REDUCING GENERATION OF BIOGENIC HYDROGEN SULFIDE IN SUGAR WASTEWATERS

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Abstract:

In the manufacture of beet sugar, improving discharge water quality is a key objective for effective reuse of water for irrigation purposes. Problems associated with the discharge water includes generation of odors from biogenic hydrogen sulfide and ammonia, sludge buildup in ponds used for water retention and treatment due to precipitation of metal sulfides, and inadequate water discharge quality, in terms of total nitrogen, ammonia, fecal coliforms count, and phosphorus concentrations. Methods used to improve water quality have focused mainly on either adding chemicals to minimize odor generation, which cause toxicity issues in the discharge water or to add external aeration, which increases dissolved oxygen concentration in the water and improves aerobic treatment rates, but increases power expenditure and hence operating cost. In this paper, use of a biocatalyst solution containing enzymes has been tested in the laboratory and at field-scale. Enzymes function by inhibiting the growth of sulfate reducing bacteria (SRBs) which are responsible for biogenic hydrogen sulfide generation. Experimental and field results show that the enzyme solution tested, ZymeOut, virtually eliminates the generation of biogenic hydrogen sulfide and introduces no water toxicity, unlike conventional biocides. It also reduces the occurrence of biofilms, improves water clarity in lagoons and significantly reduces metal sulfide sludge precipitation.

Introduction:

Lagoons, also known as stabilization ponds, are earthen facilities for the biological treatment of wastewater. Lagoons are designed to remove Biochemical Oxygen Demand (BOD) and to reduce the concentration of disease causing organisms. Large lagoons have been known to generate hydrogen sulfide odors even with extensive aeration, mainly because of inadequate dissolved oxygen dispersion in the water. Another major issue with lagoons is inadequate mixing in the water to allow adequate dissolved oxygen dispersion.

Problems associated with lagoons are:

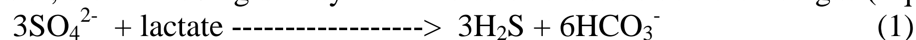
- Odor, mainly ammonia and/or hydrogen sulfide;
- Sludge build-up, requiring periodic dredging and solids removal; and
- Inadequate water discharge quality, in terms of total nitrogen, ammonia, fecal coliforms count, and phosphorus concentration

Conventional methods that have been tried with limited success includes the use of biocides (Hodges and Hanlon, 1991), which are added to the water to reduce bacterial growth. The main reason for limited success is the dissipation of biocide in reducing growth of all bacteria – aerobic, anaerobic, sulfate reducing, etc., some of which are needed for biological treatment of the water. The sulfate reducing bacteria (SRBs) responsible for generation of biogenic sulfide and hydrogen sulfide mainly reside within biofilms in the sediment, and most biocides are unable to penetrate and kill these robust biofilms (Hamilton, 1985). Another approach is aeration of the water or use oxidants to oxygenate the water, which reduces the effectiveness of SRBs. However, aeration of water is expensive and results in increased emissions of hydrogen sulfide initially as it strips the dissolved gas from the water.

Enzymes are biological catalysts that can be used to direct a chemical transformation (Aitken, 1993). They are grouped into six functional classes and numerous subclasses by the Enzyme Commission of the International Union of Biochemists, assigning each enzyme a unique four digit number. Enzyme technology has recently received extensive interest, especially in environmental treatment using biological systems, such as bacteria, fungi, or other microorganisms (Whiteley and Lee, 2006).

In recent years interest has increased in the use of specific enzymes for treatment of aqueous systems in place of live cultures. Use of living microorganisms for treatment presents several problems, which include (1) the inability of microorganisms to survive under stringent conditions, such as high temperature, low or high pH, etc.; (2) the need for nutrients and other substrates, such as oxygen, nitrogen, phosphorus, etc. for microbial growth, thereby requiring biostimulation; (3) competition from other indigenous organisms that are better adapted to the field conditions, thereby requiring bioaugmentation; (4) generation of biomass, which has to be handled as a by-product; (5) mass transfer limitations, which require mixing due to aggregation, and settling; and (6) slow degradation rates, which severely limit the practicality of microbe-based treatments.

