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Combined Utilization of Agro-Industrial Wastewaters for Non-lignocellulosic Second-Generation Bioethanol Production

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Abstract

Bioethanol is one of the most important renewable fuels, and its production from biomass is a potential substitute for fossil fuels. Second-generation technologies cover a wide range of biomass resources, from agriculture to forestry and waste materials. Although lignocellulosic residues represent an attractive feedstock for ethanol production, their processing is not economical due to problems arising from the recalcitrant nature of the biomass and the need for pretreatment. The main purpose of this work was to evaluate the feasibility of the direct use of three agro-industrial wastes as low-cost feedstock for ethanol production: wastewaters of the sugar-sweetened beverage industry as carbon source, purges of fermentation tanks of the brewery industry as inoculum and corn steep water from the corn starch processing industry as nitrogen source. Fermentations were performed in batch mode under anaerobic conditions, and the concentrations of sugar, biomass and ethanol were monitored over time. The process was optimized using surface-response methodology. Spent brewer's yeast and corn steep water supplementation at ratios of 12.4 and 8.4% v/v, respectively, were optimal for ethanol production. In these conditions, the sugar available in the soft-drink wastewater was completely depleted in less than 8 h, with an average ethanol yield of 0.45 $g_{ethanol}/g_{sugar}$. The process developed could become an alternative bioprocess for production of non-lignocellulosic bioethanol.

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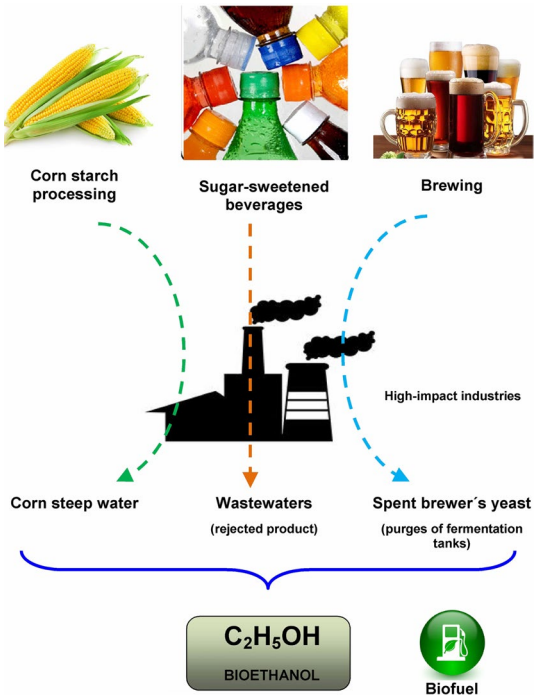
Graphical Abstract



Keywords Valorization · Fermentation · Corn-steep water · Spent brewer's yeast · Sugar-sweetened beverages

Abbreviations

CCD	Central composite design
CSW	Corn-steep water
FAN	Free amino nitrogen
FM	Fermentation medium
NCR	Nitrogen catabolite repression
RSM	Response surface methodology
SBY	Spent brewer's yeast
SDW	Soft drinks wastewaters
UP	Upstream processing step
WDGS	Wet destillers grains with solubles

Statement of Novelty

Currently, bioethanol is one of the most important green fuels, and its demand is notably increasing due to the urgent necessity of replacing fossil fuels. Although lignocellulosic biomass has received attention as a source of bioethanol, its processing conditions are obstacles to the industrial exploitation of this technology. The goal of this work was to identify and optimize the key conditions to obtain good-quality second-generation ethanol using only non-lignocellulosic agro-industrial wastes: (a) wastewaters of the sugar-sweetened beverage industry as the source of simple and fermentable sugars (glucose, fructose and sucrose), (b) purges of fermentation tanks of the brewery industry as the source of yeast, and (c) wastewaters of the corn starch processing industry as the source of nitrogen and vitamins.

