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APPLICATIONS OF ENZYME TECHNOLOGY IN BIOSALINE ENVIRONMENTS[1]

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Microorganisms from biosaline environments represent unique sources for enzyme systems since these environments experience high temperature and salinity. Potential applications of microbes grown under biosaline conditions (whole microbial cells or isolated enzymes) as biocatalysts for food, fiber, energy, and fine chemicals production are discussed. Use of such organisms and enzymes in the immobilized form is emphasized.

INTRODUCTION

The biosaline environment is characterized by high incidence of solar energy radiation and the availability of high temperature and saline (or brackish) water. These could form the basis for a renewable resources utilization program for desert lands employing microbial growth in or enzyme systems isolated from a hot, saline environment. The potential for increased future exploitation of these resources for food, fiber, energy, and fine chemicals production is examined in this communication.

The most significant developments in enzyme technology have occurred within the last decade. Spurred by early successful technology transfer to industrial processing, a rather prodigious effort is being expended in exploring new application areas of this technology. However, little attention has been paid to investigating opportunities in the biosaline environment. Therefore, it seems appropriate to review first certain relevant aspects of enzyme technology to provide a background to discussing its research priorities for biosaline environments.

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The position paper on enzyme technology prepared for the recently held biosaline workshop by Coughlin (1) covers succinctly the sources, extraction and purification, and immobilization of enzymes as well as the use of bound enzymes in continuous reactor systems. The following is a synopsis on the immobilization of whole microbial cells and isolated organelles and identification of research opportunities in the biosaline environment.

WHOLE CELL IMMOBILIZATION

The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time-consuming, and expensive steps involved in purifying intracellular enzymes. It also tends to improve the stability of the enzyme by retaining it in its natural surroundings during immobilization and subsequent continuous operation. Very special advantages may be realized when multienzyme systems and enzymes employing cofactors are considered. In the former case, purification steps for a number of enzymes are eliminated and methods to reconstruct the system in some optimum spatial arrangement are unnecessary. In the latter case, cofactor regeneration machinery included within the cell may obviate external supply of such compounds. In addition, the enzyme and cofactor may already be arranged in some optimum way within the cell, thus the possibility of retaining the structural integrity of the catalytic complex. Finally, bound cell systems offer the possibility of placing classical submerged fermentations on a heterogeneous catalysis basis.

Early work with whole cells has verified some of the above advantages—at least in simple systems involving single reactions. In fact, the concept has already become practical. Catalytic particles containing bound whole cells are being used commercially for the isomerization of glucose to fructose in the production of high-fructose corn syrup (HFCS) (2). Other applications of immobilized whole cell systems have been discussed in detail in recent reviews (3,4).

In our laboratory, we initiated studies on immobilized whole cells with simple systems such as glucose isomerization which involve a single enzymatic reaction. The excellent results obtained in this case and our strong belief that several fundamental elements of immobilized cells systems could be understood only by examining more complex reaction sequences characterized by entire metabolic pathways have steered our efforts to the study of such systems (Table 1). In all cases, reconstituted bovine hide collagen has been used as the support matrix. Immobilization procedures and the rationale for the use of collagen have been well documented (5). The complexity of the systems investigated can be appreciated from

TABLE 1. **Immobilized Whole Cell Systems**

Microbe/organelle	Substrate	Product	Comments
Streptomyces venezuelae; *Bacillus* species	Glucose	Fructose	Glucose isomerization; single enzyme process
Saccharomyces cerevisiae	Sucrose	Invert sugar	Single enzyme
Escherichia coli	Fumaric acid	L-Aspartic acid	Single enzyme
Nocardia erythropolis	Cholesterol	Δ^4-Cholestenone	Non aqueous medium; single enzyme
Corynebacterium simplex	Hydrocortisone	Prednisolone	Steroid modification; single enzyme with cofactor requirement; nonaqueous medium
Serratia marcescens	Glucose	2-Ketogluconic acid	Multienzyme
Acetobacter sp.	Ethanol	Acetic acid	Multienzyme; cofactor
Cornyebacterium lilium	Glucose	Glutamic acid	Pathway (primary metabolite)
Aspergillus niger	Sucrose	Citric acid	Primary metabolite
Chloroplast	Water	Oxygen	Immobilized organelle; first step in biophotolysis of water
Anacystis nidulans	Water	Oxygen	Immobilized algal cells
Anacystis nidulans	Nitrate	Ammonia	Biological nitrogen fixation
Streptomyces griseus	Glucose	Candicidin	Antibiotic synthesis; secondary metabolite
Pseudomonas aeruginosa	—	—	Concentration of plutonium from waste waters (bioadsorption)

the last column of Table 1. Results obtained thus far have demonstrated at least the technical feasibility of these immobilized cell-mediated processes (6).

