

A continuous alcohol oxidase bioreactor for regenerative life support

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The development of a composite fixed-bed continuous-flow immobilized alcohol oxidase bioreactor is described for use in the production of drinking water from humidity condensates collected aboard manned spacecraft. The principal organic contaminants of this wastewater stream are low molecular weight alcohols and organic acids. Alcohol oxidase catalyzes the oxidation of short chain primary alcohols to aldehydes at ambient temperatures. The resulting aldehydes are subjected to a second oxidation via the action of a heterogeneous noble metal catalyst to form carboxylic acids which are removed by sorption onto ion exchange resins. Inactivation of the enzyme by hydrogen peroxide has been significantly reduced by coimmobilization of metallic platinum onto the support. The results of ground-based testing using model contaminant solutions and true humidity condensates are reported.

Keywords: Alcohol oxidase; bioreactor; ethanol; platinum; hydrogen peroxide

Introduction

Closed-loop regenerative life-support systems are needed to maintain nominal environmental conditions required to sustain life for long-duration manned missions in space such as a space station, a Mars sprint, or a permanently manned lunar base. Water purification subsystems are required for the provision of an uninterrupted supply for drinking, food preparation, plant growth, animal husbandry, personal hygiene, laundry, and scientific experimentation. Typical aqueous wastes occurring in closed environments such as spacecraft or planetary outposts will include crew hygiene waters arising from showers, oral hygiene, and handwash facilities; laundry water, humidity condensates, and plant transpiration waters collected from the cabin atmosphere or plant growth chambers via condensing heat exchangers; and urine.¹ Additional wastewaters may be generated by animals raised for food or scientific purposes, and as the result of chemical, biological, and materials science experimentation.

Spacecraft water reclamation technologies currently in use for treatment of humidity condensate, laundry, and hygiene waters utilize either an ion exchange/sorption technology known as multifiltration (MF) or reverse osmosis (RO).^{2,3} Waters emanating from these processes are then

polished by heterogeneous catalytic oxidation of the low MW hydrophilic organics which are not efficiently separated by sorbent or membrane-based processes.⁴ Urine is treated using similar methods following a preliminary distillation separation by Vapor Compression Distillation (VCD).⁵

As shown in *Table 1*, humidity condensates contain significant levels of short-chain alcohols and diols.⁶ While heterogeneous catalytic oxidation of these contaminants has proven very effective at 125°C, simpler and less energy-intensive means for their destruction are desired. The use of enzymes offers a convenient ambient-temperature means for the catalysis of reactions which transform organics into ionic products which can be removed by sorption. The development and testing of an immobilized urease bioreactor to convert urea to more efficiently sorbed ionic species for use in the purification of urine distillate and reclaimed hygiene waters has been reported.⁷ The capability for catalytic oxidation of low MW alcohols at ambient spacecraft cabin temperature has been embodied in the development and testing of a continuous-flow immobilized alcohol oxidase bioreactor for the final polishing of the effluent from a MF train for the production of potable water from humidity condensate.⁸⁻¹¹

Oxidases are a group of enzymes with strong potential for biotechnological applications. Unlike most enzymes which catalyze oxidation-reduction reactions, the co-factor required for enzymatic activity, flavine adenine dinucleotide (FAD), is incorporated into the molecular structure of the biocatalyst and regenerated as part of the overall reac-

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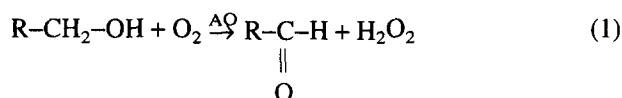
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Table 1 Major organic contaminant concentrations (mg l⁻¹) for Shuttle Orbiter humidity condensates

Contaminant	STS-47	STS-45
Ethanol	65.7	12.2
1,2-propanediol	34.2	28.0
2-propanol	26.0	7.50
ε-caprolactam	21.2	6.37
Acetic acid	11.4	8.47
Formic acid	4.41	3.50
Formaldehyde	3.16	4.16
Methanol	2.78	0.843
Propionic acid	2.44	2.19

tion.¹²⁻¹⁴ This feature has made possible the widespread application of immobilized oxidase enzymes in amperometric, fluorometric, and chemiluminescent biosensors.¹⁵

Alcohol oxidase (AO) catalyzes the reaction of primary alcohols with oxygen to form the corresponding aldehyde and hydrogen peroxide.