Introduction

The concept of *biorefiney* involves the production of chemicals from bioresources. Because petrochemical resources are finite and bioresources are renewable, the biorefinery has recently gained a lot of attention. Bioethanol is one of the most important *green* fuels. First-generation bioethanol from energy crops (mainly starch from maize and sucrose from sugar cane) seems to not be sustainable for environmental as well as economic reasons, including (a) the increase in the area of land dedicated to the energy matrix, (b) the high demand for natural resources, (c) the competition for land that could be used for food production, and (d) the high price of the sugar obtaining and their alternative applicability in the food industry [1, 2]. Since bioethanol is a bulk chemical, raw material costs are responsible for the largest part of the production cost. Therefore, it is necessary to find inexpensive carbon and nitrogen sources at the same time to reduce the production cost for satisfying the demand on the

bioethanol market. Although lignocellulosic residues represent an attractive renewable source for second-generation bioethanol production, the delignification technology and the large quantity of wastewaters produced are obstacles that still must be overcome for large-scale production [3–5]. In addition, the extreme conditions required to treat the lignocellulosic biomass before enzymatic hydrolysis result in the formation of several inhibitory compounds of yeast metabolism [6, 7]. At least until these obstacles are overcome, it is imperative to identify viable renewable sources for the production of bioethanol. Agro-industrial wastewaters seem to be good candidates [8–11]. In particular, sugar-sweetened beverage wastewaters, comprising products rejected due to quality policies during the bottling process or that are returned from the market (due to a lack of gas or having passed the expiration date), are attractive in several ways: (a) they are generated in large quantities in proportion to the high production of these beverages, (b) they exhibit a high sugar content of approximately 60–180 g/L, (c) they are renewable and low-cost raw materials, (d) they are simple in their formulation, and (e) the yeasts *Saccharomyces cerevisiae* can easily convert the employed sugars (glucose, fructose and/or sucrose) into ethanol [12, 13]. In addition, the process is environmentally friendly, and when compared to the bioethanol production from energy crops, they neither demand natural resources nor compete for land that could be used for food production. However, some aspects must be thoroughly considered to achieve both rentable and attractive process for industrial scale-up. One of these topics is the optimization of the upstream processing (UP) step. Usually, UP includes the following: the formulation of the fermentation medium (FM); sterilization of air, FM and the fermenter; inoculum preparation (aerobic proliferation and conditioned of yeast biomass); and inoculation of the FM. Nitrogen addition to the fermentation broth is necessary to achieve an adequate carbon/nitrogen ratio. Nitrogen sources can be organic, such us corn steep liquor (CSL), yeast extract and peptone, among others, or inorganic, such as ammonium salts [14]. All these operations have an impact on the final cost of the overall process.

The main purpose of this work was to evaluate viable alternatives to reduce times and costs associated with UP in yeast-mediated alcoholic fermentation of sugar-sweetened wastewaters. We focused on the inoculum preparation and formulation of the FM. The proposed scheme involved up to three industrial wastewaters (or by-products, depending of the point of view) as low-cost feedstock: (a) wastewaters of the sugar-sweetened beverage industry as carbon source; (b) *spent brewer's yeast* (SBY), in particular purges of fermentation tanks of the brewery industry, as inoculum; and (c) *corn steep water* (CSW) from the corn starch processing industry as nutritive supplement. The SBY from brewers was a good candidate as inoculum for several reasons: (a) it contains approximately 100 g/L of yeast cells; (b) it is an inexpensive feedstock; (c) it has high production and availability throughout the year; and (d) it has null or very low market price (it is mainly used as animal feed). The use of *SBY autolyzed* and *wet distiller's grains with solubles* (WDGS) were also evaluated as nitrogen sources with comparative purposes. Fermentation assays were performed on a mixture of soft-drink wastewaters (SDW), and the concentrations of biomass, sugars and ethanol were monitored over time, as was the production of other fermentation products, such as glycerol. The comparison of yeast performance using different nutritional mixtures of inorganic and organic nitrogen sources led us to identify a possible nitrogen catabolite repression (NCR) process. Finally, the fermentative process with SDW, SBY and CSW was optimized using surface–response methodology. A scheme with the experimental design followed in this work is shown as Supplementary Figure.

Materials and Methods

Raw Materials

Experimental assays were performed using materials kindly provided by leader industries located in Santa Fe, Argentina. The purges of fermentation tanks containing the surplus yeasts from a brewing industry (CCU Argentina, http://www.ccu.com.ar) were used as yeast inoculum without additional washing operations. Once collected from breweries, the SBY was stored at 10 °C. Even though the liquid fraction (supernatant) was low (approximately 10% v/v of the total sample volume), it was sufficient to handle the biomass (yeast). In addition, a portion was autolyzed at 50 °C for 24 h according to Tanguler and Erten [15]. The autolysate obtained was centrifuged at 5600×*g* for 10 min to yield a supernatant and solid residue. The supernatant was transferred to a sterile tube, and it was stored at 4 °C until use.

