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11

Microbes and Their Products as Sensors in Industrially Important Fermentations

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11.1 Introduction

Fermentation reactions have an ever-increasing role to play in the biotechnological processes involved in major industrial and environmental sectors. These applications include food, pharmaceuticals, water treatment and energy. Thus to yield an optimal result, precise control of the process conditions are important. The need of the hour for the industrial level fermentations is always rapid and sensitive on-line monitoring and control. Though the methods of spectrophotometery and chromatography are generally made for assessing the quality of the product but it is unsuitable for on-line measurements. It is in these situations that electrochemical monitoring using sensors is convenient. One such type of sensors called biosensors are used to

a large extent in the industrial level fermentations wherein the transducer for temperature, pH, optical activity is in conjunction with a biologically active system (Hikuma et al., 1979a; Karube et al., 1977; Turner, Karube, and Wilson, 1987)

The advances in the sensor technology started with the use of the biocatalysts in conjunction with the transducers, the first reports hinting this development came from Updike (Updike and Hicks, 1967). The highly specific biocatalysts coupled with the high sensitivity of the transducer systems ameliorated the entire regime of process control. The first generation of biosensors using biocatalysts paved the way to the second generation of biosensors where in whole microbe cells were utilized. The pioneerstudies by Divies in 1974 reported the use of whole microbial cells for the detection of ethanol in the year 1974. The first industrial application was reported in 1977 to determine the BOD inits waste water by Karube (Karube et al., 1977). Even though the first generation sensors using biocatalysts have an edge of specificity and response time over the microbe sensors but the latter score over the former in terms of higher tolerance to the measuring conditions, a long lifetime and cost performance. In addition, the second generation of sensors using whole microbe cells do not require downstream processes of extraction and purification and co-factors required for the first generation sensors involving only biocatalysts. Thus the cost of the second generation sensors could be economized. Over and above, these sensors could be recharged by soaking in the nutrient broth. This makesthem active for a longerperiodwith theadded advantage of reusability. The main idea of the use of the microbe as sensors revolves around their characteristics to adapt to the analyte. In the present chapter, we would discuss the different aspects of the microbes as sensors in the fermentation of major commercially viable products and environmental monitoring.

11.2 Sensors

The first microbial sensor was developed by Divies in 1974 for determination of ethanol using cells of *Acetobacter xylinum* (Divies, 1974). The working of the microbe sensors are based on the last phase of the aerobic respiration involving the electron transport chain (López-Barneo, Pardal, and Ortega-Sáenz, 2001). Another improvisation of these sensors arethe mediator based microbe sensors.In these type of sensors, the electron produced as a result of the respiration process is sequestered by the mediator, and this mediator finally passes the electron to the anode which is one of the two transducers in the system. This can be detected either by the dissolved oxygen sensor or electron transfer measuring systems (Du, Li, and Gu, 2007). The main basis of a biosensor is the intimate contact between microbe and transducer element. Microbes are immobilized to improve the contact. This immobilization can be either physical methods of adsorption, entrapment in supportsor chemical methods involving covalent binding to supports (Cassidy, Lee, and Trevors, 1996). It is the physical methods which are largely utilized for the immobilization as it facilitates microbes to retain their biological activity. The sensitivity of the physically immobilized microbe sensors also surpasses in sensitivity in detection as compared to the other methods of immobilization.

11.3 Transducers in Conjunction With Microbe Sensors

11.3.1 Dissolved Oxygen (DO) Electrode

A DO electrode is the most general transducer for the microbial sensor. The sensors used today are the improved micro-oxygen electrode developed based on semiconductor fabrication technology given by Karube et al. (Suzuki, Tamiya, and Karube, 1988). The microbial sensors are dipped in the solution saturated with DO; this causes an increase in the respiratory activity of the microorganisms which in turn results in a decrease in the DOconcentration near the membrane of the polarographic electrode. A suitable polarization voltage between the anode and cathode selectively reduces oxygen at the cathode. The results of these chemical reactions areshown as a current which is proportional to the DO concentration. Using a DO electrode, substrate concentrationcanbe measuredfrom the oxygen decrease. These changes can be estimated as concentrations of the toxic compound. The principle in this sensor type is shown in Fig. 11.1. The DO electrode