Methylotrophic yeasts such as *Pichia pastoris* or *Hansenula polymorpha*, from which the enzyme is most commonly isolated, segregate the enzyme within membrane-bound peroxisomes to prevent damage from hydrogen peroxide.^{16,17} The cell is further protected by a variety of enzymes including catalase, peroxidases, and superoxide dismutase which catalyze the decomposition of hydrogen peroxide or reactive species derived from hydrogen peroxide.

Bioreactors containing whole cells of *P. pastoris* have been used for the AO-catalyzed production of a variety of straight-chain and aromatic aldehydes including acetaldehyde and benzaldehyde in both aqueous and organic media and in the vapor phase.¹⁸⁻²² Mixtures of short-chain alcohols and alcohol oxidase have been used as a source of hydrogen peroxide in a process for the removal of aqueous aromatic alcohols and aromatic amines.²³ In this process, the aromatics are oxidized by hydrogen peroxide in reactions catalyzed by horseradish peroxidase. Immobilized AO bioreactors have been studied for the production of hydrogen peroxide and acetaldehyde in aqueous, organic, and vapor-phase reactions.^{24,25} All of these systems were found to be vulnerable to inactivation by hydrogen peroxide.

An effective means for mitigation of the loss of enzymatic activity caused by hydrogen peroxide has been identified through a series of continuous plug-flow reactor tests using small-volume packed beds of immobilized AO. The coimmobilization of catalase and horseradish peroxidase, two enzymes known to catalyze the decomposition of hydrogen peroxide, were compared to the results achieved through the predeposition of metallic catalysts. Based upon this work, metallic platinum was selected as the preferred catalyst for the decomposition of hydrogen peroxide to oxygen and water.

Further work was conducted to determine the desired platinum loading for prolonged AO bed life in a continuous-

flow bioreactor with ethanol levels typical of spacecraft humidity condensate as the influent. Building upon these results, a breadboard-composite continuous-flow AO bioreactor was designed, incorporating additional media, to provide for the oxidation of ethanol and other low MW primary alcohols to the corresponding acids and for their subsequent removal using ion-exchange resins. A platinum catalyst was incorporated for the ambient-temperature (25°C) oxidation of the aldehydes produced by the AO bed to the corresponding carboxylic acids. Additional media were identified for preconditioning of the influent stream to protect against potential influx of ionic species. Two breadboard-composite immobilized-enzyme bioreactors were fabricated. One was tested in our laboratory. The second was delivered to the George C. Marshall Space Flight Center (MSFC) where it was installed at the outlet of the potable water MF train during Stage 7 Core Module Integrated Facility (CMIF) water recovery tests.

Materials and methods

Enzymes and reagents

Alcohol oxidase (EC. 1.1.3.13) from *P. pastoris* yeast was acquired from Provista Corporation (Bartlesville, OK). Bovine erythrocyte superoxide dismutase (EC. 1.15.1.1), bovine liver catalase (EC. 1.11.1.6), and horseradish peroxidase (EC. 1.11.1.7) were obtained from Sigma Chemical Company (St. Louis, MO). Celite Biocatalyst Carrier R-648, a controlled porosity diatomaceous earth, and 580-26 activated carbon were purchased from Johns-Manville (Denver, CO) and Barnebey Cheney (Columbus, OH), respectively. Chloroplatinic acid, palladium(II) chloride, silver nitrate, copper(II) chloride, and tin(II) chloride were purchased from Aldrich (Milwaukee, WI). IRA-68 weak-base anion-exchange resin and IRN-150, a 1:1 mixture of strong-acid cation-exchange resin and strong-base anion-exchange resin on an equivalent basis, were purchased from Rohm and Haas (Philadelphia, PA). Ethanol, acetaldehyde, 2-propanol, and acetic acid were acquired from VWR Scientific (Portland, OR). Bottled hydrogen, nitrogen, and medical grade oxygen were purchased from Air Products and Chemicals (Allentown, PA). Aqueous ethanol standards were prepared in deionized water.

