a transcriptional repressor. The structure of RSP was changed from α-helix to β-sheet rich conformation when redox potential declined by adding NADH. Meanwhile, the repression of an alcohol dehydrogenase transcription caused by RSP was reversed [16]. Thioredoxin reduces cysteine moieties in the DNA-binding sites of several transcription factors and is therefore important in gene expression [17].

External redox potential correlates the net balance of intracellular reducing equivalents and the changes in the cellular redox environment can alter signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, and even regulation of the cell cycle. Thus, monitoring and controlling environmental redox potential helps to elucidate cellular physiology and intracellular metabolic interaction.

5. Redox potential and metabolic flux

Strategies to control intracellular redox potential can be developed by altering intracellular redox potential pools, consequently resulting in redistribution of metabolic profiles. However, cells have a series of built-in mechanisms to adjust their own intercellular redox balance by cofactor regeneration through the oxidoreductase-harboring genes, including mitochondrial alternative oxidase (AOX), formate dehydrogenase (FDH), cytoplasmic H_2O-forming NADH oxidase (NOX), and mitochondrial NADH kinase (POS5). Therefore, modification of these genes is a promising strategy to "design" a robust strain subjected to redox regulation through extracellular manipulation, although such an alternation may result in unexpected outcomes.

5.1. Alternative oxidase

The alternative oxidase (AOX, EC: 1.10.3.11), also named ubiquinol oxidase, forms a part of the electron transport chain in mitochondria. The function of this oxidase is believed to dissipate excess reducing power. The reaction catalyzed by AOX oxidase (ubiquinol oxidase) is shown in Reaction (4).

$$\text{ubiquinone} + H^+ + NADH = \text{ubiquinol} + NAD^+ \tag{4}$$
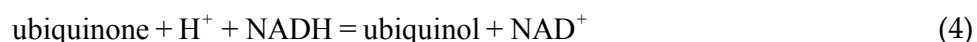
When a cell subjected to increasing glycolytic fluxes under aerobic conditions, a decrease in respiratory capacity is caused by the presence of excess glucose that repressed respiratory pathways. Introducing a heterologous alternative oxidase into *S. cerevisiae*, increased metabolic

flux toward respiration and reduced aerobic ethanol formation [18]. In other investigation, the introduction of AOX pathway improved reactive oxygen species and pyruvate levels simultaneously under stressful conditions, such as suboptimal temperature and hyperosmotic pressure [19].

5.2. Formate dehydrogenase

Formate dehydrogenases (FDH, EC: 1.2.1.2) are a set of enzymes that catalyze the oxidation of formate to carbon dioxide (see Reaction 6), donating electrons to a second substrate, such as NAD^+ or cytochrome. NAD^+-dependent formate dehydrogenases are important in methylotrophic yeast and bacteria and are vital in the catabolism of C1 compounds, such as methanol.

$$formate + NAD^+ = CO_2 + NADH + H^+ \qquad (5)$$
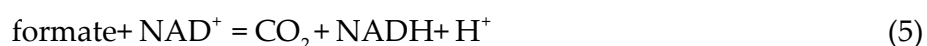
As the FDH gene from *Candida boidinii* was introduced into *Paenibacillus polymyxa*, highly expressed exogenous FDH increased $NADH/NAD^+$ and the titers of NADH-dependent products such as lactic acid and ethanol, while resulting in significantly decreased acetoin and formic acid [20]. In addition, the increased capacity of a FDH gene in *Bacillus subtilis* efficiently enhanced the production of 2,3-butanediol and decreased the formation of acetoin through increasing the availability of NADH [21]. In another case, an engineered strain for the conversion of D-fructose to allitol was developed by constructing a multienzyme coupling pathway and cofactor recycling system in *E. coli*. FDH gene was used to support the cofactor recycling system for the availability of NADH [22].