The CSW and the WDGS were provided by a corn starch processing company (Glutal S.A., http://www.glutal.com.ar) and a corn-based ethanol plant (Vicentin S.A.I.C., https://www.vicentin.com.ar), respectively. Batches were obtained from the same industrial plants approximately 3 months apart and frozen to avoid native flora proliferation before the fermentation assays. Both materials were employed as nutritional supplements without previous conditioning operations.

A mixture of soft drinks based on the marketing volumes in Argentina (65% cola type, 28% lemon-lime and 7% orange) was used as the main medium for alcoholic fermentation. This values was taken from an internal report (2015, personal communication) provided by the Argentine Chamber of the Non-Alcoholic Beverage Industry (CADIBSA,

https://copal.org.ar/cadibsa). Beverages were purchased in a local market and stored at 4 °C until use. As previously reported, additional compounds than carbohydrates present in the beverages, such as salts, preservatives, caffeine, and vegetable oils do not exert a noticeable inhibitory effect on yeast metabolism. This might be due to the negligible concentrations compared to carbohydrates and yeast inoculated [12]. Therefore, the SDW was used directly and without conditioning operations.

Fermentations

Fermentations were performed in triplicate using a 500-mL glass reactor (300-mL working volume) operated in batch mode under anaerobic conditions and at a constant temperature of 30 °C, as described previously [13]. The pH was initially adjusted to 4.50 ± 0.10, and orbital shaking (100 rpm) was maintained throughout the experiments to avoid biomass precipitation. Because brewery industries employ axenic cultures to propagate its inocula, more than one strain in an SBY is rarely observed. This aspect was confirmed by plate streaking method, i.e., only one yeast morphology was detected. Therefore, the reactors were inoculated directly with fresh SBY. In addition, the main yeast present in SBY was isolated and purified by the 5-streak technique. Stock cultures were maintained on YPD (yeast extract 5 g/L, peptone 5 g/L and D-glucose 20 g/L) agar plates at 4 °C. This strain was used to evaluate a possible NCR effect (please see Sect. 3.2) and as additional control in the fermentation assays performed with SBY.

The fermentation time was measured from the time of inoculation. All experiments were stopped 12 h after inoculation. In the feasibility assays (Sects. 3.1 and 3.2), samples were taken from reactors at 0, 4, 8 and 12 h. The measurements of the biomass, sugar and ethanol at these times were enough to achieve an approximation of the performance of the evaluated process. In the case of the optimization assays (CCD design, Sect. 3.3), the samples were taken every 1.5 h to better observe the behavior of the variables over time and to avoid failures related to the number of experimental points. The performance of yeasts was evaluated using several parameters defined in a previous work [12], such as the lag phase duration, the biomass specific growth rate, the biomass yield, the net substrate consumption, the sugar consumption specific rate, the ethanol yield and the ethanol production specific rate.

To evaluate the feasibility of using SBY as inocula, fermentations were performed on a pre-defined mixture of soft drinks supplemented at several ratios with SBY (2, 5, 10, 15, 20 and 25% v/v). Because yeast viability was not a variable that we could feasibly manage, fresh SBY batches were employed, i.e., they were immediately stored after collection. The SBY contained significant amounts of organic nitrogen compounds, magnesium and inorganic phosphate (Table 1), which are essential nutrients for successful alcoholic fermentations using the yeast *S. cerevisiae* [14]. Then, no additional nutrients were added to fermentation media. The use of low-cost nitrogenous materials, including industrial by-products and defined mixtures of inorganic salts, were evaluated here. Fermentations were performed on a mixture of soft drinks supplemented at several ratios (2, 5 and 10% v/v) with (a) corn steep water (CSW); (b) autolyzed SBY; or (c) wet distiller's grains with solubles (WDGS). A previously developed inorganic supplement comprising $(NH_4)_2HPO_4$ 10.6 g/L; $MgSO_4 \cdot 7H_2O$ 6.4 g/L and $ZnSO_4 \cdot 7H_2O$ 7.5 mg/L [14] was also evaluated.