is also used to measure the amount of the substrate as well as the toxic compounds present in the media. An improved version of the oxygen sensor based on the optical sensing device for biochemical oxygen demand (BOD) wasfirst reported by Preininger et al. in 1994 (Preininger, Klimant, and Wolfbeis, 1994). This sensor based on an oxygen-sensitive ruthenium complex [(Ru) complex] relied on the oxygen quenching of luminescence. Since the initial work, several BOD biosensors have been developed using different kinds of microbes (Chee et al., 2000; Li et al., 1994; Lin et al., 2006). Even though the additionof mediators in these biosensors can enhance the electrontransfer, these biosensors have poor stability because of thetoxicity of mediators. Recently, mediator-less microbial fuelcells have been exploited to fabricate novel BOD sensors forcontinuous and real-time monitoring (Chang et al., 2004). Furthermore, Kim et al. reported that the performance of a microbial fuelcell as BOD sensor was improved using respiratory inhibitors (Chang, Moon, Jang, & Kim, 2005).

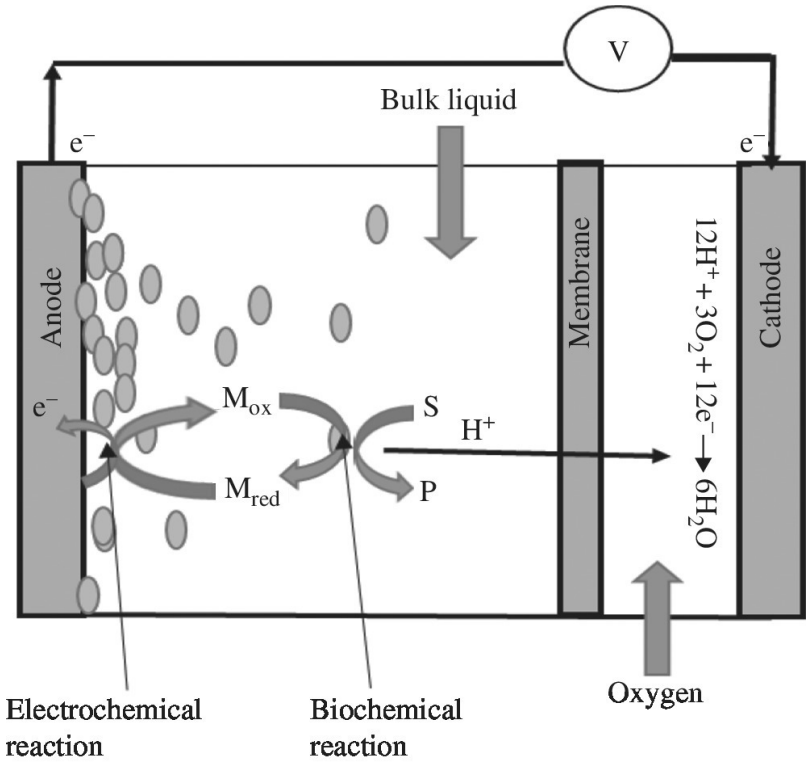

Figure 11.1 Principle of the Oxygen Sensor based on the respiration activity. (*See insert for color representation of this figure.*)

11.3.2 Electron Transfer Measuring Systems

In these types of sensors, the electron shuttles or mediators or color indicators are used between the microbes and the electrode. The principle of this sensor type is shownin Fig. 11.2. The mediator most commonly used for the microbial sensors is potassium hexacyanoferrate (III) [HCF (III)]. In the aerobic respiration, the end point acceptor for anelectron is oxygen. However competition is imposed by the HCF (III) when present in the reaction medium. Thus it acts as the electron acceptor and is preferentially reduced to HCF (II) during the metabolic oxidation of organic substances. The reduced HCF (III) is then reoxidized at a working electrode. This was initially tried with Pseudomonas sp. (Yoshida et al., 2001)with similar report on with a consortium of microbes (Catterall et al., 2003). The sensor system utilizing electron transfer has many advantages over the DO electrode. Firstly, the solubility of mediator or color indicator is much higher than that of DO. Secondly, the sensor system does not require an aeration system and can be greatly simplified to a mobile type device. Thirdly, the detectable potential of the mediator is lower compared tothat of DO. A further

improvement of the electron transfer system sensor is a combination of microbe and the redox color indicators (RCI). This sytem allows simultaneous measurements simultaneous spectroscopic measurement using 96 or 364 microwells (Yoshida et al., 2002).