5.3. NADH oxidase

NADH oxidase (NOX, EC: 1.6.3.4) is a membrane-associated enzyme that catalyzes the production of superoxide, a reactive free radical, by transferring one electron from NADH to oxygen as the electron acceptor (see Reaction 7). It is considered one of the major sources of producing superoxide anions in humans as well as bacteria, subsequently used in oxygen-dependent killing mechanisms for invading pathogens.

$$2H^+ + 2NADH + O_2 = 2H_2O + 2NAD^+ \qquad (6)$$

Glycerol is a main by-product in the 2,3-butanediol metabolic pathways. To minimize glycerol accumulation by an engineered *S. cerevisiae*, the *Lactococcus lactis* NOX gene was inserted and expressed, resulting in substantial decreases in intracellular $NADH/NAD^+$ ratio. As a result, the carbon flux was redistributed from glycerol to 2,3-butanediol [23]. NADH oxidase was also expressed with L-arabinitol dehydrogenase in *E. coli* to efficiently produce L-xylulose. Thus, the efficiency above 96% for the conversion of L-arabinitol into L-xylulose was achieved under optimized conditions [24].

5.4. NADH kinase

NADH kinase (like POS5, EC: 2.7.1.86) catalyzes the replacement reaction with two substrates ATP and NADH and two products ADP and NADPH (see Reaction 8). It provides a key source of the important cellular antioxidant NADPH.

$$ATP + NADH = ADP + NADPH \tag{7}$$

NADPH is a key cofactor for carotenoid biosynthesis. *Corynebacterium glutamicum* was always used for the production of amino acids, such as L-isoleucine. By implementing NADPH-supplying strategies based on NAD kinase (PpnK), NADH kinase, glucose-6-phosphate dehydrogenase (Zwf), and PpnK coupling with Zwf, the expression of all genes increased both the intracellular NADPH concentration and the L-isoleucine production [25]. Researchers constructed the NADPH regenerators of heterologous NADH kinase to increase the availability of NADPH and resulted in a superior S-adenosylmethionine production in *E. coli* without requiring L-methionine addition [26]. When a *S. cerevisiae* strain-producing carotenoid was constructed by overexpressing glucose-6-phosphate dehydrogenase and NADH kinase individually, the final product β-carotene yield increased by 18.8% and 65.6%, respectively. Thus, NADPH supply improved by overexpression of NADH kinase is more important than glucose-6-phosphate dehydrogenase [27].

6. Application of redox potential to fermentation processes

Controlling redox potential at a desired level alters the intracellular metabolic flow in order to favor the formation of desired product(s). Many researches have been conducted in this regard with a large number of examples for enhanced production of metabolites under redox potential–controlled conditions. Most studied metabolites using redox potential–controlled approaches are hydrogen, pyruvate, 1,3-propanediol, butanol, and 2,3-butanediol, and the following metabolites are reviewed but provided with references: acetoin [28], succinic acid [29], xylitol [30], and so on.

6.1. Hydrogen

Hydrogen, as a clean and high-combustion energy in widespread areas, can be generated by fermentative anaerobes. Hydrogen production from anaerobic fermentation by bacteria demands reducing level because the standard redox potential of H_2/H^+ is low. Zhang et al. [8] showed that the addition of NAD^+ during hydrogen fermentation by *Enterobacter aerogenes* resulted in the increase of overall hydrogen. Nakashimada et al. [31] investigated *E. aerogenes* for its hydrogen production under different intracellular redox state through the utilization of different substrates bearing various reduction degrees. Low redox potential accelerated the NAD(P)H-dependent hydrogenase activity in membrane and favors high H_2 evolution capability. Ren et al. [32] assessed H_2 production during butyric acid fermentation, propionic

acid fermentation, and ethanol fermentation by controlling redox potential and pH simultaneously. Besides, the NAD^+ synthetase encoded by nadE gene was homologously overexpressed in *E. aerogenes* to decrease the $NADH/NAD^+$ ratio and thus enhanced hydrogen yield [33].

6.2. Pyruvate

Pyruvate, a product of glycolysis, serves as an effective starting material for the synthesis of many drugs and agrochemicals and is presently used in the food industry. By combining adaptive evolution and cofactor engineering, a series of engineered yeasts that can produce pyruvate using glucose as the sole carbon source was obtained. Consequently, the constructed strains were able to produce 75.1 g/L pyruvate, increased by 21% compared with the wild strain. The production yield of this strain reached 0.63 g pyruvate/g glucose [34].

