

# Treatment of High-Strength Wastewater from the Sugar-Sweetened Beverage Industry by an Alcoholic Fermentation Process

Raúl N. Comelli,<sup>†</sup> Lisandro G. Seluy,<sup>†,‡</sup> Ignacio E. Grossmann,<sup>§</sup> and Miguel A. Isla<sup>\*,†,‡</sup>

<sup>†</sup>Departamento de Medio Ambiente de la Facultad de Ingeniería y Ciencias Hídricas (FICH), Universidad Nacional del Litoral y Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Ciudad Universitaria CC 242 Paraje El Pozo, 3000 Santa Fe, Argentina

<sup>‡</sup>Instituto de Desarrollo Tecnológico para la Industria Química (INTEC), General Güemes 3450, 3000 Santa Fe, Argentina

<sup>§</sup>Chemical Engineering Department, Carnegie Mellon University, 5000 Forbes Avenue, Pittsburgh, Pennsylvania 15213, United States

**ABSTRACT:** Certain wastewaters from the nonalcoholic sugar-sweetened beverage industry, particularly those discarded because of deficient bottling processes or those returned from the market because of quality constraints, exhibit chemical oxygen demand (COD) levels as high as 135000 mg of O<sub>2</sub>/L because of their sugar content: 60–119 g/L, depending on the beverage. Thus, treating such wastewaters before discharging them into the environment involves high investment and operating costs. Therefore, any treatment process capable of transforming the sugars into other compounds that are easy to separate from the treated wastewater, such as ethanol or CO<sub>2</sub>, should be regarded as an interesting alternative. In this work, a process that comprises an alcoholic fermentation of sugar-sweetened beverage industry wastewaters followed by ethanol and biomass separation and subsequent aerobic propagation of yeast was developed, and its technical feasibility was studied. The proposed process was found to allow 98% of the COD to be depleted.

## 1. INTRODUCTION

The nonalcoholic sugar-sweetened beverage industry produces traditional beverages (e.g., soft drinks) and “new wave” beverages, such as fortified/functional (FF) drinks containing ingredients such as herbs, vitamins, minerals, amino acids, raw fruits, and vegetables. FF beverages are often claimed to provide specific benefits, such as heart and joint health improvement, immunity and digestion improvement, and energy boosts.<sup>1–3</sup> Examples of FF drinks are enhanced waters, super fruit juices (concentrates, ready to drink or in powdered form), sports and performance drinks, and energy and herb drinks, among others.

This industry produces approximately 4350 million L/year of soft drinks in Argentina.<sup>4</sup> A portion of the beverages produced (2–5%) is discarded during the bottling process because of quality policies or returned from the market because of a lack of gas content or an exceeded expiration date. The sugar content of these beverages (60–150 g/L) confers on the resulting effluents a high chemical oxygen demand (COD), which can reach levels of approximately 150000 mg of O<sub>2</sub>/L. Therefore, the effluents must be treated prior to their discharge into the environment. Conventional treatments involve anaerobic processes with high residence times.<sup>5–8</sup> Moreover, the seasonal nature of the production and consumption of nonalcoholic beverages and the low rate of degradation of the anaerobic process make it necessary to store large volumes of the effluents to avoid saturation of treatment plants during periods of high production.

In this work, an alternative treatment process for these effluents is proposed. This process comprises the alcoholic fermentation of the sugars contained in the wastewater, separation of the biomass and ethanol produced, and finally removal of glycerol generated during alcoholic fermentation through an aerobic fermentation using a yeast. A flowchart of the process is shown in Figure 1. The main steps of the process are

the following: (1) collection and storage of high-strength nonalcoholic sugar-sweetened beverage industry wastewaters, (2) batch alcoholic fermentation, (3) yeast separation, (4) holding in a storage tank, (5) ethanol removal by continuous distillation, (6) continuous rectification to obtain an ethanol concentration of 92% (w/w), (7) cooling, (8) holding in a storage tank, (9) batch aerobic propagation of an adapted glycerol-growing *S. cerevisiae* strain, and (10) yeast separation.