Analytical Determinations

During experiments, samples (1 mL) were taken in duplicate and immediately centrifuged for 5 min at 1200×*g*. The pellet (yeasts) was washed with distilled water and resuspended 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 biomass concentration was indirectly determined by turbidity measurements at 600 nm using a VIS spectrophotometer (DR/2010, HACH, USA), as described previously [12]. The total sugar content was determined using the phenol–sulfuric acid colorimetric method [16], and the reducing sugar content was measured using the Miller colorimetric method [17]. The ethanol concentration was determined using a device based on a SnO_2 sensor (TGS Figaro 2620; Figaro Engineering Inc., Osaka, Japan) as described in previous works [12–14]. Ammonium, magnesium and inorganic phosphorus were

Table 1 Raw material characterization

Parameter[a]	SBY	CSW
Initial soluble COD (mg of O_2/L)	135,000 ± 5,000	140,000 ± 5,000
Total sugar content (g/L)	40.0 ± 8.0	9.3 ± 0.7
Reducing sugars content (g/L)	5.0 ± 1.0	6.5 ± 0.8
Total protein content[b] (mg/L)	760 ± 40	3,500 ± 250
Free amino nitrogen content (mg/L)	270 ± 60	1,200 ± 100
Ammonium (mg/L)	Not detected	Not detected
Ethanol (g/L)	34 ± 2	1.0 ± 0.5
Glycerol (g/L)	4.2 ± 0.5	1.1 ± 0.4
Magnesium (mg/L)	45 ± 5	60 ± 10
Phosphate (mg/L)	400 ± 50	340 ± 90
pH	4.5 ± 0.5	4.0 ± 0.5

[a]The values denote the mean (± SD) of duplicate analysis from three independent batches

[b]Determined by the Bradford colorimetric method (1976) using Bovine Serum Albumin as standard

determined by colorimetric methods according to the manufacturer's instructions (Wiener Lab., Rosario, Argentina) and a standard colorimetric technique was used for chemical oxygen demand (COD) determinations [18]. The free amino acid nitrogen (FAN) content was evaluated according to the ninhydrin-staining method using glycine (Biopack, Buenos Aires, Argentina) as the standard [19]. Glycerol was measured using an enzymatic kit (SB Lab., Santa Fe, Argentina) whereas CO_2 produced during fermentation was measured online using a mass flowmeter with a transducer (Matheson, East Rutherford, NJ), and the total CO_2 production was estimated by integration as described in a previous work [13]. The glycerol concentrations were measured at the beginning and end of each experiment, whereas the CO_2 produced was only monitored for the fermentations performed with the optimized values obtained by the CCD (Sect. 3.3). These data were useful to make a carbon balance.

All the used reagents were of analytical grade. The DNS was purchased directly from Sigma-Aldrich (USA), whereas the rest of the reagents including carbohydrates, ethanol, glycine, and inorganic salts were purchased from Biopack (Buenos Aires, Argentina). All culture media and ingredients were commercially available (Britania, Buenos Aires, Argentina).

Statistical Design and Optimization

The SBY and CSW supplementation ratios (% v/v) were selected as independent variables (factors) for the optimization analysis. A three-level central composite design (CCD) was performed to maximize the ethanol yields in fermentations from SDW. The results commented on Sects. 3.1 and 3.2 allowed delimiting the range of useful concentrations of SBY and CSW to be evaluated at this point. Because the best results for both SBY and CSW were obtained at supplementation ratios of approximately 10% v/v, these values were taken as the central values, and the upper and lower limits were ± 50% of these. Therefore, the ratios of supplementation for each raw material were 5, 10 and 15%. For predicting the optimal point, a second-order polynomial function was fitted to examine the correlation between the independent variables and the response. This method was successfully used in a previous work [14]. The statistical analysis was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities p(t) were determined by Student's t-test. A 95% significance level (alpha) was used for the ANOVA analysis. All statistical analyses of experimental designs and results were performed using Statgraphics® Centurion XV v15.2.06 (StatPoint Inc., USA).