6.3. Propanediol

1,3-propanediol, made from glycerol under anaerobic condition, is a monomer for producing various industrial polymers. Du et al. [35] demonstrated that controlling redox potential at −190 mV was preferable for *Klebsiella pneumoniae* to ferment glycerol into 1,3-propanediol. They further developed a redox potential–based strategy for screening high productivity strain using the correlation between redox potential level and growth rate [36]. Zheng et al. [37] regulated redox potential under low levels (−200 and −400 mV) during 1,3-propanediol fermentation in order to avoid the accumulation of by-product. Wu et al. [38] engineered the pathways of 2,3-butanediol and formic acid in a recombinant *K. pneumonia* to improve 1,3-propanediol production. The intracellular metabolic flux was redistributed pronouncedly by shrinking all nonvolatile by-products and supplying the availability of NADH. Jain et al. [39] established novel metabolic pathways for 1,2-propanediol in *E. coli* by disrupting the major competing pathways for acetate production as well as the ubiquinone biosynthesis pathway that conserved more NADH.

6.4. Butanol

Butanol attracts public attentions due to its favorable physicochemical properties for blending with or for directly substituting for gasoline. Fermentation of butanol by *C. acetobutylicum* is generally a biphasic process consisting of acidogenesis and solventogenesis. It has been reported that an earlier initiation of solvent genesis under redox potential control at −290 mV could increase solvent production by 35% [40]. Li et al. [41] supplemented nicotinic acid, the precursor of NADH and NADPH, into the growth medium, and led to a significant increase of NADH and NADPH levels for a wild-type *Clostridium sporogenes* strain. As a result, the metabolic pattern was shifted toward the production of more reduced metabolites, in which butanol production was then enhanced. Bui et al. [42] constructed the recombinant *K. pneumoniae* by overexpressing the genes *kivD*, *leuABCD*, and *adhE1*, with several NADH regeneration strategies to overcome redox imbalance, including the introduction of NAD^+-dependent enzymes or elimination of the NADH competition pathway (1,3-propanediol synthesis). The $NADH/NAD^+$ ratio was increased resulting in butanol titer increase [42].

6.5. Butanediol

2,3-butanediol (2,3-BD) is a promising bulk chemical with extensive industry applications. In order to enhance the production of 2,3-BD, various strategies for increasing the NADH availability were developed through regulation of low dissolved oxygen, supplement of reducing substrates and gene modification. An *udhA* encoding transhydrogenase was introduced and more NADH from NADPH was provided to allow the enhancement of production [43]. For the same reason, two NADH regeneration enzymes, glucose dehydrogenase and formate dehydrogenase, were introduced into *E. coli* with 2,3-butanediol dehydrogenase, respectively [44]. In other case, an engineered *S. cerevisiae* harboring NADH oxidase gene (noxE) from *L. lactis* minimized glycerol accumulation, because intracellular NADH/NAD$^+$ ratio was decreased substantially and carbon flux was redirected to 2,3-BD from glycerol [23].

7. Redox potential process design: a case study of ethanol fermentation

Fuel ethanol, the most successful renewable energy so far, is produced worldwide and applied in transportation as alternative to fossil fuel. However, the high cost associated with bioethanol production urges researchers to innovate new fermentation technologies like redox potential–controlled ethanol fermentation. In this section, the role of redox potential in *S. cerevisiae* pathways, the correlation between yeast growth and redox potential, and the application of redox potential to very high gravity fermentation will be reviewed.

7.1. The role of redox potential in yeast pathway

S. cerevisiae has been considered as a model microorganism, whose genome, proteome, and relevant pathway information are almost unveiled. As illustrated in **Figure 4**, glucose is converted into small molecules through the coupling of redox reactions, in which NADH plays an essential role in key metabolites production such as ethanol, glycerol, and lactate. In this process, glucose is oxidized by NAD$^+$ to make pyruvate and NADH. The surplus of reducing power is then balanced by the formation of glycerol and ethanol, where NAD$^+$ is restored. When the growth environment favors the production of acetic acid, the implementation of redox potential control can alter the trend, leading to a more reduced state toward ethanol production.