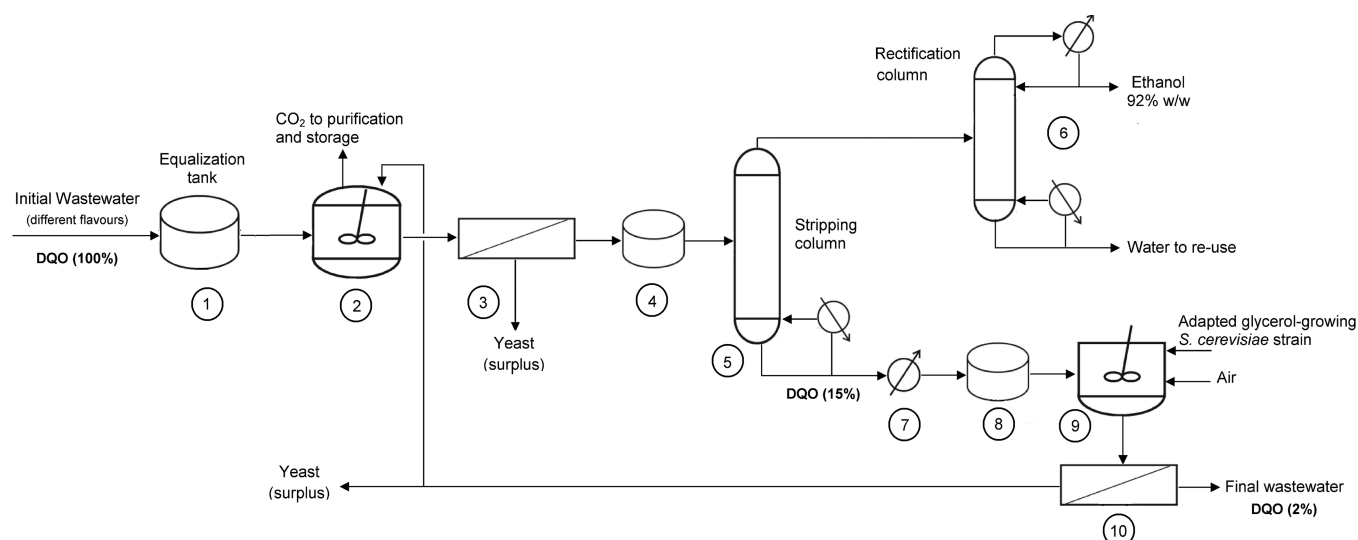
The proposed process is based on the same principle as biological treatment processes: the conversion of dissolved organic matter into compounds that can be easily removed from the medium. In conventional processes, these compounds are gases, which separate spontaneously, and biomass, which can be separated by decantation or filtration. In addition to gases and biomass, the proposed process yields another compound, ethanol, that can be easily separated by distillation. Ethanol is produced by yeast-mediated fermentation of the sugars, mainly sucrose and/or high-fructose corn syrup, contained in the sugar-sweetened beverages.

The ability of the yeast *Saccharomyces cerevisiae* to ferment the sugars in several nonalcoholic sugar-sweetened beverages produced by worldwide leading brands was evaluated in this work. Fermentation assays were performed on enhanced water, fruit juices, sports and energy drinks, and a mixture of soft drinks. The concentrations of biomass, sugars, and ethanol were monitored over time, and the production of other fermentation products, such as carbon dioxide and glycerol, was also measured. Once the fermentation was completed, biomass and ethanol

**Received:** February 10, 2015

**Revised:** June 9, 2015

**Accepted:** July 21, 2015



**Figure 1.** Flowchart of the treatment process of certain sugar-sweetened beverage effluents to reduce the organic load while producing ethanol, CO<sub>2</sub>, and biomass.

were removed by filtration and distillation, respectively, and the residual COD was determined. Glycerol is the principal coproduct of the alcoholic fermentation of sugars after ethanol and CO<sub>2</sub> and remains in the medium after distillation. An attractive strategy based on an aerobic process that uses an adapted yeast strain as an inoculum for the removal of the glycerol produced was also studied.

## 2. MATERIALS AND METHODS

**2.1. Strain and Culture Media.** A commercial yeast strain, *Saccharomyces cerevisiae* var. Windsor (Lallemand Brewing Co., Felixstowe, UK), was used for all assays. The yeast was maintained on YPD solid medium (5 g/L yeast extract, 5 g/L peptone, and 20 g/L D-glucose supplemented with agar–agar at 15 g/L as a solidifying agent) and stored at 4 °C. The culture was transferred to fresh medium monthly. To obtain a strain with improved capability of growing on glycerol, sequential subcultivations (up to 10 times) on fresh medium containing 25 g/L glycerol as the sole carbon source (YPGly) in agitated flasks under aerobic conditions were carried out as described by Ochoa-Estopier et al.<sup>9</sup> Before being used as an inoculum for fermentation, the initial yeast culture was propagated aerobically on YPD or YPGly medium and then separated by centrifugation.

Various media were used in the fermentation assays, including individual functional drinks (enhanced water, fruit juices, energy drinks, and sports drinks), a mixture of soft drinks (cola, orange, and lemon–lime), and synthetic media containing 100 g/L sucrose, used as a control. All beverages were purchased in a local market.

**2.2. Fermentation Conditions.** Samples of the beverages (300 mL) were fermented in batch mode in 500 mL glass flasks under anaerobic conditions at a constant temperature of 30 °C. The experiments were performed in triplicate. Following the recommendations of a previous study,<sup>10</sup> inorganic salts were added to the media: (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (10.5 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (6 g/L), and ZnSO<sub>4</sub>·7H<sub>2</sub>O (7.5 mg/L). The pH was measured using a sensor (ThermoOrion 105A; Thermo Fisher Scientific, Madrid, Spain) and was adjusted to 5.00 before the biomass inoculation. The fermentations were initiated under micro-aerophilic conditions, and the initial concentration of the yeast in each assay was 5.00 ± 0.25 g/L.

**2.3. Analytical Determinations.** During fermentation assays, samples of 1 mL were taken in duplicate and immediately centrifuged for 5 min at 4500 rpm. The pellet (yeasts) was washed five times using phosphate buffer and resuspended in distilled water to the starting volume prior to biomass determination. The initial supernatants were transferred to sterile 1.5 mL tubes and stored at –20 °C until the corresponding determination.

The concentration of volatile suspended solids was chosen as a measure of the biomass concentration. To build the calibration curve, yeast was grown for 12–18 h in YPD medium, harvested, washed, and suspended in distilled water prior to spectrophotometric measurements at 600 nm, conducted using a visible spectrophotometer (DR/2010, HACH, Loveland, CO) according to the standard technique.<sup>11</sup>

The total sugar (sucrose, glucose, and fructose) content was determined using the phenol–sulfuric acid colorimetric method,<sup>12</sup> and the reducing sugar (glucose and fructose) content was measured using the Miller colorimetric method.<sup>13</sup> The sugar concentration was calculated indirectly using a standard curve constructed from various concentrations of D-glucose (Merck, Whitehouse Station, NJ).