Enzyme immobilization

The diatomaceous earth support was prepared for enzyme immobilization using the titanium activation technique²⁶ with ethylene diamine and glutaraldehyde bridging groups as illustrated in Figure 1. A batch of 100 g diatomaceous earth was mixed with 200 ml of 15% TiCl₄ solution in 10% HCl. The mixture was taken to dryness, washed with methanol, and maintained at 45°C for two h in 125 ml of 1.25% ethylenediamine in CCl₄. The CCl₄ was decanted and the support was rinsed first with methanol and then water. The diatomaceous earth was then suspended in 200 ml of 5% glutaraldehyde dissolved in a borate buffer pH 9, transferred into a closed plastic container, and mixed overnight on a roller mill. The supernatant was decanted and the support washed with water and then air dried. In this form, the enzyme immobilization support can be stored in the dry state for prolonged periods.

The enzymes were subsequently immobilized by Schiff-base formation between an amino terminus or lysine residue of the enzyme and the free aldehyde group linked to the support.²⁷ The aqueous enzyme preparations were added directly to the activated supports as received from the suppliers. The mixtures were allowed to stand at 25°C for 24-48 h. Coimmobilizations of AO

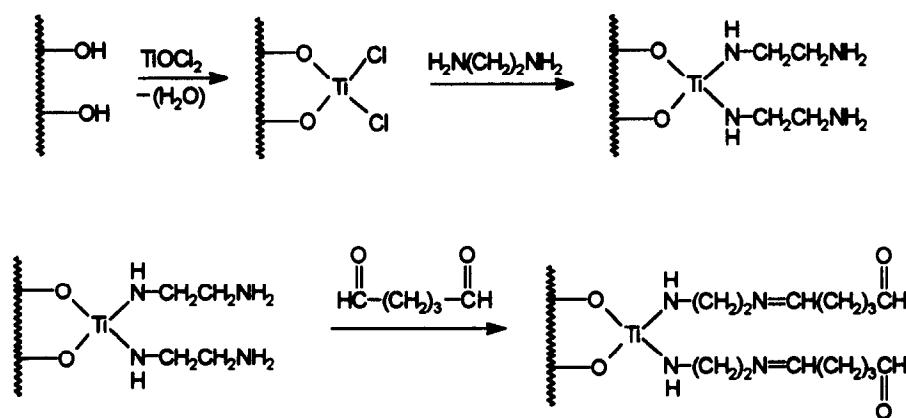


Figure 1 Support preparation for enzyme immobilization by titanium linkage to silanol groups on diatomaceous earth

with catalase, horseradish peroxidase, and superoxide dismutase were conducted by separately immobilizing each enzyme, followed by mixing of the two preparations.

Immobilized enzyme supports with copper, platinum, palladium, silver, or tin loadings were prepared by direct impregnation of copper(II) chloride, chloroplatinic acid, palladium(II) chloride, silver nitrate, and stannous chloride from acidic solutions onto diatomaceous earth prior to immobilization of the enzyme. The metals were then reduced at 400°C in a hydrogen stream. Metallic loadings were determined by weight.

Catalyst preparation

The 2% platinum catalyst was prepared by direct impregnation of chloroplatinic acid from an acidic solution onto activated carbon, followed by drying and reduction at 400°C in a hydrogen atmosphere. Platinum loadings were determined by weight.