Compared with other control parameters, such as temperature, pH, and the ingredients of medium, redox potential has less influence on improving fermentation results. Hence, the implementation of redox potential control in ethanol fermentation was not popular until the new concept of "very high gravity (VHG)" was proposed. VHG is generally regarded as the final ethanol concentration is greater than 15% (v/v) or initial glucose concentration is greater than 250 g/L. VHG is a promising technology to reduce energy consumption and labor cost, as well as elevate the efficiency of the fermenter. However, high sugar concentration depresses cell growth and bioconversion. Redox potential control helps cells survive from osmotic pressure and ethanol toxicity by constructing healthier membranes or other potential mechanisms. Yeast grown under VHG condition without redox potential control requires much

longer fermentation times in order to completely utilize substrate [45]; therefore, the improvement of ethanol production by redox potential control would be expected.
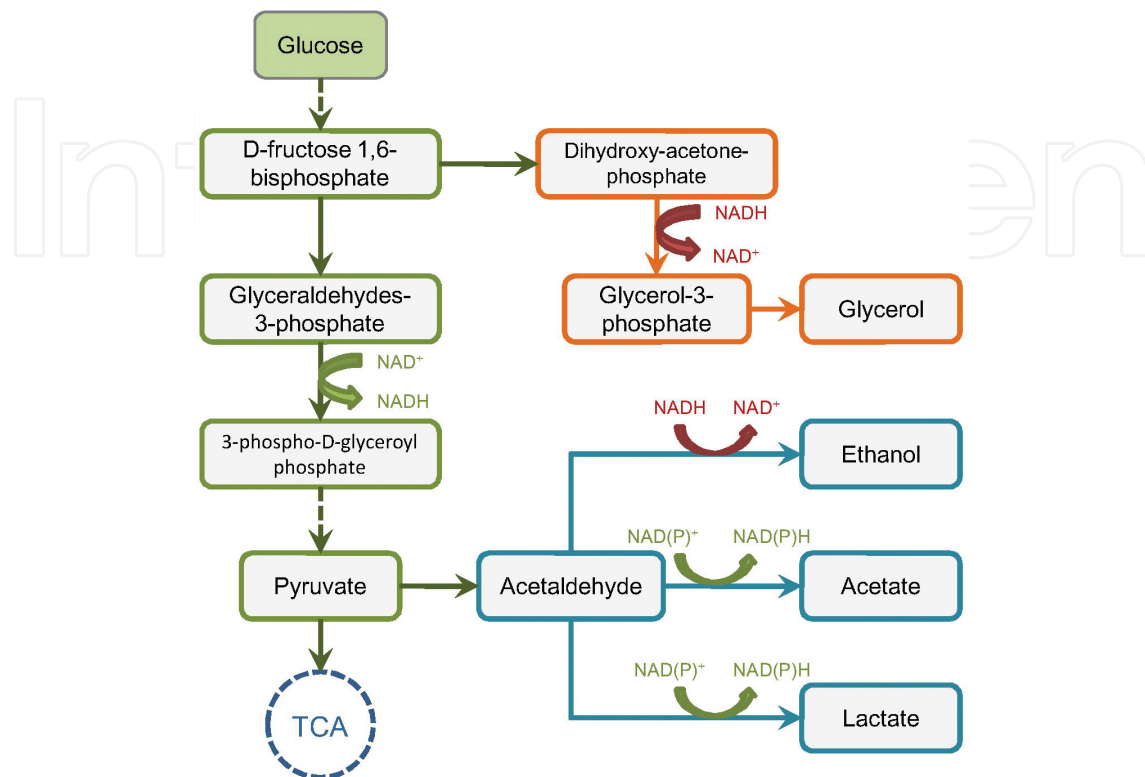

Figure 4. Metabolic pathway of glucose degradation in *Saccharomyces cerevisiae*.