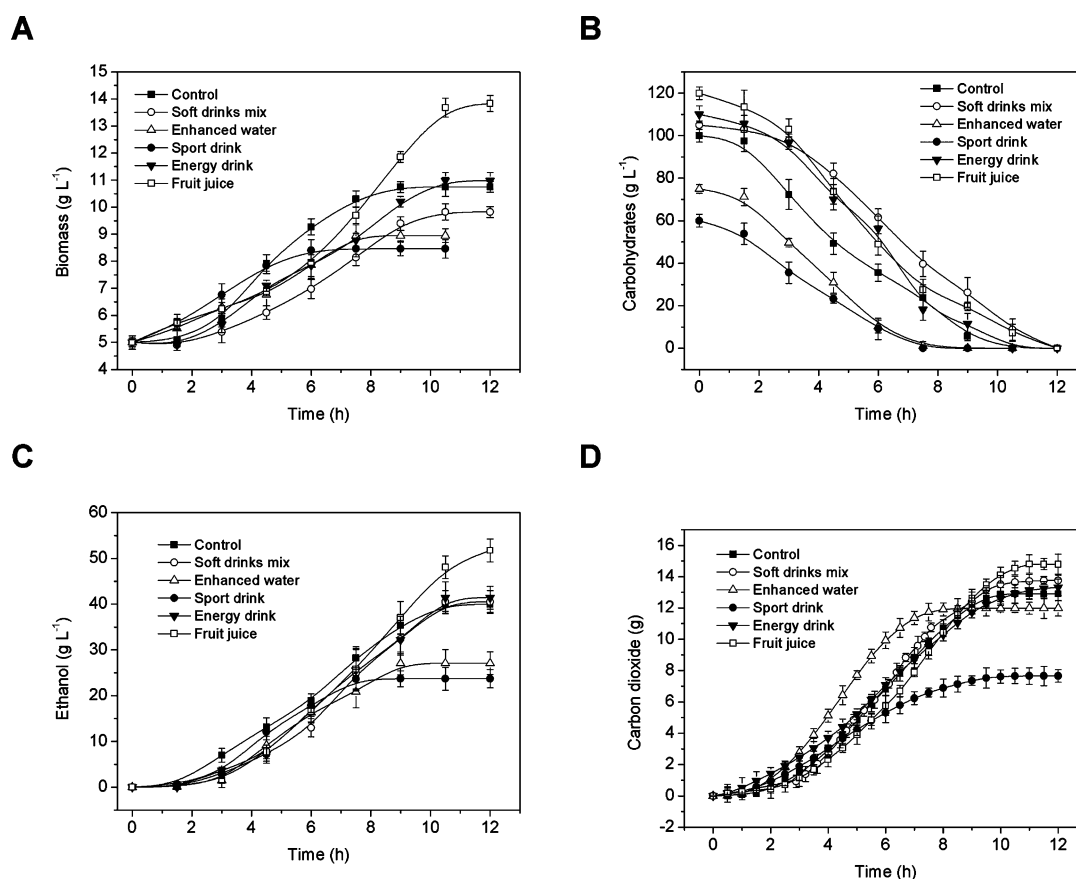
The ethanol concentration during fermentation was determined using a device based on a SnO<sub>2</sub> sensor (TGS Figaro 2620; Figaro Engineering Inc., Osaka, Japan), as described in a previous work.<sup>14</sup> The CO<sub>2</sub> produced during fermentation was measured online using a mass flowmeter with a transducer (Matheson, East Rutherford, NJ), and the total CO<sub>2</sub> production was estimated by integration. The glycerol concentration after ethanol had been removed by distillation was measured using an enzymatic kit (SB Lab, Santa Fe, Argentina).

A standard colorimetric technique<sup>11</sup> was used for chemical oxygen demand (COD) determinations. Initial (medium before inoculation) and final COD were measured in triplicate. At the end of the experiments, the biomass was separated by centrifugation. The supernatant was filtered in a vacuum using diatomaceous earth and a cellulose nitrate membrane filter (0.45 μm; Biopore, Buenos Aires, Argentina). Prior to the COD determination, the ethanol was removed from the filtrate by distillation.

**Table 1.** Yeast Performance for Various Nonalcoholic Sugar-Sweetened Beverages and for a Synthetic Medium That Was Used As a Control<sup>a</sup>