Several important process engineering parameters need to be examined in order to design efficient means to utilize the immobilized cell system. These include (a) determination of the physiological state and viability of the microbial cells within the carrier—whether they are living, resting, or dead cells, (b) determination of whether or not the cells undergo autolysis on immobilization, (c) development of better immobilization conditions to minimize enzyme inactivation during immobilization, (d) identification and control of microbial contamination problems, if any, and their abatement,

(e) determination of the role of dissolved oxygen and development of the means to augment oxygen transfer to the reactor system, (f) determination of the fate of cofactors, (h) investigation of side reactions, if any, (i) external and internal mass transfer problems, and (j) determination of the energetics of the fixed cells. These aspects have been discussed in some detail elsewhere (6,7).

IMMOBILIZATION OF ISOLATED ORGANELLES

In the cell, organelles represent highly ordered compartments where specific reaction sequences are carried out. The spatial and conformational arrangements of such multienzyme complexes have been recognized as crucially important factors in mediating these reactions very efficiently. Respiration and phosphorylation in the mitochondria are examples of such reaction schemes. Unlike the intact cells, isolated organelles are not protected by a thick cell wall and are therefore very fragile. Organelle immobilization thus presents additional problems. Special techniques must be used to preserve not only the activities of the individual enzymes but also their structural and conformational integrity. Some success has been achieved in the immobilization and stabilization of spinach leaf chloroplasts (6).

Referring back to Table 1, two processes are of particular importance with respect to biosaline research applications: biophotolytic production of hydrogen and microbial synthesis of ammonia. Since the biosaline environment experiences a particularly high incidence of solar radiation, it lends itself as the most logical place to carry out these solar energy-driven reaction schemes. Therefore, they are discussed here in some detail.

BIOPHOTOLYSIS OF WATER

Photosynthesis is first and foremost a process for converting the radiant energy of sunlight into physiological chemical energy, i.e., reducing power and high-energy phosphate. The primary source of electrons for reducing power in algae and higher plants is water, these organisms being the only ones which can split water into hydrogen and oxygen by radiation at the energy level of visible light. This process—called biophotolysis of water—generates two electrons for each water molecule split, which are then transferred through a complex sequence of photosynthetic electron transfer system (PETS) to suitable electron acceptors such as iron-sulfur proteins (also called ferredoxins) or nicotinamide adenine dinucleotide phosphate ($NADP^+$), depending on the kind of organism. These substances are then

used by the cells to reduce oxidized inorganic compounds such as carbon dioxide, sulfates, nitrates, and, in some algae, even atmospheric nitrogen. These compounds, once in the reduced form, are used in metabolic processes.

Photosynthetic production of chemical energy and reducing power in green algal cells is a highly efficient structure-dependent process. The great complexity of these physicochemical processes and the structural organization involved are the main current difficulties in mimicking this process *in vitro* and turning it into a valuable source of energy. In spite of the abovementioned difficulties, research into the theoretical problems, conversion efficiencies, and potential economics of the process are both timely and of a long-term economic importance. As fossil fuels become exhausted, new forms of energy will have to be brought into service. Conceptually, photosynthesis offers the possibility of an inexhaustible energy supply, but there is still a long way to go before it can be realized economically.

Figure 1 shows possible approaches to the utilization of the reducing power generated in nonviable cells, cells with altered metabolism, or subcellular particles (chloroplasts or thylakoid membranes), represented in the form of a "black box." Incident light triggers the splitting of water molecules with the evolution of molecular oxygen, accompanied by the production of hydrogen ion and reduced ferredoxin (Fd). In blue-green algae, Fd is the only electron donor used in reduction processes. But in other algae which can utilize NO_3 as nitrogen source and in all higher plants the

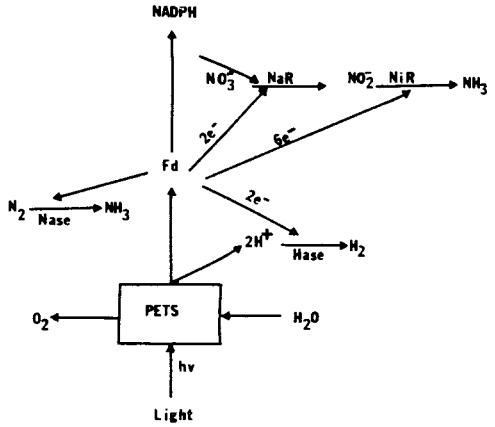

FIG. 1. Schematic of the photosynthetic electron transport system and possible approaches to produce hydrogen and ammonia. Key: NaR, nitrate reductase; NiR, nitrite reductase; Hase, hydrogenase; Nase, nitrogenase; Fd, ferredoxin; PETS, photosynthetic electron transport system.

electron donor for NaR is NADPH. The transfer of electrons from Fd to $NADP^+$ is mediated by a flavoprotein, Fd-NADP reductase. Some algae contain the enzyme hydrogenase, which catalyzes the Fd-dependent reduction of $2H^+$ to H_2. This enzyme has also been found in a number of nonphotosynthetic bacteria but not in higher plants. In addition, a limited number of blue-green algae contain the enzyme nitrogenase, which catalyzes the reduction of molecular nitrogen to ammonia, a strongly endergonic process which requires ATP.