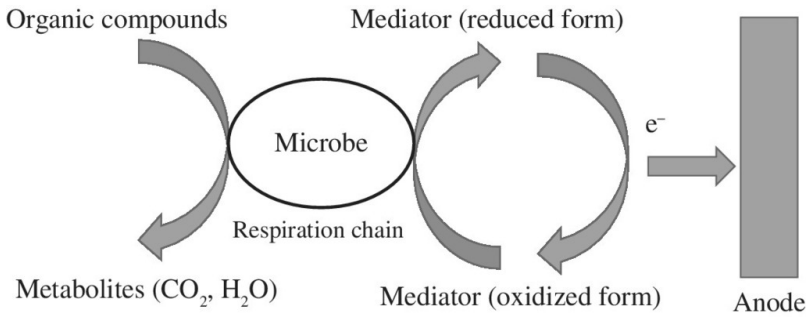

Figure 11.2 Principle of the Oxygen Sensor based on the Electron Transfer Measuring Systems. (*See insert for color representation of this figure*.)

11.4 Metabolite Measuring Systems

In addition to the whole cell DO electrode and electron transfer systems, electrically active metabolites, such as H_2, CO_2, NH_3 and organic acidssecreted from microorganisms, can also be used as the microbialsensors. This type of sensor mostly uses a gas-permeable membrane todetect gaseous compounds in aqueous or gaseous samples. The principle ofthis sensor type is shown in Fig. 11.3. In this sensor type, the microbes employed can be either aerobes or anaerobes.

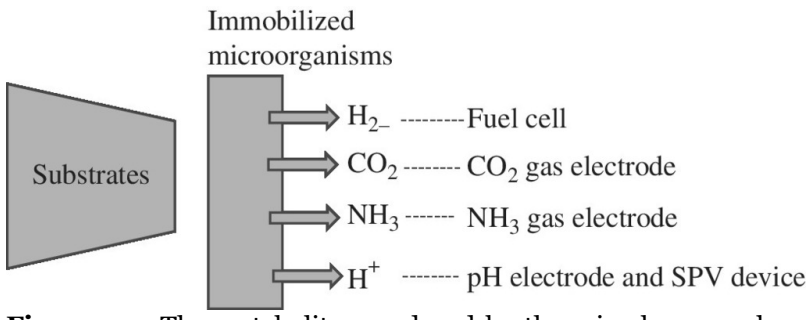

Figure 11.3 The metabolites produced by the microbes can also use as sensors. (*See insert for color representation of this figure*.)

The transducers for H_2 detection have been fuel cell type, CO_2 electrodes, NH_3 electrodes, or pH electrodes (including ion-sensitive field-effect transistors (ISFETs)). Most of these sensors are based on potentiometry. Though they have a large measurable range, there have a limitation of lower detection limits

11.5 Other Measuring Systems

In addition to the metabolites that the microbes secrete, several other parameters can also be usedof for the sensors. For example,to measurethe metabolic heat evolved by the immobilized microorganisms, microbialsensors can be constructed by placing them in proximity to a thermistor.Another method of detecting is to use photobacteriain conjunction with a photodetector (e.g. aphotomultiplier (PMT) or photodiode (PD). The luminescence intensity of photobacteria also known as luminobacteria isdependent on its metabolic activity. Therefore, nutrients like, glucose, amino acids) supplied to the bacteria can easily be detected. Also,

the inhibitors,e.g. toxicants, heavy metals which deter the metabolic rate of the bacteriacould alsobe detected using this type of device. In general, luminescence intensity isa more sensitive parameter for metabolic activity than respiration activity orheat generation. Obviously, only photobacteria can be used for this purpose.The modulation in optical properties such as UV–vis absorption, bio- and chemi-luminescence, reflectance and fluorescence brought by the interaction of the biocatalyst with the target analyte is the basis for optical microbial biosensor (Mulchandani and Rogers, 1998; Tran-Minh, 1993; Turner et al., 1987). Opticalbased biosensors offer advantages of compactness, flexibility, resistance to electrical noise, and a small probe size. Some representative bioluminescence and fluorescence based microbial biosensors.