Results and Discussion

Fermentation of Wastewaters of the Soft Drink Industry Using Spent Brewer's Yeast as Inoculum

The technical feasibility of the use of wastewaters of the soft drink industry as medium for alcoholic fermentations mediated by industrial yeasts was previously demonstrated [12–14]. Because a proper inoculum demands viable yeasts, the first approach was the identification of an inexpensive source of yeasts able to be directly used as inoculum. Although the SBY from brewers could be a good candidate as inoculum, a critical point seems to be yeast's performance in a new fermentation round. To evaluate this quality, fermentations were performed on a pre-defined mixture of soft drinks supplemented at several ratios with SBY. The concentrations of sugar, biomass and ethanol were monitored over time in each assay. The experimental results are shown in Fig. 1. Some fermentation parameters are also reported in Table 2 for comparative purposes.

As expected, the initial yeast concentrations in runs with 2% v/v and 5% v/v of SBY supplementation were 1.86 ± 0.42 and 5.20 ± 0.51 g/L, respectively. Although these values are largely appropriate in fermentations with freshly inoculum [12, 13, 20], the null biomass growth and the poor consumption rates of sugars suggested that a great portion of the biomass was dead or had severe metabolic injuries. We think this was mainly due to the "age" of the inocula, the previous fermentation process for beer production and/or the presence of preservatives such as sodium benzoate or potassium sorbate in SDW [12].

Nevertheless, the sugar consumption was complete in less than 12 h during the experiments with supplementation ratios equal or higher than 10% v/v (Fig. 1b). This highlights the feasibility of using SBY as inoculum for alcoholic fermentations. For some perspective on the process, a brief analysis could be performed at this point. Supplementations higher than 10% v/v are suitable from an *environmental point of view*, i.e., as a wastewater treatment process [13]. Since the main principle of biological treatment processes is the conversion of dissolved organic matter into easily removable compounds (mainly gases and biomass), the inoculation of soft-drink wastewaters with SBY depleted all sugar and produced ethanol, which could be easily separated by distillation. However, from a *production point of view*, the goal must be the maximization of the conversion of the sugars into ethanol. In the experiments with 10 and 15% v/v SBY, the ethanol yields were 0.39 ± 0.03 and 0.37 ± 0.04 $g_{ethanol}/g_{sugar}$, respectively, which were close to 80% of the theoretical value. SBY supplementation ratios higher than 15% v/v reduced process

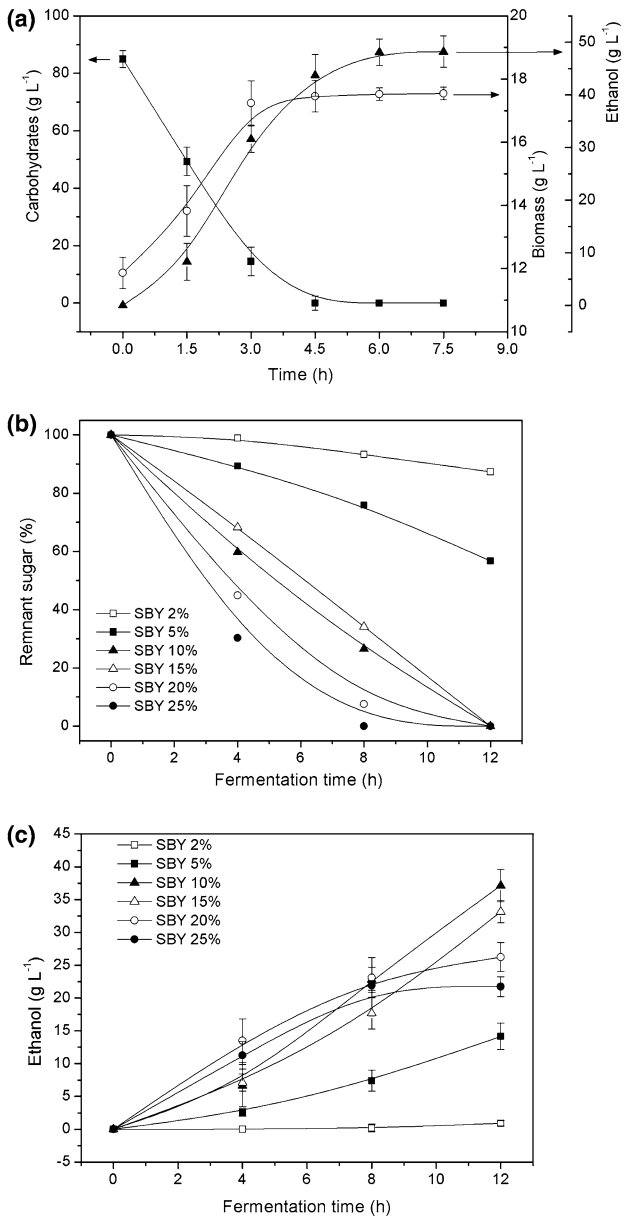


Fig. 1 SBY as inoculum in batch fermentation assays performed using a mixture of soft drinks (65% cola, 28% lemon-lime and 7% orange). Evolution of the concentrations of biomass (**a**), sugar (**b**) and ethanol (**c**). The values denote the mean of three independent experiments (SDs were intentionally omitted in some panels for ease of reading)