A great deal of effort is being devoted currently toward the production of molecular hydrogen, which could become a major source of energy in the future. The reduction of nitrate to ammonia also offers an interesting possibility of utilizing solar energy. The third alternative, i.e., the biological photofixation of nitrogen, is the most difficult to accomplish because of its high ATP requirement. The success of any future application of water biophotolysis rests on the "black box" of Fig. 1. Application of the process as a practical means of utilizing solar energy for fuel requires the development of a reliable system capable of withstanding numerous environmental perturbation factors such as high intensity of sunlight and temperature fluctuations for a reasonably long period. Lien and San Pietro (8) have reviewed the major factors affecting the stability of PETS as well as the numerous attempts made to stabilize the various activities associated with it.

Benemann et al. (9) studied hydrogen release from spinach chloroplast preparations mixed with hydrogenase and ferredoxin from *Clostridium kluveri*. Without any other additive, they found a rate of H_2 production of 0.02 μmol/mg chlorophyll/min. In the presence of certain additives which remove oxygen, the rate was 0.26 μmol/mg chlorophyll/min. (These differences illustrate another serious problem associated with in vitro production of H_2: extreme sensitivity of isolated hydrogenase toward oxygen.) Fry et al. (10), using again spinach chloroplast together with hydrogenase from *Escherischia coli* and ferredoxin from the unicellular alga *Spirulina maxima*, reported a rate of H_2 production of 0.17 μmol H_2/mg chlorophyll/min, which could be sustained for 6 hr. In order to achieve a practically meaningful process, the "active life" of the isolated catalytic system should be substantially greater than a few hours (11). One effective technique to decrease the rate of activity loss of chloroplast and hydrogenase is microencapsulation of chlorophyll reaction centers in semipermeable membranes (12). Other researchers have succeeded in extending the life of hydrogenase to more than 30 days by immobilization on glass beads (13). Another paper in this workshop by Krasner (14) discusses successful approaches to combining biological water splitting with inorganic metal catalysis (platinum or palladium) to produce hydrogen. Some success has

been achieved in our laboratory on the immobilization of chloroplasts and algal cells (*Anacystis nidulans*) for effecting biophotolysis of water (15).

Typical activities of free and immobilized chloroplasts and algal cells (*A. nidulans*) are shown in Table 2. Immobilized chloroplasts retain about half of the activity of free chloroplasts, while the activity retention is about 27% in the case of bound algae cells. Normal photosynthetic rate in an actively growing plant is about 450 μmol O_2/mg chlorophyll/hr. The observed activities thus represent an energy recovery efficiency of 1.5–3.0% when compared with a growing plant. Storage stability at 4°C of a stripped chloroplast suspension has also been compared with that of a collagen-chloroplast membrane. Intermittent batch assays conducted at 25°C using potassium ferricyanide as electron acceptor indicate that the fixed chloroplast preparation is active after 15 days of storage. These results bear on a primary aspect of the feasibility of water splitting by immobilized systems in a preliminary way. An intensive effort is now under way to increase the activity and stability of these preparations.

MICROBIAL SYNTHESIS OF AMMONIA

Considerably less attention has been paid to the reduction of nitrate to ammonia via PETS compared to hydrogen production. In the long term, ammonia can be considered as a possible source of H_2 in addition to being the raw material for a number of commercial fertilizers. It is easy to separate it from the reaction mixture by distillation at high pH. Other advantages of ammonia are its ease of handling, transport, and storage. A cyclic process is conceivable in which ammonia produced by photosynthetic reduction of

TABLE 2. **Typical Activities of Collagen–Chloroplast and Collagen–A. Nidulans Membranes**[a]

Sample	Chlorophyll content	Specific acitivity (μmol O_2 mg chlorophyll-hr)
Stripped chloroplast preparation	2.5 mg chl/ml	35.4
Collagen-chloroplast membrane	11.8 mg chl/g	18.5
Algal cells	11.9 mg chl/g	29.1
Collagen-algal cells membrane	3.6 mg chl/g	7.68

[a] Assay conditions: 25°C, light intensity 40,000 Lux; ca. 4-mil-thick membranes; 70% of dry weight of the membrane was collagen; assay medium consisted of pH 7.4, 0.34 M sucrose or sorbitol, 0.01 M NaCl, 0.6 mM phenylene-diamine, and 1.5–3.0 mM potassium ferricyanide. All assays were short-term assays (3–8 min).

nitrate is again reoxidized to nitrate. The oxidation reactions have been well studied, and, in fact, they form the backbone of industrial nitric acid technology. These are highly exothermic reactions and the total high potential energy (800°C) liberated is about 70 kcal/mole.