| parameter   | control       | nonalcoholic beverages |               |               |               |               |
|---|---------------|------------------------|---------------|---------------|---------------|---------------|
|   |               | M                      | E             | W             | FJ            | Sp            |
| initial sugar content (g L <sup>-1</sup> )  | 100 ± 2       | 105 ± 3                | 110 ± 2       | 75 ± 1        | 119 ± 5       | 60 ± 2        |
| time of complete sugar removal (h)  | 9.20 ± 0.03   | 11.25 ± 0.05           | 9.25 ± 0.02   | 7.50 ± 0.04   | 11.75 ± 0.08  | 6.00 ± 0.05   |
| initial COD (mg of O <sub>2</sub> L <sup>-1</sup> )   | 107000 ± 500  | 135000 ± 800           | 122000 ± 500  | 82000 ± 400   | 130000 ± 500  | 68000 ± 700   |
| theoretical contribution of sugar to initial COD (mg of O <sub>2</sub> L <sup>-1</sup> )                          | 106700        | 112000                 | 117400        | 80000         | 127000        | 64000         |
| final COD <sup>b</sup> (mg of O <sub>2</sub> L <sup>-1</sup> )  | 13000 ± 300   | 19000 ± 400            | 16000 ± 200   | 10500 ± 500   | 19000 ± 300   | 10500 ± 200   |
| COD removal (%)   | 87.90 ± 0.04  | 85.90 ± 0.02           | 86.90 ± 0.05  | 87.20 ± 0.02  | 85.40 ± 0.09  | 84.60 ± 0.05  |
| mean COD removal rate <sup>c</sup> (mg of O <sub>2</sub> L <sup>-1</sup> h <sup>-1</sup> )                        | 10200 ± 100   | 10300 ± 100            | 11450 ± 50    | 9500 ± 150    | 9450 ± 150    | 9550 ± 50     |
| mean specific COD removal rate <sup>d</sup> [mg of O <sub>2</sub> (mg of biomass) <sup>-1</sup> h <sup>-1</sup> ] | 1.28 ± 0.06   | 1.35 ± 0.04            | 1.38 ± 0.03   | 1.38 ± 0.07   | 1.03 ± 0.09   | 1.40 ± 0.05   |
| biomass yield [g of biomass (g of consumed sugar <sup>-1</sup> )]   | 0.060 ± 0.005 | 0.050 ± 0.008          | 0.060 ± 0.006 | 0.050 ± 0.005 | 0.070 ± 0.008 | 0.060 ± 0.004 |
| ethanol yield [g of ethanol (g of consumed sugar <sup>-1</sup> )]   | 0.40 ± 0.02   | 0.39 ± 0.04            | 0.38 ± 0.05   | 0.36 ± 0.04   | 0.43 ± 0.02   | 0.39 ± 0.03   |
| CO <sub>2</sub> yield [g of CO <sub>2</sub> (g of consumed sugar <sup>-1</sup> )]                                 | 0.43 ± 0.02   | 0.44 ± 0.04            | 0.42 ± 0.05   | 0.47 ± 0.03   | 0.41 ± 0.04   | 0.43 ± 0.02   |
| glycerol yield [g of glycerol (g of consumed sugar <sup>-1</sup> )]   | 0.100 ± 0.005 | 0.120 ± 0.008          | 0.090 ± 0.007 | 0.090 ± 0.004 | 0.110 ± 0.006 | 0.110 ± 0.002 |

<sup>a</sup>Abbreviations: M, mixture of soft drinks (65% cola, 28% lemon–lime, and 7% orange); E, energy drinks; W, enhanced water; FJ, fruit juice; and Sp, sports drinks. <sup>b</sup>After removal of biomass and ethanol. <sup>c</sup>Calculated as (initial COD – final COD)/(time of complete sugar removal). <sup>d</sup>Calculated as the ratio between the mean COD removal rate and the mean biomass concentration.

**Figure 2.** Yeast performance during batch fermentation assays performed using various nonalcoholic sugar-sweetened drinks. Evolution of the concentrations of (A) biomass, (B) total sugar, and (C) ethanol and (D) evolution of the carbon dioxide production.

### 3. RESULTS AND DISCUSSION

**3.1. Alcoholic Fermentation Followed by Distillation Reduced the COD of Certain Effluents of the Sugar-Sweetened Beverage Industry by 85%.** Because of deficient bottling processes and returns from the market due to quality policies, a portion of the total volume of produced sugar-sweetened beverages must be treated prior to disposal by

discharge to the environment. The sugars present in these beverages are the main reason for their COD levels, which can be as high as 135000 mg of O<sub>2</sub>/L (Table 1).

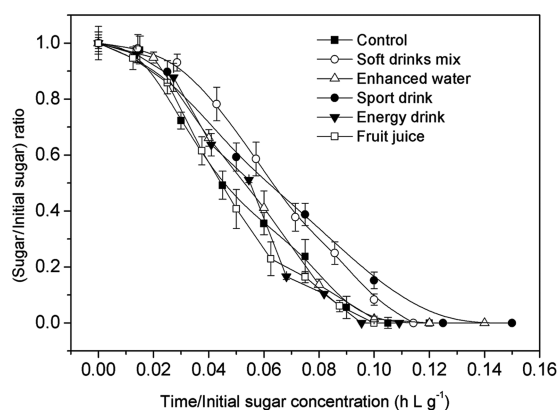
In this work, an alternative to conventional anaerobic treatment processes was studied. This treatment process is based on the production of ethanol by yeast-mediated fermentation, followed by the separation of ethanol by

distillation. The technical feasibility of the fermentation process has already been demonstrated for soft drinks supplemented with yeast extract.<sup>14</sup> In the present work, a mixture of salts reported in another study to be optimal<sup>10</sup> was used rather than yeast extract, and other types of sweetened beverages in addition to soft drinks were assayed. In addition to its economic benefits, the use of salts as nutrients offers the advantage of not increasing the COD of the media.