times but also reduced ethanol yields. More diluted fermentation medium reduces the sugar/yeast ratio, and the Pasteur effect would be predominant over the Crabtree effect. In this scenario, a portion of the sugars would not be metabolized down by the fermentative pathway, and they would be used for cell maintenance [21, 22].

Because the obtained ethanol yields were less than the expected, additional experiments were performed to evaluate the nutritional status of the inoculum. Fermentations were carried out with supplementation of 10 g/L of commercial yeast extract, and SBY at 10% v/v was chosen as inoculum. A control without yeast extract was also included. The evolution of the concentrations of sugar, biomass and ethanol is shown in Fig. 2. The addition of yeast extract increased ethanol yield by approximately 15% v/v, yielding an average of 0.46 ± 0.02 $g_{ethanol}/g_{sugar}$ versus the 0.39 ± 0.03 $g_{ethanol}/g_{sugar}$ obtained in the control without supplementation. The specific rate of biomass growth (r_b, $g_{biomass}$/L/h) was clearly increased when nutritive supplement was added: 0.047 ± 0.003 in comparison to the 0.032 ± 0.04 obtained to the control without supplementation. Regarding the specific rate of ethanol production (r_e, $g_{ethanol}$/L/h), the medium with yeast extract supplementation showed a value of 5.4 ± 0.03, i.e., slightly higher than the control (4.2 ± 0.05). *Saccharomyces cerevisiae* is a non-diazotrophic and non-proteolytic organism, i.e., it cannot fix nitrogen or use proteins as a nitrogen source [21]. The yeast extract provides specific nutrients, such as organic nitrogen (in the form of amino acids and dipeptides), trace elements and vitamins. The obtained results suggest that the SBY could be a deficient source of nitrogen or trace elements that are essential to yeast growth. Therefore, the fermentation medium should be supplemented with readily utilizable sources of nitrogen, which can be provided by ammonium salts or amino acids.

Finally, the storage times of the SBY in a potential bioethanol plant have to be considered. Once collected from breweries, the SBY could be kept at 10 °C for up to 2 weeks without loss of fermentation performance. Nevertheless, prolonged storage times (longer than 3 weeks) severely affected the yeast metabolism and reduced ethanol yield by approximately 20% (not shown).

The results in this section show that it would be possible to develop an ethanol production process using two low-cost raw materials (SBY and SDW). However, the challenge will be the identification of a good and economic raw material containing readily utilizable sources of nitrogen by yeasts. This would increase both the ethanol yield and the economical attractiveness of the proposed process.

Fermentation of Wastewaters of the Soft Drink Industry Using Corn Steep Water as an Inexpensive Nitrogen Source

The nitrogen source is one of the critical components affecting productivity and economy of fermentation processes. So, different nitrogen sources were evaluated. All media were inoculated with 10% v/v of SBY, and the concentrations of biomass, sugar and ethanol were monitored over time. A set of parameters of yeast performance were calculated from the experimental results (Table 3).

Because no changes with respect to control were obtained with WDGS as supplement (not shown), this waste seems

Table 2 Parameters of fermentation performance on assays performed using a mixture of soft drinks supplemented at different SBY ratios