Small-column continuous-flow tests

Small-column continuous-flow tests were performed using small-volume ($\approx 5 \text{ cm}^3$) packed beds of the immobilized alcohol oxidase and related media confined within borosilicate glass tubes of 1 cm internal diameter. This diameter was sufficiently small to ensure length-to-diameter ratios (L/D) of at least two. The test apparatus is illustrated schematically in Figure 2. The packed beds were held in place using glass wool end plugs and capped at the ends with septa fitted with tubing adapters. The packed beds were positioned vertically with flow in the upward direction. Ambient-temperature (25°C) challenge solutions containing varying ethanol concentrations were pumped at a constant flow rate through the enzyme beds using a Barnart Model 7553-60 pump. Challenge solution flow

rates were monitored and controlled to provide the desired space velocities. Cumulative flow through the beds was recorded as the ratio of total flow in l to packed bed volume in cm^3 ($l \text{ cm}^{-3}$). Continuous-flow tests were generally conducted on a 24 h day⁻¹ basis, Monday through Friday, and discontinued over the weekends. Oxygen saturation for the influent to the beds was provided by sparging with medical grade O_2 twice daily.

Instrumentation

Analyses for total organic carbon (TOC) were performed using an Astro International Model 2001 carbon analyzer based upon UV-persulfate oxidation with infrared-absorption detection. Ethanol, methanol, acetaldehyde, 2-propanol, and acetone were determined using a Hewlett-Packard Model 5710A gas chromatograph with a 1% SP-1000 on Carbowax B column (Alltech, Deerfield, IL), nitrogen carrier gas, and flame-ionization detection.

Results and discussion

An absolute requirement for the development of a continuous-flow alcohol bioreactor was a means to mitigate enzyme deactivation by the hydrogen peroxide by-product of ethanol oxidation. Several options are available to promote the decomposition of peroxide to water and molecular oxygen.



Hang *et al.*²⁵ have suggested the use of coimmobilized catalase or peroxidase for this purpose. The decomposition of peroxide is also catalyzed at metallic surfaces such as platinum, osmium, iridium,²⁸ silver,²⁹ and manganese.³⁰ The decomposition can be promoted by various metal oxides,³¹⁻³⁴ including MnO_2 , Co_2O_3 , Ru_2O_3 , CuO , NiO , and Ag_2O . The free-radical mechanism proposed by Weiss³⁵ for this process is given in Eq. (3-8) where Σ represents the catalytically active surface site.

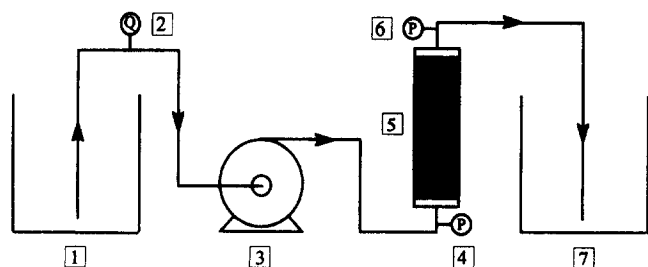
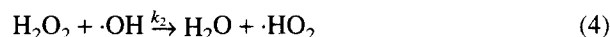
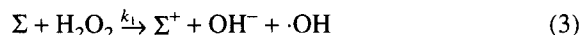


Figure 2 Small-column continuous-flow test apparatus. Influent reservoir, 1; flow meter, 2; pump, 3; inlet pressure gauge, 4; immobilized-enzyme fixed bed, 5; outlet pressure gauge, 6; effluent reservoir, 7

The formation of superoxide during peroxide decomposition has been confirmed on both metal oxides and supported metal surfaces by electron-spin resonance studies.^{36,37} Both the peroxy radical and the hydroxyl radical are extremely reactive, suggesting that production of these species can possibly assist the immobilized enzyme in the oxidation of ethanol.