Reduction of sulfate to sulfide (Lens, 1998) requires an organic electron donor molecule, e.g., lactic acid, which is used by the SRBs, such as *Desulfovibrio* and *Desulfuromonas* species, to reduce sulfate to hydrogen sulfide and concomitantly form bicarbonate, which results in an increase in pH (Equation 1). Soluble metal salts react with the sulfide ion *in-situ* to produce insoluble metal sulfides (Equation 2), thereby reducing the metal (M) concentrations in the water and forming black sludge precipitates. Bicarbonate ions react with the protons to form carbon dioxide and water, thus removing acidity from the solution as carbon dioxide gas (Equation 3).



$$3SO_4^{2-} + \text{lactate} \text{ ------------------> } 3H_2S + 6HCO_3^- \quad (1)$$

$$H_2S + M_2^+ \text{ -----------------> } MS(\text{precipitate}) + 2H^+ \quad (2)$$

$$HCO_3^- + H^+ \text{ -----------------> } CO_2 \text{ (gas)} + H_2O \quad (3)$$

Enzymes Involved in Biogenic Sulfate Reduction:

Several enzymatic reactions are known to be involved in sulfate reduction. For example, adenosine 5'-phosphosulfate (APS), which is synthesized from sulfate and adenosine triphosphate (ATP) by the enzyme ATP sulphurylase (Enzyme classification 2.7.7, Table 1), serves as a nucleoside sulfate donor in sulfate reduction. APS is then broken down into sulfite and adenosine monophosphate (AMP) by APS reductase (Enzyme Classification 1.8.99, Table 1), followed by reduction to sulfide by sulfite reductase (Enzyme Classification 1.8.99, Table 1).

Table 1. Listing of Enzymes Involved in Microbial Sulfate Reduction.

No. (Reference)	Classification (Properties)	Reaction
1.7.2.2	Nitrite Reductase Donors: Nitro compounds Acceptors: cytochrome or copper	$R\text{-}NO_2$ -------> R-H Aerobic 3NAD(P)H -------> 3NAD $R\text{-}NO_2$ -------> $R\text{-}NH_2$ Anaerobic 3NAD(P)H -------> 3NAD
1.13.11.18	Sulfur dioxygenase	MS_n -----> M^{++} + S^{2-} ----> S_8 ---> SO_4^{2-} MS_n ------> $S_2O_3^{-}$ -----> SO_4^{2-} Fe^{2+} ---> Fe^{3+}
1.8.99	Sulfite reductases	SO_4^{2-} -----> SO_3^{2-} SO_3^{2-} -----> HS^{-}
1.1.1	Oxidoreductases	R-C-OH ------> $R\text{-}CO_3^{-}$ NADH -----> NAD
2.7.7	Transfers phosphate to OH; Donor: ATP; Acceptor: OH	CH_2OH ---------> CH_2OP ATP -------> ADP

Inhibition of Sulfate Reduction:

Inhibition of biogenic sulfide production is typically attempted using one or more of the following approaches: (1) application of biocides; (2) use of nitrate; and (3) use of nitrite. Biocides that reportedly have been used for inhibiting sulfide-producing bacteria include benzalkonium chloride, glutaraldehyde, formaldehyde, cocodiamine (1-(C_6-C_{18})alkyl-1,3 propane diamine acetate), nitrite salts, and molybdate salts. Their reported mechanisms of action are summarized in Table 2. As the treatment level data in Table 2 indicate, very high levels of these biocides are required to inhibit hydrogen sulfide production (e.g., 50 to 500 parts-per-thousand), making these treatments expensive and environmentally undesirable (Jack and Westlake, 1995).