Parameter	SBY ratio (% v/v)					
	2%	5%	10%	15%	20%	25%
ΔS_{12h} (g_s)	13 ± 2	43 ± 5	100	100	100	100
Y_b (g_b/g_s)	–	0.032 ± 0.002	0.033 ± 0.003	0.046 ± 0.003	0.042 ± 0.005	0.034 ± 0.003
r_b (g_b/L/h)	–	0.12 ± 0.02	0.32 ± 0.04	0.38 ± 0.03	0.42 ± 0.03	0.29 ± 0.05
r_s (g_s/L/h)	1.44 ± 0.05	3.58 ± 0.05	8.31 ± 0.03	8.35 ± 0.07	11.59 ± 0.05	17.8 ± 0.08
r_e (g_e/L/h)	–	1.52 ± 0.05	4.23 ± 0.05	3.93 ± 0.04	3.61 ± 0.07	3.63 ± 0.02
Y_e (g_e/g_s)	–	0.14 ± 0.02	0.39 ± 0.03	0.37 ± 0.04	0.31 ± 0.02	0.28 ± 0.03

ΔS sugar (s) consumption percentage at the end of experiment (12 h); Y_b biomass (b) yield; r_b biomass specific growth rate; r_s sugar consumption specific rate; r_e ethanol (e) production specific rate; and Y_e ethanol yield. The tabulated values denote the mean of the parameters calculated from three independent experiments

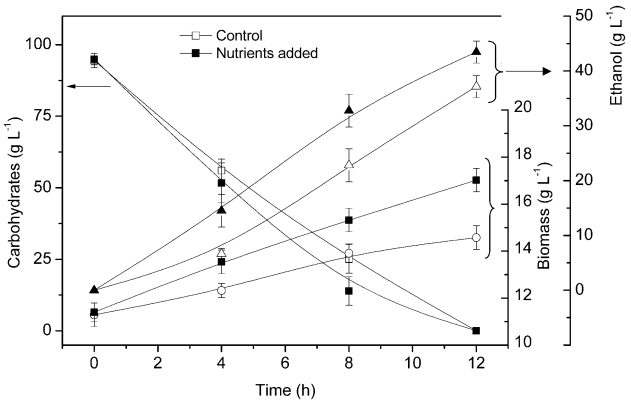


Fig. 2 SBY is deficient as a nutritional supplement. Evolution of the concentrations of biomass, sugar and ethanol on batch fermentation assays performed using a mixture of soft drinks (65% cola, 28% lemon-lime and 7% orange) with 10% v/v of SBY and supplemented with yeast extract (closed symbols). A control without yeast extract (open symbols) was also included. The values denote the mean (± SD) of three independent experiments

to be inadequate to support yeast growth and ethanol production, and thus, no further experiments were performed with this waste. On the other hand, both the CSW and the autolyzed SBY improved the alcoholic performance of the yeasts. Ethanol production increased according to the ratio of supplementation, and the highest ethanol yields (Ye) were obtained employing CSW as supplement. Both wastes contributed a negligible amount of carbohydrate to the fermentation medium. The increase in ethanol yield was directly associated with a more efficient conversion into ethanol of the sugars provided by soft-drink wastewaters. This improvement in the fermentative efficiency of the yeasts was related to both the availability and the quality of the nitrogen source. CSW was a better nutritional supplement than the others evaluated, and the wastes showed substantial differences in the organic nitrogen compound concentrations. CSW had a high FAN content (1200 ± 100 mg/L), whereas FAN was five times lower in autolyzed yeast (290 ± 50 mg/L) and

Table 3 Parameters of fermentations performed on SDW supplemented with different nitrogen sources

Parameter[a]	Control	Nitrogen source supplementation							
		Corn steep water (% v/v)			Autolyzed SBY (% v/v)			Inorganic salts[b]	YE[c] 10 g/L
		2%	5%	10%	2%	5%	10%		
$\Delta S_{12\,h}$ (g_s)	100	100	100	100	100	100	100	95 ± 3	100
Y_b (g_b/g_s)	0.033 ± 0.003	0.044 ± 0.005	0.058 ± 0.004	0.071 ± 0.004	0.035 ± 0.003	0.42 ± 0.002	0.051 ± 0.005	0.031 ± 0.002	0.056 ± 0.004
r_b (g_b/L/h)	0.32 ± 0.04	0.85 ± 0.01	1.12 ± 0.04	1.54 ± 0.03	0.33 ± 0.02	0.64 ± 0.03	0.88 ± 0.05	0.28 ± 0.02	0.79 ± 0.03
r_s (g_s L/h)	8.31 ± 0.03	11.4 ± 0.05	16.8 ± 0.02	21.2 ± 0.04	8.7 ± 0.02	9.2 ± 0.05	11.9 ± 0.04	7.50 ± 0.04	12.13 ± 0.06
r_e (g_e L/h)	4.23 ± 0.05	7.5 ± 0.04	9.8 ± 0.02	10.1 ± 0.03	4.7 ± 0.02	6.2 ± 0.023	8.1 ± 0.05	3.85 ± 0.05	5.4 ± 0.03
Y_e (g_e/g_s)	0.39 ± 0.03	0.40 ± 0.02	0.43 ± 0.04	0.45 ± 0.02	0.38 ± 0.01	0.39 ± 0.03	0.41 ± 0.02	0.35 ± 0.02	0.46 ± 0.02