Small continuous-flow test columns were prepared using bienzyme mixed beds composed of 8 g AO mixed with 1.2 g of horseradish peroxidase, catalase, and superoxide dismutase, respectively. In addition, immobilized AO columns were prepared from a series of supports which had been previously impregnated with 1% metallic loadings of platinum, palladium, copper, tin, and silver, respectively. Continuous-flow tests were conducted such that each of these columns was challenged with 16 mg l⁻¹ aqueous ethanol and compared to the performance of an immobilized AO control. The coimmobilization of platinum yielded the most prolonged AO bed life. Among the metals, the general relation was Pt > Cu > Pd > Sn. Tin showed marginal improvement over the bed life of the AO control. Coimmobilization of silver resulted in the complete inactivation of the enzyme. The bienzyme small columns showed only marginal improvement in bed life as compared to the AO control column. The very high level of life extension resulting from the impregnation of the AO support with platinum is shown in Figure 3 which compares acetaldehyde production as a function of cumulative influent flow for the bienzyme and the platinum-impregnated AO bioreactors at a processing rate of 2 ml min⁻¹. Impregnation of the support with 1% platinum resulted in a half-life of approximately 275 h for the enzyme. No significant improvement was found when hydrogen-peroxide decomposing enzymes were simultaneously coimmobilized from aqueous mixtures with alcohol oxidase.

In the optimization of the AO bioreactor life, it is important to maintain an acceptable level of enzymatic activity. Given that coimmobilized enzymes and impregnated metallic species must necessarily occupy surface sites on the support which otherwise might be occupied by AO, it is important to achieve a balance between the loading of AO

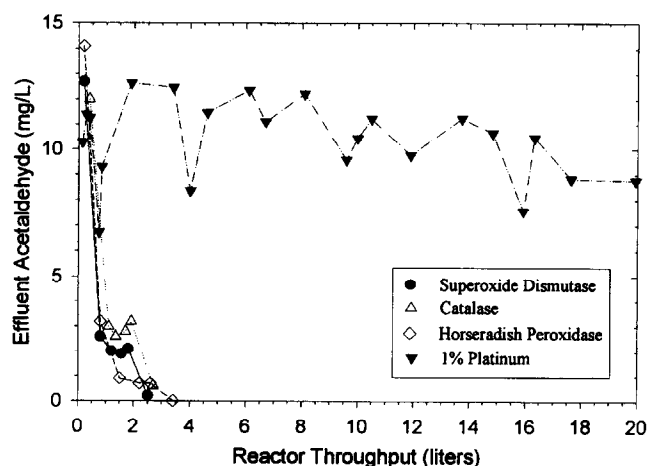


Figure 3 Comparison of coimmobilized enzymes and metallic platinum for prevention of H₂O₂ deactivation of alcohol oxidase

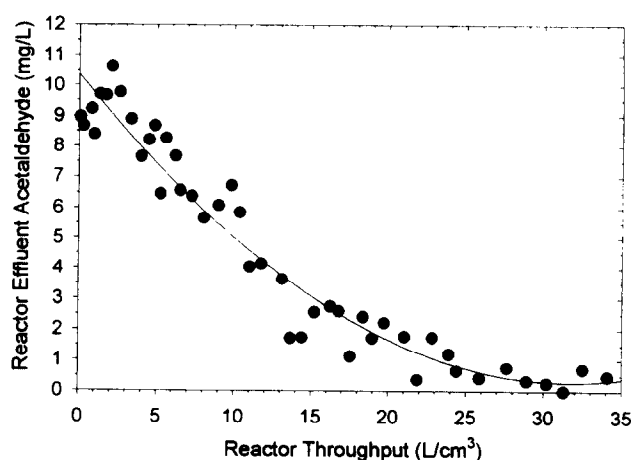


Figure 4 Effluent acetaldehyde with 16 mg l⁻¹ ethanol influent

and other species. This was investigated in a series of small-column continuous-flow tests using AO supports with a range of platinum loadings between 0.5–4.5%. Small-column continuous-flow tests were conducted using 16 mg l⁻¹ aqueous ethanol as influent to determine the preferred level of metallic loading for the optimal enzymatic activity and maximum achievable extension of bed life. Effluent ethanol and acetaldehyde levels were monitored. The best results were achieved using a 2% platinum loading. These results are illustrated in Figures 4 and 5 which present effluent acetaldehyde and ethanol concentrations, respectively, for a 6.7 cm³ AO–2% Pt bed taken to near-complete enzymatic inactivation at a processing rate of 2 ml min⁻¹. The diminishing acetaldehyde and rising ethanol concentrations in the bioreactor effluent indicates the progressive loss of activity with an approximate half-life of 1,500 h.