Table 2. Biocides for Inhibiting Sulfide-Producing or Sulfate Reducing Bacteria.

Biocide	**Chemical Nature and Mechanism of action**	**Minimum Concentration that prevents sulfide production**
Benzalkonium chloride	Quarternary ammonium cationic surfactant; Solubilizes cell membranes, allowing uptake of other antimicrobials	50 mg/L
Glutaraldehyde	Aldehyde; Crosslinks amino and sulfhydryl groups of proteins	500 mg/L
Formaldehyde	Aldehyde; Cross links amino groups of proteins	180 mg/L
Cocodiamine	Cationic surfactant at low pH; acts similarly to benzalkonium chloride	
Nitrite	Sulfite analog; inhibitory of sulfite reductase enzymes	230 mg/L
Molybdate	Sulfate analog; depletes ATP reserves	120 mg/L

Enzymatic Composition for Inhibition of Sulfate Reducers:

The enzymatic composition that was studied in this work (ZymeOut) is comprised of several enzymes prepared by growing a nitrate-reducing sulfide-oxidizing (NR-SO) bacterial culture in a nutrient medium that preferably contains a sulfate salt, an oxidized nitrogenous inorganic salt (e.g., a nitrate salt) and one or more organic salts (e.g., a lactate salt, a citrate salt, and the like), preferably for about 3-5 days. A bacterial growth inhibitor (e.g., a nitrite salt) is then added to the culture in an amount sufficient to substantially arrest bacterial growth in the mixture, after which a sufficient amount of water is removed from the resulting mixture to form a solid composition. Preferably, the majority of the water is removed by reverse osmosis to concentrate the mixture, and then the obtained concentrate is further dried, e.g., in an oven, plate dryer, or rotary drier.

One suitable NR-SO bacterial culture is putative *Campylobacter* sp. strain CVO, which is described in U.S. Patent No. 5,686,293 (Jenneman *et al*. 1997) and reportedly was deposited under the provisions of the Budapest Treaty on June 20, 1995 at the Agricultural Research Service Culture Collection of the United State Department of Agriculture, National Center For Agricultural Utilization Research, formerly known as the Northern Regional Research Laboratory (NRRL), Peoria , Illinois, and was assigned NRRL Accession No. B-21472. Preferably, the bacterial culture has a sulfide oxidizing activity similar to strain CVO. A particularly preferred bacterial strain is a mixed culture comprising *Halothiobacillus*, *Burkholderia*, *Rhizoblum*, *Ensifer* and *Aminobacter* species.

Effect of Enzymatic Composition on Biofilms:

Biofilms are complex mixtures of mixed cultures primarily dominated by sulfate reducers at the solid-biofilm interface, facultative bacteria covering the sulfate reducers and aerobic bacteria at the biofilm-water interface (Wolfaardt et al., 2000). This stratification

protects the sulfate reducers (sulfide generators) from dissolved oxygen as well as biocides, and explains the robustness of these biofilms for sulfide generation even after aggressive treatment with biocides or extensive aeration. The enzymatic composition only inhibits the sulfate reducers and does not impact the facultative and the aerobic layers of the biofilm. This specificity of the enzymes renders it very effective in inhibiting sulfide generation at low concentrations in the aqueous phase. Since the inhibited SRBs are unable to derive adequate metabolic energy through sulfate reduction, they decay naturally, thereby allowing the biofilms to detach from the immersed solid surfaces, since these biofilms are anchored by the SRBs. This is a major benefit of using the enzyme composition, since most of the sulfide generation occurs within these biofilms. As noted earlier, biocides and/or aeration (dissolved oxygen) does not sufficiently penetrate the biofilms to effectively reach the SRB layer, and sulfide generation generally continues or is inhibited temporarily. The enzymatic composition is able to specifically inhibit the metabolic rates of SRBs sufficiently to enable their natural decay rate to exceed their growth rate, resulting in a natural decay of the SRBs followed by the physical sloughing-off of the biofilm from the solid surface.