11.5.1 Bioluminescence Biosensor

Bioluminescence is associated with the emission of light by living microorganisms, and it plays a very important role in realtime process monitoring. The bacterial luminescence *lux* gene has been widely applied as a reporter either in an inducible or constitutive manner. In an inducible manner, the reporter *lux* gene is fused to a promoter regulated by the concentration of a compound of interest. As a result, the concentration of the compound can be quantitatively analyzed by detecting the bioluminescence intensity (Rensing and Maier, 2003; Sagi et al., 2003). In a constitutive manner, the reporter gene is fused to promoters that are continuously expressed as long as the organism is alive and metabolically active (Rensing and Maier, 2003). This kind of reporter is good for evaluating the total toxicity of contaminant. Both types of reporters have been shown to be useful for biosensor development. Heavy metal-mediated toxicity in the environment is dependanton bioavailable metal concentrations. Bioluminescent microbial biosensors have been extensively investigated to monitor bio available metal. *Ralstonia eutropha* AE2515 was constructed by transcriptionally fusing *cnr* YXH regulatory genesto the bioluminescent *lux* CDABE report system to fabricate a whole cell biosensor for the detection of the bioavailable concentration of Ni^{2+} and Co^{2+} in soil (Ivask, Rõlova, and Kahru, 2009). Several optical biosensors consisting of bacteria that contain gene fusion between the regulatory region of the *mer* operon (*mer*R) and *lux* CDABE havebeen developed to quantitatively response to Hg^{2+}. The *mer* promoter is activated when Hg^{2+} binds to *Mer* R, then result inthe transcription of the *lux* reporter gene and subsequent lightemission (Hansen and Sørensen, 2001; Omura, Kiyono, and Pan-Hou, 2004). Bioavailable copper in soil is also monitoredby using engineered *P. fluorescens* through mutagenesis of *P. fluorescens* containing copper-induced gene and *Tn5::lux*AB Promoter probe transposon (Bereza-Malcolm, Mann, and Franks, 2014). To monitor nutrients in an aquatic ecosystem, a biosensor for monitoring phosphorus bioavailability to *Cyanobacteria* (*Synechococcus* PCC 7942) was developed (Schreiter et al., 2001). The reporter strain *Synechococcus* harbors the gene coding the reporter protein luciferase under the control of aninducible alkaline phosphatase promoter, which can be induced under phosphorous limitation and shows improvement to conventional phosphorus detection methods (Schreiter et al., 2001). Bioluminescent microbial biosensors using the inducible reporter gene have also been developed for the measurement of bioavailable NAPH

Thus from the above applications, we can generalize that the microbial species chosen for biosensor development must fulfil at least one of the two criteria:

i. aerobic uptake of oxygen in the respiratory process for assimilation of the substrates (in this case the microbial sensor is constructed by coupling the microorganisms with an amperometric oxygen electrode); or
ii. electrode-active products liberated derived from reactions of the microbial metabolism, e.g. protons, ammonium ions, H_2S, CO,and H,O_2,which can be detected by potentiometric or amperometric electrodes.