At 25°C, deionized water in equilibrium with the atmosphere will have a dissolved O₂ concentration of approximately 8 mg l⁻¹. For efficient-reaction kinetics, it is desirable to have a substantial excess of dissolved O₂ to promote zero-order kinetics with respect to the oxidant. In order for the immobilized enzyme bioreactor to accommodate higher-influent alcohol loads, a hollow-fiber membrane saturator was evaluated as a means to provide excess O₂. A cylindrical polycarbonate housing 2.54 cm in diameter and 22 cm long was constructed as an O₂ saturator consisting of 50 nonporous polydimethylsiloxane hollow fibers of 0.03 cm internal diameter and 0.017 cm wall thickness. The influent alcoholic stream was routed through the interior of the hollow-fiber membranes. Through a side inlet, O₂ at pressures up to 300 kPa was applied to the cartridge from an external-pressurized oxygen cylinder, thereby providing dissolved O₂ concentrations up to 120 mg l⁻¹. A dead volume of 10 cm³ was provided at the inlet to allow equilibration of the influent stream with the elevated external pO₂. A 15 cm³ packed bed of immobilized AO followed the dead volume. The combined saturator/bioreactor was then challenged with an influent containing 32 mg l⁻¹ ethanol in deionized water at a flow rate of 2.0 cm³ min⁻¹. The reactor processed 13 l of this solution and produced consistent effluent ethanol concentrations of ≤0.5 mg l⁻¹.

Information gained during the preliminary phase of test-

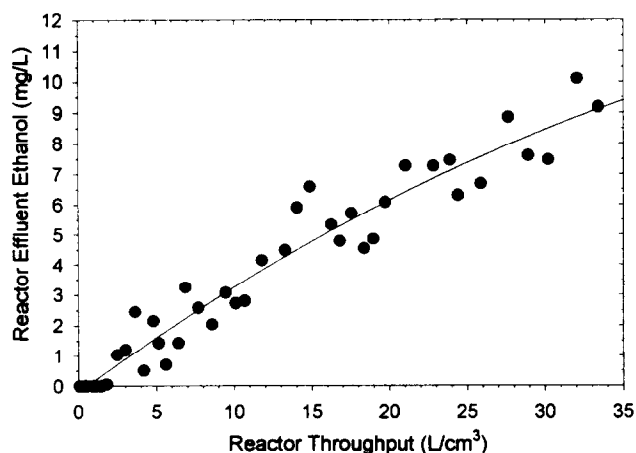


Figure 5 Effluent ethanol with 16 mg l⁻¹ ethanol influent

ing was then used in the design of a breadboard-composite immobilized enzyme bioreactor for use in posttreatment of the effluent from a multifiltration train used in the production of potable water from humidity condensate according to the then-current potable water processor design for Space Station *Freedom*. The bioreactor was sized to process 25 ml min⁻¹ flow from the potable water processor, then undergoing Phase III CMIF water recovery testing at MSFC. Additional operational constraints included a maximum system pressure of 377 kPa and a maximum head loss across the bioreactor of 34.5 kPa. Given the internal diameter, the minimum allowable length to diameter (L D⁻¹) ratio required a minimum bed volume of 60 cm³. The resulting design is illustrated schematically in Figure 6. The 1090 cm³ composite AO bioreactor consists of a sequence of six separate media segregated into discrete layers and separated by polypropylene screen spacers. In sequential order from inflow face to outflow face, these include: IRN-150 mixed anion-cation exchange resin (60 cm³), immobilized AO-Pt (200 cm³), supported platinum catalyst (360 cm³), weak-acid ion-exchange resin (350 cm³), mixed resin (60 cm³), and microbial check-valve (MCV) resin (60 cm³). The mixed resins at the inflow face serve to protect the immobilized enzyme and heterogeneous catalyst beds from inactivation by aqueous salts in the unlikely event of a process upset in the upstream multifiltration train. In the AO bed, low MW straight-chain alcohols are oxidized to aldehydes and the decomposition of hydrogen peroxide to elemental oxygen and water is promoted. In the next bed, aldehydes are oxidized to carboxylic acids by the 1% platinum catalyst. These weak organic acids (pK_a ≈ 4) are then removed by weak-acid anion-exchange resin. The downstream-mixed resin bed serves to guard against a potentially dam-