Another interesting alternative is to start from nitrite instead of nitrate. After oxidation of the ammonia to nitric oxides, nitrite can be regenerated by the reaction

$$2NaOH + NO + NO_2 \rightarrow 2NaNO_2 + H_2O$$

The use of nitrite presents the following advantages:

1. Only one enzyme, i.e., nitrite reductase, is involved.
2. In vivo, nitrite reduction is about 20 times faster than the reduction of $NO_3^- \rightarrow NO_2^-$.

Nitrite reductase is a surprisingly stable enzyme capable of withstanding heating at 100°C at pH 1.0 for 30 min (16).

Nitrate or nitrite reduction is more feasible than H_2 production because there is no need for auxiliary enzymes; the same organisms which split water contain both enzymes required for NO_3 reduction. These are key enzymes in the metabolism (in contrast to hydrogenase), and they are present or can be induced at high concentrations.

Both enzymes have been studied thoroughly using artificial electron donors to carry out the reduction to ammonia. The only data reported up to the present on the coupled photolysis of water and nitrate reduction are those of Candar et al. (17). The rate of ammonia synthesis in this case was 0.04 μmol/mg chlorophyll/min. In preliminary experiments performed in our laboratory using a 14-liter laboratory fermentor, a production rate of 50 μmol NH_3/mg chlorophyll/min has been obtained for the blue-green alga *Anacystis nidulans* (18).

BIOSALINE RESEARCH OPPORTUNITIES

From the foregoing discussion, it is apparent that organisms isolated from the biosaline environment could possess unique advantages (e.g., greater thermal stability) which would enable them to be more efficient catalytic systems for capturing solar energy—for the production of either hydrogen or ammonia. There are but a few microorganisms which possess the enzymatic systems for both water splitting and hydrogen production, i.e., hydrogenase. Search for such algae still continues actively (19). Perhaps a

more appropriate place to look for such organisms is the biosaline environment. Algal cells with higher hydrogen or reduced nitrogen (e.g., ammonia) production capability can be used either in fermentation or in the immobilized state to design continuous processes. Many fundamental problems of organelles and whole cell immobilization need to be studied in this connection. Results from such a project would also be beneficial to the practical processes employing bound whole cells or bound organelles.

A further approach to cell-free photosynthesis is the possibility of coupling water biophotolysis to another biochemical process. The rationale here is that the reductive power generated in biophotolysis could be tapped to drive economically more valuable reactions such as steroid dehydrogenation. This would involve, in the simplest scheme, controlled cultivation of mixed cultures.

Many biosaline organisms are known to accumulate valuable chemicals such as glycerol, sorbitol, and mannitol. Microbial synthesis of such products through the use of biosaline organisms represents a unique approach to tap the potential of renewable resources.

Because of their ability to grow under extreme conditions, it might be possible to use the biosaline organisms in practical processing in unique ways. A ready example relates to sterilization. In traditional fermentations, media and air sterilization are laborious and expensive steps. It might be possible to eliminate sterilization when growing biosaline organisms under hot, saline conditions since such environments would be hostile to many types of contaminating microbes. Indeed, in our work with *Anacystis nidulans* neither media nor air sterilization is routinely practiced (19). In a related context, biosaline organisms could be particularly suitable for whole cell immobilization because the immobilization process itself is often conducted under nonsterile conditions. Identification and characterization of thermo- and halotolerant organisms are of particular significance in this regard. Enhancing the tolerance of known enzymes to high-temperature and/or high-salinity conditions could form a related project.

Finally, a very interesting possibility exists in the use of organisms such as *Halobium halophilum*, a halophilic bacteria which grows in very-high-salinity conditions. The "purple membrane" of such halophils has been demonstrated to act as a light-driven proton pump (20). In other words, under certain conditions these organisms can use visible light (510 nm) for the production of their chemical energy in the form of ATP (21). The energy transduction system involved here is extremely simple compared to the photosynthetic apparatus, easy to isolate in large scale, and very stable. Combinations of the ATP-generating system of *Halobium* and the reducing power coming from photosynthesis present exciting new lines of research.

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