11.6 Applications of Microbe Sensors in Some Commercially Important Products

The microbial biosensors based on DO and Electron transfer mediators have been utilized for the food analysis and other fermentation processes like brewing and fuel production. During the process of brewing, the alcohol concentration is important. The works of Hikumaetal., in 1979 showed that using Trichosporonbrassicae, the detection of the analyte i.e. ethanol in the range of 2–22.5 mg/L was possible. In another study with Clostridium butyricum the levels of formic acid in the range of 10–1000 mg/L could be detected (Matsunaga, Karube, and Suzuki, 1980). In another study, the gaseous ethanol was determined using by *T. brassicae* or *Gluconobacteroxydans* with ferricyanide mediation (Vostiar et al., 2002). The details are listed in Table 11.1. In all these examples from the brewing and the food industry, simply DO electrodes have been employed to detect the analyte. In recent times many improvements of the DO sensors have been realized. One such improvisationis the inclusion of magnetite-functionalized Bacillus subtilis that can be immobilized and regenerated on an ultramicroelectrode array (UMEA). Modification and regeneration are magnetically controlled. The assay can be performed within 5 min owing to the fast mass transfer of the magnetite-functionalized microbes on the surface of the UMEA.These sensors have been successful applied to the determination of BOD in spiked water samples (Wang et al., 2017).

Table 11.1 A list of the sensors and their applications in the fermentation industry.

Analyte	Microbe Used as Sensor	Electrode Type	Measuring time(min)	Stability (in days)	Reference
Ethanol	*Trichosporon brassicae*	Amperometric	<10	>21	(Hikuma, Kubo, Yasuda, Karube, and Suzuki, 1979b)
Formic acid	*Clostridium butyricum*	Amperometric	20	>20	(Matsunaga et al., 1980)
Acetic acid	Trichosporon brassicae.	Amperometric	8	>20	(Hikuma et al., 1979a)
Glutamic acid	Escherichia coli	Potentiometric	5	>21	(Hikuma, Obana, Yasuda, Karube, and Suzuki, 1980)
Aspartic acid	Pseudomomas				
Glucose	*Pseudomonas fluorescens*	Amperometric	10	>14	(Karube, Mitsuda, and Suzuki, 1979)
Ammonia	*Nitrifying bacteria*	Amperometric	4	>10	(Ikeda, Hachiya, Ito, Asano, and Imato, 1998)
Vitamin B12	*E.coli* 215	Amperometric	120	25	(Karube, Wang, Tamiya, and Kawarai, 1987)

11.6.1 Red Wine

Wines, particularly red wines, contain numerous biologically active compounds, the most important of which are phenolic compounds. The nutritional importance of phenolic compounds is attributed to their antioxidant properties. In particular, flavonoids and related phenolic compounds which are naturally found in red wines have gained increasing interest (Campanella et al., 2004). Red wines have been reported to be preventive of many ailments, and they play a possible role in reducing thrombotic and anthrogenic processes. Phenolic compounds also contribute substantially to the quality of wines and affect their color, flavor, stability and aging behavior (Luximon-Ramma et al., 2005). See Figure 11.4. In one such study, *Acaligense sp.*-immobilized biosensor based on CdS-MWNT and Cu2S-MWNT supports prepared by a one-step radiation

reaction were fabricated. The sensing range of the*Acaligense sp*.-immobilized biosensor based on CdS-MWNT and Cu2S-MWNT supports for phenol was in the range of 0.5 ~ 5.0 mM and 0.7 ~ 10 mM, respectively. Both biosensors exhibited a wide linear range, high sensitivity, and good stability. The prepared biosensors were used to the determination of phenolics in commercial red wines. The results showed that the amount of phenolic. Compounds in commercial red wines were in the range of 926.1 ~ 1,018 mg/L, calculated from the calibration curve of phenol measured by the *Acaligense sp*.-immobilized biosensor based on QD-MWNT supports The relatively high amounts of phenolic compounds in Blue Nun are responsible for the bitter taste of the red win (Kim, Kwen, and Choi, 2011).