Figure 6 Block diagram of composite assembly incorporating AO bioreactor. Inlet, 1; mixed resin, 2; AO/Pt, 3; Pt/C, 4; weak-base resin, 5; mixed resin, 6; MCV[®] resin, 7; outlet, 8

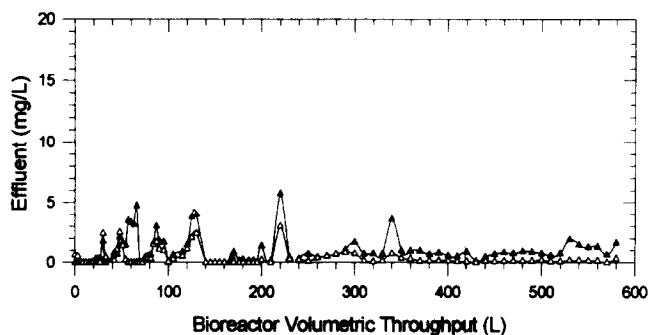


Figure 7 Breadboard bioreactor effluent ethanol (▲) and acetaldehyde (△)

aging influx of elemental iodine under backflow conditions. The final bed of MCV resin serves to add elemental iodine as a biocide for the maintenance of product-water potability.

This design was incorporated into a cartridge containing 1090 cm³ of media. Two identical breadboard bioreactors were fabricated within polycarbonate, 5 cm³ × 75 cm³ cylindrical housings using polypropylene lip seals to separate media layers and stainless-steel quick disconnection fittings at inflow and outflow faces to facilitate integration into the CMIF potable water processor at MSFC. One unit was tested in our laboratory at the design processing rate of 25 ml min⁻¹ under continuous-flow conditions with an influent consisting of: 16 mg l⁻¹ ethanol, 8 mg l⁻¹ methanol, and 1.6 mg l⁻¹ 2-propanol in deionized water. This corresponds to an influent challenge of approximately 15 mg l⁻¹ as total organic carbon (TOC). The test was conducted for a total of approximately 600 l cumulative flow. This was limited by the time constraints of the project. As expected, the unit exhibited little effect on 2-propanol. The test results showing effluent ethanol and acetaldehyde, and effluent methanol and TOC, are given in Figures 7 and 8, respectively. Anomalous high effluent concentrations are attributed to transients during bioreactor start-up following weekend shutdowns. Exclusive of these anomalies, the effluent concentrations of methanol (0.5 mg l⁻¹), ethanol (1.3 mg l⁻¹), acetaldehyde (0.2 mg l⁻¹), 2-propanol (1.0 mg l⁻¹), and TOC (1.8 mg l⁻¹) at the conclusion of the test were indicative of adequate bioreactor performance. The bioreactor was not intended to treat 2-propanol, and the data indicate only a partial ability to do so.