Three measurements of the COD were made in each assay: (a) in the effluent; (b) after the addition of nutrients; and (c) at the end of the experiment, once the ethanol and biomass had been separated by distillation and filtration, respectively. Several drinks were assayed, including enhanced water, energy drinks, fruit juices, sport drinks, and a mixture of soft drinks in the same proportions as their trading volumes. To evaluate the influence of the various compounds present in the beverages (salts in high concentrations in sport drinks, preservatives such as sodium benzoate and potassium sorbate, caffeine, and vegetable extracts, among others) on the fermentation process, assays were also performed on a synthetic medium containing sucrose that was used as a control.

The concentrations of total sugar, biomass, and ethanol and the CO<sub>2</sub> production were monitored over time in each assay. The glycerol concentrations at the beginning and end of each experiment were also determined. The experimental results for the assayed beverages are shown in Figure 2. A synthetic medium was used as the control. All media were supplemented with 10.5 g/L (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 6 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, and 7.5 mg/L ZnSO<sub>4</sub>·7H<sub>2</sub>O. The values denote the means of three independent experiments performed using each medium (standard deviations were intentionally excluded to simplify the reading). Note that, in all cases, the yeast was able to completely consume the total sugars in a short time period: less than 12 h when the medium was inoculated with 5 g/L of yeast.

Obviously, the time necessary for the complete depletion of the total sugar depends on its initial concentration in addition to the amount of yeast inoculated. The latter was the same for all assays; therefore, the evolution of the total sugar content over time, with both variables scaled to the initial total sugar content, is plotted in Figure 3. It can be observed that all of the assayed media exhibited similar behaviors. As can be seen from Table 1, the reported yields of biomass, ethanol, CO<sub>2</sub>, and glycerol were very close to the values obtained using the control media. This, in addition to the results depicted in Figure 3, provide evidence that



**Figure 3.** Evolution of the ratio of the sugar content to the initial sugar content versus the ratio of the time to the initial sugar content for all of the assayed media.

other compounds present in the assayed beverages, such as salts in high concentrations, preservatives, caffeine, and vegetable extracts, do not exert a noticeable inhibitory effect on yeast metabolism. This might be due to the negligible concentrations of these products when compared with the yeast concentration.

The most important parameters calculated from the experimental data are summarized in Table 1. In all cases, the media were supplemented with 10.5 g/L (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 6 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, and 7.5 mg/L ZnSO<sub>4</sub>·7H<sub>2</sub>O. The tabulated values denote the means ( $\pm$  standard deviation) of the parameters calculated from three independent experiments. Note that, once the ethanol and biomass were removed, 84.6–87.9% of the COD was removed for all of the assayed effluents.

To check the consistency of the experimental data, a carbon balance was performed for each assayed medium. The formulas of the carbonaceous compounds involved in the fermentation and their respective carbon fractions are listed in Table 2. The

**Table 2. Carbonaceous Compounds Involved in the Fermentation Process**

| compound        | formula   | molecular weight (g/mol) | carbon fraction [g of C (g of compound) <sup>-1</sup> ] |
|-----------------|---|--------------------------|---|
| glucose         | C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>             | 180                      | 0.40  |
| biomass (yeast) | CH <sub>1.613</sub> O <sub>0.557</sub> N <sub>0.158</sub> | 24.7                     | 0.48  |
| ethanol         | C <sub>2</sub> H <sub>6</sub> O                           | 46                       | 0.52  |
| carbon dioxide  | CO <sub>2</sub>   | 44                       | 0.27  |
| glycerol        | C <sub>3</sub> H <sub>8</sub> O <sub>3</sub>              | 92                       | 0.39  |

yeast “formula” was taken to be CH<sub>1.613</sub>O<sub>0.557</sub>N<sub>0.158</sub>.<sup>15</sup> The sums of the yields reported in Table 1 multiplied by the respective carbon fractions, in grams of carbon per gram of sugar, were 0.39 (control), 0.39 (mixture of soft drinks), 0.37 (energy drinks), 0.37 (enhanced water), 0.41 (fruit juice), and 0.39 (sports drinks). These values reasonably satisfy the C balance (the theoretical value is 0.40 g of carbon/g of sugar). This confirms the reliability and consistency of the experimental data, as well as the fact that no carbonaceous compounds other than biomass, ethanol, carbon dioxide, and glycerol were produced in significant amounts in the fermentation process.

**3.2. Proposed Process Outperforms Conventional Treatment Processes.** The process presented in this work is an interesting alternative that outperforms the conventional treatments because (a) it accomplishes the complete and rapid removal of sugars present in wastewaters from the sugar-sweetened nonalcoholic beverage industry and (b) it produces ethanol, which can be easily separated by distillation, and CO<sub>2</sub>, which separates spontaneously. Both compounds are value-added products.

The mean specific COD removal rates achieved in this work fell within the range of 1.03–1.40 mg of O<sub>2</sub> (mg of biomass)<sup>-1</sup> h<sup>-1</sup> (Table 1), which are significantly higher than the rates that have been reported for conventional anaerobic treatments of similar effluents, namely, 0.03–0.05 mg of O<sub>2</sub> (mg of biomass)<sup>-1</sup> h<sup>-1</sup>.<sup>6,7,16,17</sup> To confirm these rates, assays using the sludge of an anaerobic reactor in operation (courtesy of Compañía Industrial Cervecería S.A., Santa Fe, Argentina) as the inoculum were made on the following samples: (a) the mixture of soft drinks; (b) the synthetic medium; and (c) the synthetic medium diluted to achieve a COD of approximately 10000 mg of O<sub>2</sub>/L, which is a typical value for upflow anaerobic sludge blanket (UASB) reactor

influent that is adjusted in industrial practice by recycling of treated effluent. As expected, the mean specific COD removal rates obtained in these assays fell within the range reported in the literature for similar effluents: 0.04–0.08 mg of O<sub>2</sub> (mg of biomass)<sup>-1</sup> h<sup>-1</sup>.