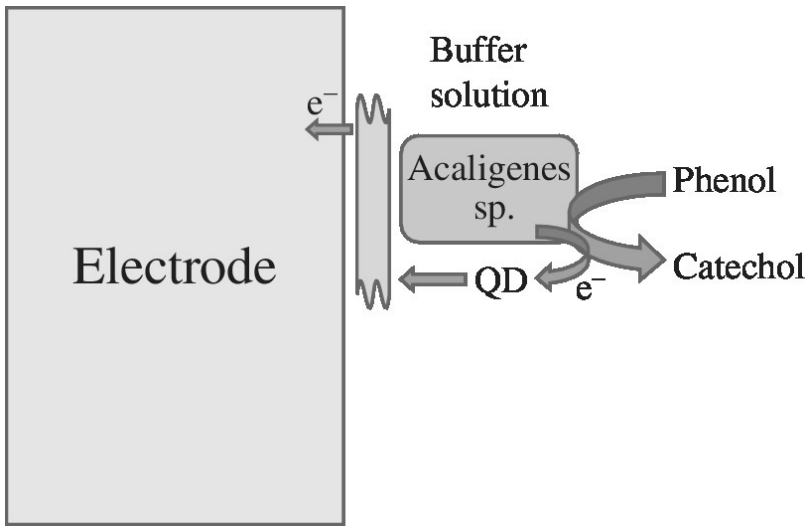

Figure 11.4 Application of microbe sensor in the quality control of red wine. *Source:* Adapted from Kim et al., 2011. (*See insert for color representation of this figure*.)

11.6.2 Fermentation of Cereal Products

The main volatile compounds reported by studies on cereal-based liquid fermentation have been mainly associated with the carbohydrate (mainly carboxylic acids and aldehydes, ketones, esters) and amino acid metabolism (mainly aldehydes and alcohols). Diacetyl (butane-2,3-dione) is a ketone responsible for a butterscotch-like aroma, and the verylow odor threshold (0.005 mg/l in water) is often exceeded during LAB fermentation of cereal substrates ((Burdock, 2002). This volatile is mainly formed from the oxidative decarboxylation of α-acetolactate (α-AL), an intermediate metabolite formed mainly duringsugar, citrate and amino acid catabolism (Hugenholtz et al., 2000). Metabolically related todiacetyl are the less flavoursome acetoin, formed by the reduction of diacetyl or afterenzymatic decarboxylation of α-AL, and 2,3-butanediol, which results from the reduction ofacetoin (Montel, Masson, and Talon, 1998). Although considered as being off-flavours in beer (Bokulich & Bamforth, 2013), these low-molecular-weight compounds are also responsible for mellowing theflavour during cereal fermentation (Mugula et al., 2003). Only some LAB strains, e.g. *L. lactis, Lb. Plantarum, Oenococcusoeni*, can metabolize citrate to pyruvate that can be eventually re-directed into the acetoin/diacetyl pathway. This pathway is responsible for the high accumulation of α-AL during LAB fermentation of milk-based matrices (citrate concentration of ca. 1500 mg/l)(Garde et al., 2002). Although citrate in cereal malt gruels has been reported as being rather low (170 mg/l) (Owuama, Chiangi, and Adeyemo, 2011), higher levels (871 mg/kg) have been reported in a sorghum-based liquid substrate (Acetaldehyde is a highly volatile aldehyde formed from pyruvate or threonine catabolism(Ardö, 2006). It has been described as delivering a pungent, fruity (green apples) flavor withsweet notes (odor threshold of 0.027 mg/l in water) (Salmerón et al., 2015)) showed that the high level of acetaldehyde (1.36 mg/l) found after fermentation of a malt-based beverage with *Lb.Plantarum* NCIMB 8826 positively contributed to the high acceptance of the beverage.

11.6.3 Mevalonate Production

In whole-cell biosensing, changes in cellular metabolism, pH, and gene expression have been quantified as a response of the sensing elements to the presence of target molecules (Ivask et al., 2009; Pfleger et al., 2007; Schultheiss et al., 2008). Microbialauxotrophy has been used used to monitor growth-limiting small molecules. For example, Pfleger et al. constructed an autotrophic *E. coli* strain for the detection and quantification of mevalonate,an intermediate in the biosynthesis of isoprenoids, a large class of industrially important secondary metabolites that includes flavor, fragrance, anti-oxidants, steroids, and the anti-malarial drug artemisinin.Since mevalonate is a key precursor whose production must be increased toimprove the production of isoprenoids, optimizing the level of mevalonate is important in developing recombinant strains for enhanced isoprenoid production. The authors deleted the native pathway for the production of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) that are necessary for growth, and incorporating the mevalonate-utilizing pathway Hence, using simple growth monitoring, the concentrations of mevalonate could be determined.