The second of these units was tested at MSFC during

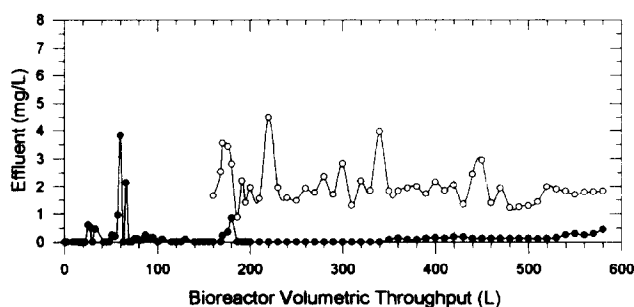


Figure 8 Breadboard bioreactor effluent methanol (●) and TOC (○)

Table 2 Breadboard AO bioreactor test results (mg l⁻¹) of Core Module Integrated Facility

Day	TOC in	TOC out	EtOH in	EtOH out	MeOH in	MeOH out
1	—	—	—	—	—	—
2	7.2	1.22	3.91	<0.1	5.50	0.98
3	4.92	0.86	2.66	<0.1	<0.41	<0.41
4	5.61	0.72	2.76	<0.1	0.70	<0.41
5	5.72	0.66	2.52	<0.1	0.83	<0.41
6	5.74	0.93	1.85	<0.1	<0.41	<0.41
7	5.10	<1.0	0.90	<0.1	0.67	<0.41
8	—	—	—	—	—	—
9	4.51	<1.0	0.61	<0.1	1.33	<0.41
10	—	1.47	—	<0.1	—	<0.41
11	3.86	1.30	0.30	<0.1	<0.41	<0.41

Stage 7 of the Phase III CMIF Water Recovery Tests. The unit was integrated into the potable-water processor downstream of the multifiltration adsorption train. An immobilized urease bioreactor⁷ and a hollow-fiber membrane-based O₂ saturator were placed into the system immediately upstream of the AO bioreactor. The oxygen saturator, operating at 300 kPa, was designed to add a nominal 120 mg l⁻¹ O₂ to the bioreactor influent. The contaminated water stream influent to the potable water processor consisted of humidity condensate collected within the End-Use Equipment Facility (EEF), a sealed chamber in which crewmen exercised on a rotating schedule. Humidity condensate was collected using a condensing heat exchanger. The composite AO bioreactors were tested for 11 days. The test results are summarized in Table 2. While TOC levels influent to the multifiltration train ranged between 18.1–35.9 mg l⁻¹, bioreactor influent levels were substantially less (3.86–7.2 mg l⁻¹), indicating partial sorption of organics by activated carbons and nonfunctionalized resins upstream of the immobilized enzymes. AO bioreactor effluent TOC values ranged between 0.66–1.22 mg l⁻¹. With one exception, ethanol, methanol, and 2-propanol concentrations were below their respective lower limits of detection. On Day 2, an effluent of 0.98 mg l⁻¹ methanol was observed.

The technical feasibility of a continuous-flow fixed-bed bioreactor utilizing alcohol oxidase in conjunction with a heterogeneous catalyst and sorption media for the removal of low MW primary alcohols from humidity condensate has been demonstrated. The predeposition of platinum onto the diatomaceous earth enzyme support for the destruction of the hydrogen peroxide reaction by-product has sufficiently prolonged enzymatic activity to make the use of a continuous flow AO bioreactor practical. The ability of the reactor to process alcohols in wastewater is limited by the dissolved O₂ content of the influent. This limitation is overcome by oxygenation of the influent stream using a hollow-fiber membrane saturator. Using this device, the successful processing of higher influent organic loads by equilibration of the influent with above-ambient oxygen partial pressures has been demonstrated.

In the current configuration, dependent upon the composition of the influent stream, the useful life of the bioreactor may be limited by the bed size of expendable media particularly the ion-exchange resins. In ground-based systems,

this limitation can be easily overcome using conventional ion-exchange resin regeneration techniques. Regeneration of resins in spacecraft, however, can be extremely problematic due to the containment requirement for strongly acidic and basic regenerants and the resultant highly contaminated brines. To achieve an optimally designed nonregenerable fixed-bed system, a more thorough characterization of the influent concentrations of all relevant contaminants which will be processed by the reactor must be undertaken.

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