The effluent of the process, once ethanol and biomass had been removed, exhibited residual COD values that were approximately 15% that of untreated wastewater. This COD cannot be attributed to other compounds present in very low concentrations in the assayed drinks (colorants, preservatives, plant extracts, etc.), but rather must be due to nonvolatile byproducts of the alcoholic fermentation, such as glycerol and acetic, pyruvic, and succinic acids.<sup>18–20</sup> As Table 1 shows, approximately 0.10 g of glycerol was produced for every gram of sugar consumed, which is similar to the results reported in the literature for similar media supplemented with ammonium salts.<sup>21</sup> As the COD of glycerol is 1.22 mg of O<sub>2</sub> (mg of glycerol)<sup>-1</sup>, simple calculations made using the data in Table 1 show that this compound is primarily responsible for the residual COD of the assayed media: 94% (control media), 81% (mixture of soft drinks), 75% (energy drinks), 78% (enhanced water), 84% (fruit juice), and 77% (sports drinks). This finding, in addition to the satisfactory carbon balance obtained when glycerol was included, leads us to conclude that glycerol is the main carbonaceous compound that remains in solution once fermentation is finished and ethanol and biomass are removed. This effluent can be treated using conventional methods<sup>22</sup> or with alternative valorization processes.<sup>23</sup>

Finally, this process could be applied to the treatment of effluents from other beverage industries (e.g., coffee, tea, and milk) and even other food industries (e.g., candy and jams), thereby expanding the spectrum of potential users in the industrial application of the process. It is also important to emphasize that the process can be carried out using conventional equipment (fermenters, distillation columns, filters, and decanters).

**3.3. Adapted Glycerol-Growing *S. cerevisiae* Strain Can Improve Overall Process of Wastewater Treatment.** Glycerol synthesis plays a significant physiological role in the metabolism of yeast, including osmoregulation and maintenance of the intracellular redox balance under anaerobic conditions. A surplus of NADH, formed during the synthesis of biomass and secondary fermentation products, such as pyruvic and succinic acids, is reoxidized to NAD<sup>+</sup> by glycerol production during fermentation, because ethanol formation from glucose is a redox-neutral process.<sup>18,21</sup> Moreover, *S. cerevisiae* can utilize certain nonfermentable compounds (e.g., ethanol, acetate, and glycerol) as its sole source of carbon and energy. Glycerol is able to diffuse freely across the yeast plasma membrane, and it also crosses the plasma membrane through protein-mediated transport systems, to finally convert glycerol into dihydroxyacetone phosphate, an intermediate of glycolysis pathway.<sup>19</sup>

As already mentioned, once ethanol and biomass are removed, the glycerol produced by yeasts during fermentation remains in the medium. Because this compound is the main compound responsible for the residual COD, a complementary process able to remove the glycerol, such as aerobic yeast propagation, should be of interest. As the yeast *S. cerevisiae* grows slowly on media containing solely nonfermentable carbon sources such as glycerol, the fermentative capacity of a strain adapted for improved growth on glycerol was evaluated. For this purpose, the same *S. cerevisiae* var. Windsor strain as used in the fermentation assays was repeatedly transferred into fresh medium containing

only glycerol as the carbon source and grown under full aeration (i.e., avoiding any oxygen limitation during the culture).<sup>9</sup> The “adapted” biomass was inoculated on the reactors containing the effluents already free of ethanol and biomass and grown under aerobic conditions. To evaluate whether the adapted strain retained its ethanol production capacity from the sugar contained in the assayed wastewaters, a portion of the biomass in exponential-growth stage was collected and directly used as the inoculum in a new set of fermentation assays on (a) the synthetic medium, (b) the mixture of soft drinks, and (c) enhanced water. The remaining biomass was grown to starvation, and the aerobic growth experiments were finished when the CO<sub>2</sub> production stopped.

The fermentation assays were performed in triplicate, and the concentrations of biomass, sugars, ethanol, and glycerol were monitored over time. As expected, the depletion of sugars was complete for all assays, and the corresponding ethanol yields (in grams of ethanol per gram of consumed sugar) were 0.39 ± 0.1 (control), 0.40 ± 0.2 (mixture of soft drinks), and 0.35 ± 0.2 (enhanced water). Note that the ethanol yields were very close to those obtained with the original (“wild-type”) *S. cerevisiae* var. Windsor under comparable conditions (Table 1). Therefore, the fermentative capacity of the adapted strain remained intact when brought back to anaerobic (fermentative) conditions.

At the end of the aerobic propagation, the biomass was removed, and both the remaining COD and the glycerol concentration in each assayed medium were measured. No glycerol was detected in all assays, confirming that the aerobic propagation using an adapted glycerol-growing strain is an excellent strategy for glycerol depletion. The final values of COD (mg of O<sub>2</sub>/L) were around 500 (control), 3000 (mixture of soft drinks), and 1400 (enhanced water). These values allowed the resulting media to be treated by conventional aerobic treatment processes once the proposed process was applied. The overall COD removal percentages (%) were 99.5 (control), 97.8 (mixture of soft drinks), and 98.3 (enhanced water).