Experimental Studies:

Preparation of Enzymatic Composition

A mixed bacterial culture was grown in a modified Coleville Synthetic Brine (mCSB), containing about 12 mM sodium sulfate, about 30 mM sodium lactate and about 10 mM sodium nitrate. After the strain CVO had grown for about 4 days, the bacterial growth was terminated by adding about 100 ppm of sodium nitrite, and then passing the mixture through a cellulose acetate reverse osmosis membrane (H1312-075/K/C, Osmonics, Livermore, California) to remove about 98% of the water, leaving behind a concentrated, enzymatically active mixture of culture containing inorganic salts. The so-obtained concentrated mixture was then dried in an oven maintained at about 55°C to form a powder.

The mixed bacterial culture utilized in this Example was deposited with the American Type Culture Collection (ATCC; Manassas, Virginia) on July 10, 2007, under the provisions of the Budapest Treaty, and was assigned ATCC Accession Number PTA-8448.
Culture No. ATCC PTA-8448 was genetically characterized using denaturing gradient electrophoresis (DGGE). The bands listed in Table 3 were observed and identified based on similarity to DNA sequences in the Ribosomal Database Project (RDP). Similarity indices above about 0.9 are considered excellent matches; similarity indices in the range of 0.7 to about 0.8 are considered good, while similarity indices below about 0.6 are considered to be unique sequences. The similarity indices listed in Table 3 range from about 0.861 to 1.00, indicating a very good to excellent match to sequences of the listed genus. Bacteria must constitute about 1-2 percent or more of the total bacterial community to form a visible band. Phylogenic affiliations are presented in Table 4, along with the GenBank Accession No. of each matching sequence.

Testing of Enzymatic Composition in Anaerobic Digesters:

A test was conducted at bench scale using four sealed 5-gallon (about 19 liter) containers. Each 5-gallon container was filled with about 20 pounds (about 9.1 Kg) of waste drywall (calcium sulfate) along with a nutrient source comprising about 4 teaspoons of sugar (about 16 grams), about 2 gallons (about 7.7 liters) of leachate from a landfill (containing SRB) that had

hydrogen sulfide odor issues, and about 1.5 gallons (about 5.8 liters) of water. The leachate resulted in strong active SRB colonies within the containers. Nitrogen gas was continually passed through the containers to eliminate any oxygen that could suppress hydrogen sulfide production and to provide a gas flow in which hydrogen sulfide produced in the containers could be measured. Various levels of the composition of Example 1 were then added to selected containers and the hydrogen sulfide levels were monitored in the nitrogen stream from each of the containers over time (Table 5). As shown in Table 5, a 100 ppm level of the composition of Example 1 controlled SRB growth and lowered hydrogen sulfide production for one day; a 300 ppm level controlled SRB growth and lowered hydrogen sulfide production for 2 to 6 days, while a 10000 ppm level completely stopped all SRB activity, as measured by hydrogen sulfide production, for greater than 90 days. As a comparison, Trosan BK-86 biocide was added to one container with similar results - all SRB activity, as measured by hydrogen sulfide level, was controlled for greater than 30 days at 3000 ppm and greater than 90 days at 10,000 ppm.

Table 3. Bacterial Characterization of Mixed Culture used in preparation of Enzymatic Composition.

Band	Similar Genus	Similarity Index	Donors	Acceptors	Description
1.3	*Halothiobacillus*	0.954	$S_2O_3^{-2}$	nitrate, sulfate	shallow water aerobes
1.4	*Halothiobacillus*	1.00	$S_2O_3^{-2}$	nitrate, sulfate	shallow water aerobes
1.5	*Burkholderia*	0.861	organics	oxygen	freshwater soil
1.6	*Rhizoblum*	1.00	complex organics	oxygen	contains the formerly valid genera *Allorrhyzoblum* and *Agrobacterium*
1.6	*Ensifer*	1.00			
1.6	*Aminobacter*	1.00			

Table 4. Phylogenic Affiliations of Mixed Culture used in preparation of Enzymatic Composition.