Lin et al. [45] controlled redox potential under −150 mV, −100 mV, and no control conditions and demonstrated that VHG ethanol fermentation under −150 mV resulted in the highest final ethanol concentration and the highest ethanol-to-glucose yield. Compared with the case of 200g glucose/L, the effect of redox potential control becomes significant under VHG conditions [45]. Jeon and Park [46] cultivated *Zymomonas mobilis* and *S. cerevisiae* to produce ethanol in two separate compartments of an electrochemical bioreactor. The results showed that *Z. mobilis* favors the reducing environment, but *S. cerevisiae* produced more ethanol under higher redox potential conditions [46]. Na et al. [47] observed that ethanol production was enhanced in the anode compartment than in the cathode one, although the reduced environment would be better for fermentation process.

7.2. Correlation between cell growth and redox potential

During ethanol fermentation, changes of redox potential are caused by two major substances, electron donor NAD(P)H resulting from dissimilatory processes (e.g. glycolysis) and assimilatory processes (e.g. biomass formation), and electron acceptor oxygen dissolved from sparging and/or agitation. The redox potential profiles are thus correlated to cellular activities and oxygen tension.

A typical redox potential profile resembles a bathtub curve. In the beginning, yeast was inoculated into the autoclaved medium where redox potential is as high as normal oxygen tension. Yeast consumes oxygen as the final electron acceptor during respiration process for rapid propagation, causing a steep fall of redox potential (Stage I, **Figure 5**). When dissolved oxygen is nearly depleted, yeast modulates the respiratory requirement from aerobic to anaerobic stages where a short transition is seen in order to alter relevant gene expression and pathways (between Stage I and II, **Figure 5**). After adjustment, yeast cells accelerate their growth rate in the exponential phase with rapid glucose utilization. Although ethanol production is a redox neutral process in theory, the use of reducing substrate like sugar tends to lower fermentation redox potential. The trend of decline in redox potential continues as fermentation proceeds and could drop as low as to −300 mV if there is no other oxidizing reagent present in the fermentation broth (Stage II–III, **Figure 5**). Due to the substrate depletion and the decline of cell viability attributed to ethanol toxicity, the lowest trough in redox potential level is observed (Stage III, **Figure 5**). Near the end of fermentation, an abrupt increase in redox potential is attributed to constant aeration or well agitation. Technically, an uprising curve appearing reveals that the fermentation is about to finish (Stage IV, **Figure 5**).
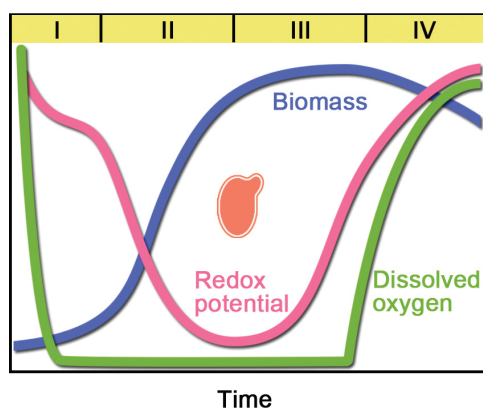

Figure 5. Profiles of redox potential, biomass, and dissolved oxygen.

7.3. Process design using redox potential

The performance of VHG ethanol fermentation can be further improved by (1) searching for the optimal redox potential setting and (2) extending redox potential control period to prolong the exponential growth phase. Three redox potential control schemes are collected [48]. The simple aeration-controlled scheme (ACS) has a short redox potential–controlled period. For glucose-controlled feeding scheme (GCFS), glucose was supplemented along with dissolved oxygen presented in the feed stream. For combined chemostat and aeration-controlled scheme (CCACS), a constant glucose was fed along with air supply determined by redox potential–controlled device. The GCFS extends the redox potential–controlled period by offering enough glucose for yeast propagation and maintaining the low residual glucose. As a result, the ethanol yield is increased noticeably. The operation of GCFS as a fed batch, as such the buildup of ethanol causes yeast cessation, resulting in incomplete fermentation. The CCACS is a set of

continuous equipment that feeds the fresh medium into a fermenter and discharge spent broth into aging vessels at a constant dilution rate. Sterilized air was used to adjust the fermentation redox potential at a predetermined level. In the chemostat fermenter, both intracellular and extracellular factors should reach their respective steady states. Thus, constant growth rate and yeast viability are sustained under a preset redox potential level, which is helpful to prolong the redox potential–controlled duration and to maximize the benefits from redox potential control. The CCACS achieved the longest controlled period and the highest ethanol yield among all three schemes. However, a chemostat device alone could not result in zero glucose discharge. The incorporation of aging vessel design into fermentation operation thus was developed [49].