The results obtained in this work show that the proposed process, namely, alcoholic fermentation of high-strength sugar-sweetened beverage industry wastewaters followed by ethanol and biomass separation and subsequent aerobic propagation of yeast, is a technically feasible treatment alternative for these effluents. In addition, it allows the typical problems of anaerobic conventional treatment processes (high residence times, large storage volumes, offensive odors, competition/inhibition between different groups of bacteria, and particular nutritional/biochemical requirements of each of them) to be avoided.

Note that the COD removal levels obtained, once the aerobic propagation of yeast was performed, were greater than the expected theoretical values if only the COD due to glycerol was considered. This would indicate that, under aerobic conditions, the yeasts are capable of consuming other nonfermentable carbon sources remaining in the media after alcoholic fermentation. These could be fermentation byproducts (e.g., acetic, pyruvic, or other organics acids) or ingredients of each beverage formulation (acidulants, preservatives, etc.).

Glycerol removal by the aerobic proliferation of adapted yeast could be of interest in treating other wastewaters containing glycerol, such as those generated in biodiesel production processes<sup>24</sup> or in the treatment of brewery vinasses.<sup>25</sup>

**3.4. Bioethanol and CO<sub>2</sub> Produced by Yeast Are Value-Added Products.** Finally, it should be noted that the studied process produces ethanol and CO<sub>2</sub>, compounds with added value. Ethanol can be rectified to produce a food-grade product

or dehydrated for use as biofuel. Ethanol is one of the most important renewable fuels, and it can be added to gasoline to reduce the negative environmental impacts of fossil fuels.<sup>26,27</sup>

The CO<sub>2</sub> produced by the process is also a value-added product. Among other uses, it is an important raw material for the carbonated drinks industry, where it is added to obtain a dissolved CO<sub>2</sub> concentration in soft drinks in the range of 5–10 g/L.<sup>28</sup>

Finally, the biomass produced during the fermentation process should not be regarded as a waste material because yeast is used in animal food formulations.<sup>29</sup>

#### 4. CONCLUSIONS

A process for COD removal from high-strength wastewaters of the nonalcoholic sugar-sweetened beverage industry by yeast fermentation was studied. The main products of the fermentation are ethanol, which can be recovered by distillation, and CO<sub>2</sub>, which separates spontaneously and can be collected. The yeast can be removed by filtration. The alcoholic fermentation step of the process was tested using several beverages and exhibited high specific COD removal rates, in the range of 1.03–1.40 mg of O<sub>2</sub> (mg of biomass)<sup>-1</sup> h<sup>-1</sup>, which is far above the typical range for conventional anaerobic processes [0.03–0.05 mg of O<sub>2</sub> (mg of biomass)<sup>-1</sup> h<sup>-1</sup>]. The addition of simple nutrients to the media inoculated with 5 g/L of yeast, along with pH adjustment, enabled the total consumption of the sugars contained in these effluents in less than 12 h. Once ethanol and biomass were removed, 85% COD depletion was achieved. Most of the residual COD was found to be due to the presence of glycerol, which is a byproduct of the fermentation. This nonfermentable compound can be used as a carbon source for aerobic propagation of yeast, which can then be used as the inoculum in the next cycle of fermentation. COD removal values close to 98% were achieved by the integral process.

In addition to the high rate of COD removal, the process offers the advantages of requiring only conventional equipment and producing value-added products, such as ethanol and CO<sub>2</sub>, at ratios of approximately 0.40 g of ethanol and 0.44 g of CO<sub>2</sub> per gram of removed COD.

#### AUTHOR INFORMATION

##### Corresponding Author

\*Tel.: +54 342 4559177, ext. 2147. Fax: +54 342 45509 44. E-mail: [misla@santafe-conicet.gov.ar](mailto:misla@santafe-conicet.gov.ar).

##### Notes

The authors declare no competing financial interest.

#### ACKNOWLEDGMENTS

This research was supported by the Universidad Nacional del Litoral (UNL) via the CAI+D Program and the Nuevo Banco de Santa Fe Foundation (NBSF). The collaboration of undergraduate students K. J. Schanton and J. M. Villalba in the realization of some assays is gratefully acknowledged.

#### REFERENCES

- (1) Brouns, F.; Kovacs, E. Functional drinks for athletes. *Trends Food Sci. Technol.* **1997**, *8*, 414–421.
- (2) Siró, L.; Kapolna, E.; Kapolna, B.; Lugasi, A. Functional food. Product development, marketing and consumer acceptance - A review. *Appetite* **2008**, *51*, 456–467.
- (3) Zbakh, H.; El Abbassi, A. Potential use of olive mill wastewater in the preparation of functional beverages: A review. *J. Funct. Foods* **2012**, *4*, 53–65.

- (4) [http://www.indec.mecon.ar/nivel4\\_default.asp?id\\_tema\\_1=3&id\\_tema\\_2=6&id\\_tema\\_3=18](http://www.indec.mecon.ar/nivel4_default.asp?id_tema_1=3&id_tema_2=6&id_tema_3=18) (in Spanish) (accessed Feb 9, 2015).

- (5) Tebai, L.; Hadjivassilis, I. Soft Drinks Industry Wastewater Treatment. *Water Sci. Technol.* **1992**, *25*, 45–51.