Band	Similar Genus	Similarity Index	GenBank Accession No.
1.3	*Halothiobacillus*	0.954	AF173169; AY096035; AY487255
1.4	*Halothiobacillus*	1.00	AF173169; AY096035; AY487255
1.5	*Burkholderia*	0.861	AY497470
1.6	*Rhizoblum*	1.00	X74915
1.6	*Ensifer*	1.00	Z78204
1.6	*Aminobacter*	1.00	AJ011760

Table 5. Effect of Various Levels of the Composition of Example 1 on Hydrogen Sulfide Generation in Anaerobic Reactors

Level of Enzymatic Composition (ppm)	H_2S Control Period (days)
100	<1
300	2-6
3000	>30
10000	>90

<u>Field Testing of Enzymatic Composition in Beet Sugar Wastewaters:</u>

The enzymatic composition of Example 1 was field tested in a lagoon receiving wastewater from a Beet Sugar factory in Colorado, USA. Wastewater was generated during beet washing, transportation and beet sugar production. The wastewater flowed into a nearby lagoon for treatment and eventually flowed into a local creek. The main issue was strong smell of hydrogen sulfide near the lagoon during the production season and precipitation of black sludge in the lagoon requiring periodic dredging.

The enzymatic composition was added to the wastewater as it flowed into the lagoon at an average flow rate of about 4-10 gallons per minute (15 – 38 L/min). The enzymatic composition was added to mix with the wastewater resulting in a enzymatic concentration in the range of 10-25 ppm in the wastewater. It was observed that after this addition had been continued for about 20 days, the smell of hydrogen sulfide became less and after 60 days, there was no detectable hydrogen sulfide smell near the lagoon. The lagoon water also became very clear and there was no black precipitate settling at the bottom.

<u>Toxicity Evaluation of Enzymatic Composition:</u>

A 450,000 ppm aqueous solution of the so-formed powdered enzymatically active composition was tested by an independent laboratory for acute oral toxicity, acute skin irritation, and acute eye irritation according to standard U.S. Environmental Protection Agency (EPA) Health Effects Guidelines, OPPTS 870.1100, OPPTS 870.2500, and OPPTS 870.240, respectively. Results were as follows: Oral LD50 in Rats: > 5000mg/kg (considered non-toxic under the protocol); Primary Dermal Irritation in Rabbits: Not a skin irritant; Acute Eye Irritation in Rabbits: Minor eye irritant.

<u>Economics of Enzymatic Composition Application:</u>

The average cost of using the enzymatic composition commercially has been found to be in the range of $1-20/1,000 gallon of wastewater treated. If the wastewater is recycled and re-used within the plant, lower application costs result from the presence of the enzymes in the recycled water.

<u>Conclusions:</u>

An enzymatic composition was prepared from a mixed bacterial culture and was assigned ATCC Accession Number PTA-8448. This enzymatic composition has been found to selectively

inhibit growth of sulfate reducing bacteria (SRBs) which are primarily responsible for sulfide generation in wastewater, resulting in air emission of hydrogen sulfide, growth of fungi in the water and increased sulfide corrosion. Through inhibition of SRB growth, the natural metabolic decay rate of SRBs exceeds their growth rate, resulting in elimination of SRBs from the biofilms and eventual sloughing-off of the biofilms from the immersed solid surfaces. This eliminates the generation of sulfide within the biofilms that are otherwise fairly well protected from biocides and dissolved oxygen. Laboratory and field studies conducted using the enzymatic composition have shown that the enzyme mixture, dissolved in water, can be easily and economically added to significantly reduce sulfide generation in sugar production wastewaters.

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