8. Future work of redox potential and fermentation

Although many fermentation processes have been well developed with long-term operability, cost saving is an endless effort, particularly for the production of biofuels and bio-based chemicals at bulk quantity. Every penny in cost savings is destined to bring huge economic returns. Since redox reactions and homeostasis are the basis for intracellular metabolism, monitoring and controlling redox potential status inside a cell could potentially re-route metabolic material and energy flow. Numerous works have been done and confirmed that proper redox potential control could alter cellular metabolism, thereby enhancing the conversion of targeted metabolites.
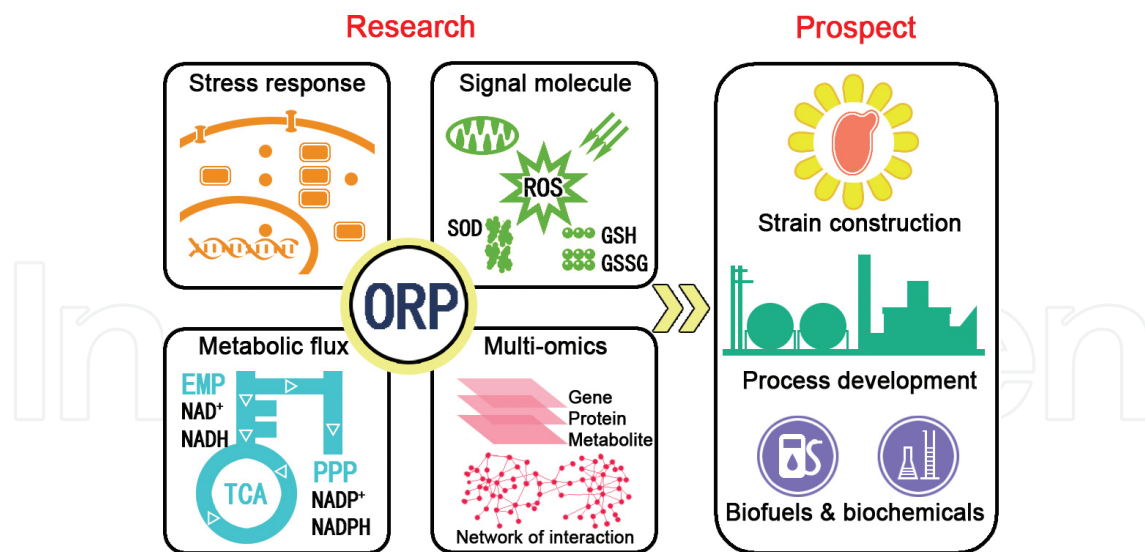

Figure 6. Research and prospect in redox potential–controlled fermentation.

With the availability of technologies that can detect intracellular redox potential levels, an integrated approach, including gene expression, protein biosynthesis, and biomolecular interacting network, should be employed to identify effects of redox potential control on the multiple hierarchy (**Figure 6**). The underlying mechanism of this phenomenon can then be

elucidated at molecular and bioprocess engineering levels. The more details obtained, the better applications of redox potential control can be exploited. Consequently, robust strains and optimized processes can be developed toward high-yield production.

Future perspective of redox potential control is attractive. Fermentation will be carried out using gene-modified strains featuring tailor-made redox potential balance. The strain will be subjected to tight regulation through precise redox potential level. Metabolic flux profiles obtained at different redox potential levels will be quantified to achieve the maximum production of various desired metabolites or used to locate potential bottleneck for strain improvement. Benefits from the development of new redox potential–controlled fermentation technology are thus anticipated.

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