- (6) Borja, R.; Banks, C. J. Kinetics of anaerobic digestion of soft drink wastewater in immobilized cell bioreactors. *J. Chem. Technol. Biotechnol.* **1994**, *60*, 327–334.

- (7) Kalyuzhnyi, S. V.; Valadez Saucedo, J.; Rodriguez Martinez, J. The Anaerobic Treatment of Soft Drink Wastewater in UASB and Hybrid Reactors. *Appl. Biochem. Biotechnol.* **1997**, *66*, 291–301.

- (8) Redzwan, G.; Banks, C. J. An evaluation of soft-drink wastewater treatment by anaerobic digestion process. *Malays. J. Sci.* **2007**, *26* (1), 35–42.

- (9) Ochoa-Estopier, A.; Lesage, J.; Gorret, N.; Guillouet, S. E. Kinetic analysis of a *Saccharomyces cerevisiae* strain adapted for improved growth on glycerol: Implications for the development of yeast bioprocesses on glycerol. *Bioresour. Technol.* **2011**, *102*, 1521–1527.

- (10) Comelli, R. N.; Seluy, L. G.; Ingaramo, D. L.; D'Andrea, M. F.; Isla, M. A. Sustainable bioethanol production from industry wastewater. Presented at the 4th Iberoamerican Congress on Hydrogen and Sustainable Sources of Energy (HYFUSEN 2013). Cordoba, Argentina, Jun 10–14, 2013.

- (11) Eaton, A. D.; Clescer, L. S.; Greenberg, A. E. *Standard Methods for the Examination of Water and Wastewater*, twentieth ed., American Public Health Association, USA, 2005.

- (12) DuBois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356.

- (13) Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428.

- (14) Isla, M. A.; Comelli, R. N.; Seluy, L. G. Wastewater from the soft drinks industry as a source for bioethanol production. *Bioresour. Technol.* **2013**, *136*, 140–147.

- (15) Von Stockar, U.; Liu, J. S. Does microbial life always feed on negative entropy? Thermodynamic analysis of microbial growth. *Biochim. Biophys. Acta, Bioenerg.* **1999**, *1412*, 191–211.

- (16) Guiot, S. R.; Podrutzny, M. F.; McLean, D. D. Assessment of macroenergetic parameters for an anaerobic upflow biomass bed and filter (UBF) reactor. *Biotechnol. Bioeng.* **1989**, *34*, 1277–1288.

- (17) Poh, P. E.; Yong, W. J.; Chong, M. F. Palm Oil Mill Effluent (POME) Characteristic in High Crop Season and the Applicability of High-Rate Anaerobic Bioreactors for the Treatment of POME. *Ind. Eng. Chem. Res.* **2010**, *49*, 11732–11740.

- (18) Hohmann, S. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 300–372.

- (19) Dickinson, J. R.; Schweizer, M. *The Metabolism and Molecular Physiology of Saccharomyces cerevisiae*, second ed., CRC Press, USA, 2004.

- (20) Zhang, A.; Chen, X. Improve Ethanol Yield through Minimizing Glycerol Yield in Ethanol Fermentation of *Saccharomyces cerevisiae*. *Chin. J. Chem. Eng.* **2008**, *16*, 620–625.

- (21) Albers, E.; Larsson, C.; Liden, G.; Niklasson, C.; Gustafsson, L. Influence of the Nitrogen Source on *Saccharomyces cerevisiae* Anaerobic Growth and Product Formation. *Appl. Environ. Microbiol.* **1996**, *62* (9), 3187–3195.

- (22) Hutián, M.; Kolesárová, N.; Bodík, I.; Czölderová, M. Long-term monodigestion of crude glycerol in a UASB reactor. *Bioresour. Technol.* **2013**, *130*, 88–96.

- (23) Faria, R. P. V.; Pereira, C. S. M.; Silva, V. M. T. M.; Loureiro, J. M.; Rodrigues, A. E. Glycerol Valorization as Biofuel: Thermodynamic and Kinetic Study of the Acetalization of Glycerol with Acetaldehyde. *Ind. Eng. Chem. Res.* **2013**, *52*, 1538–1547.

- (24) Severson, K.; Martin, M.; Grossmann, I. E. Optimal biodiesel production using bioethanol: Towards process integration. *AIChE J.* **2013**, *59*, 834–844.

- (25) Seluy, L. G.; Isla, M. A. A Process to Treat High-Strength Brewery Wastewater via Ethanol Recovery and Vinasse Fermentation. *Ind. Eng. Chem. Res.* **2014**, *53*, 17043–17050.

(26) Stichnothe, H.; Azapagic, A. Bioethanol from waste: Life cycle estimation of the greenhouse gas saving potential. *Resour. Conserv. Recycling*. **2009**, *53*, 624–630.

(27) Martín, M.; Grossmann, I. E. Energy optimization of bioethanol production via hydrolysis of switchgrass. *AIChE J.* **2012**, *58*, 1538–1549.

(28) Ljunggren, E.; Karlberg, B. Determination of total carbon dioxide in beer and soft drinks by gas diffusion and flow injection analysis. *J. Autom. Chem.* **1995**, *17*, 105–108.

(29) Ferreira, I.; Pinho, O.; Vieira, E.; Taveira, J. G. Brewers *Saccharomyces* yeast biomass: characteristics and potential applications. *Trends Food Sci. Technol.* **2010**, *21*, 77–84.