11.6.4 Bioaerosols

The recent advances in analytical techniques open a new door for the chemical characterisation of bioaerosol. Specifically, chemical analysis of microbial volatile organic compounds (MVOCs) can be a reliable and rapid assessment of the nature of ambient bioaerosols (Lemfack et al., 2014) as microbial communities express different MVOCs profiles depending in which environment they are in Ref.(Konuma et al., 2015). Further to this, it has been shown that species-specific volatiles may serve as marker compounds for the selective detection of pathogenic microbial species in indoor and outdoor environments (Lemfack et al., 2014). MVOCs are secondary metabolites produced by fermentation and are volatile due to their physicochemical properties (low molecular weight, low boiling point, and high vapor pressure)(Schenkel et al., 2015). Characterizing and quantifying MVOCs can also be used as a proxy approach to estimate microbial concentration (Araki et al., 2012)and to detect invasive *Aspergillus fumigatus* from breath samples (Chambers et al., 2009).

11.6.5 Aptamers

Metabolites and sensitivity and have a long history as detectors in complex samples in the food, environmental and medicinal sectors. The design of new biosensors is now facilitated by the availability of increasingly sophisticated methods for the recruitment of aptamers that comprise RNA(Ozer, Pagano, and Lis, 2014). Similar to antibodies or enzymes, aptamers feature a high affinity for both small molecules and larger structures (Garde, 2002, Hansen, 2001, He, 2016, Hikuma, 1979a,b) such as proteins. Aptamers can, however, can be converted into genetic actuators called riboswitches by combining them with another RNA sequence referred to as an expression platform (van der Meer and Belkin, 2010).These switches undergo conformational changes on ligand binding that result in a switch between states that are OFF (no translationof an adjacent open reading frame) and ON (translation occurs of an adjacent open reading frame encoding, for example,a green fluorescent protein (GFP)). The combination of highly specific ribo switches with the appropriate microbial sensor chassis thus allows the generation of highly specific microbial sensors. Taking advantage of this sensing principle, here we identify *Bacillus subtilis* strains that secrete particularly high levels of vitamin B2 when using cellobiose as a carbon source. Cellobiose is one of the main components of cellulosic biomass hydrolysates, which makes it a potential future feedstock for industrial biotechnology products. B2 secreted by a *B. subtilis* cell is taken up by metabolically active, but growth-arrested, E. coli sensor cells. The sensor cells then transform B2 into flavin mononucleotide (FMN) which binds with high affinity to a specifically developed hammer head riboswitch, switching its conformation to ON and triggering GFP production in the sensor cells (Fig. 11.5) in a concentration-dependent manner (Meyer et al., 2015).

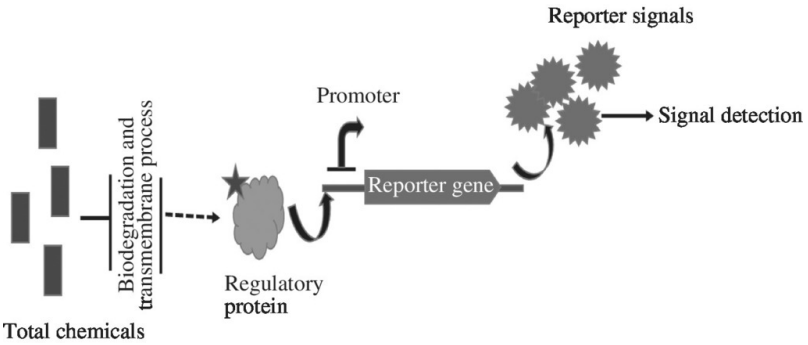

Figure 11.5 The regulation, construction and organization of the Genetically engineered microbial whole-cell biosensors. *Source*: Adapted from He et al. (2016). (*See insert for color representation of this figure*.)

11.7 Conclusions

In this chapter, an attempt has been made to introduce various transducer systems available at an industrial scale. In addition, major applications of these sensors in various industries have been highlighted. The main motive of the chapter was to help in analyzing the sensitivity of each sensor type to help extend these sensors in the field of research.

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