All media were inoculated with 10% v/v SBY

ΔS sugar (s) consumption percentage at the end of experiment (12 h); Y_b biomass (b) yield; r_b biomass specific growth rate; r_s sugar consumption specific rate; r_e ethanol (e) production specific rate; and Y_e ethanol yield. The tabulated values denote the mean of the parameters calculated from three independent experiments

[b] $(NH_4)_2HPO_4$ 10.6 g/L, $MgSO_4 \cdot 7H_2O$ 6.4 g/L and $ZnSO_4 \cdot 7H_2O$ 7.5 mg/L [14]

[c] Commercial yeast extract (Britania Lab., Argentina)

negligible in WDGS. The enlarged characterization of the CSW is shown in Table 1.

Traditionally, CSW is concentrated at 5.5–7.5% w/v of solids to obtain the corn steep liquor. This is the major by-product of the corn wet-milling industry, and it contains a rich complement of organic nitrogen, vitamins, minerals and growth stimulants. It is capable of replacing yeast extract in a variety of fermentation processes. The corn steep liquor has been successfully used in the production of enzymes and organic acids [23–26]. Note that the CSW was used directly in the presented experiments. This might be relevant at the industrial scale. Because the concentration operations are cost- and time-consuming, the direct use of CSW as an inexpensive nitrogen source would reduce the overall costs of the fermentative process in the industrial production of bioethanol from sugar-sweetened wastewaters.

Because ammonia salts are good nitrogen sources for yeast metabolism, a previously developed inorganic medium was also evaluated [14]. The addition of inorganic salts did not enhance the fermentative performance of the SBY. Rather, it seemed to hinder it: a clear decrease in ethanol yield was obtained, among other affected fermentation parameters (Table 3, last column). Inorganic salt supplementation reduced the ethanol yield by approximately 10% in comparison to control medium (without salts). A brief explanation of this phenomenon could be as follows. The two main nitrogen sources available for yeast growth are amino acids and ammonium ions. Not all nitrogen sources support yeast growth equally. In particular, *S. cerevisiae* selects nitrogen sources by a mechanism called nitrogen catabolite repression (NCR) to achieve an efficient utilization of them and prevent the operation of useless pathways under a particular set of environmental conditions. Amino acids (organic nitrogen source) are transported into the cell by general and specific transport systems. Several permeases are nitrogen-regulated and become down-regulated at the transcriptional as well as the post-translational level in response to high-quality nitrogen sources such as ammonium. Ammonium availability is sensed by the ammonium-specific permeases named Mep, and the MEP genes are also subject to nitrogen control [27, 28]. Because SBY provided amino acids and the inorganic supplement incorporated ammonium in the medium, it is possible that an NCR mechanism was operating. To investigate this possibility, simple additional experiments were performed. The main yeast strain present in the SBY was isolated and purified to obtain an axenic culture. Fermentations were performed in triplicate on SDW inoculated with 2 g/L of yeast and supplemented with different nitrogen sources. As expected, the sugar consumption was complete in all cases in less than 12 h (not shown). Ethanol yields (Y_e, $g_{ethanol}/g_{sugar}$) lower than 0.40 were obtained when inorganic nitrogen was added to the fermentation medium: 0.29 ± 0.02 when $(NH_4)_2SO_4$ 10.6 g/L was added, 0.31 ± 0.03 when $(NH_4)_2HPO_4$ 10.6 g/L was added and 0.38 ± 0.02 when $(NH_4)_2HPO_4$ 10.6 g/L, $MgSO_4.7H_2O$ 6.4 g/L and $ZnSO_4.7H_2O$ 7.5 mg/L [14] was added. Yeast extract supplementation at 5% w/v yielded more ethanol (0.49 ± 0.03 $g_{ethanol}/g_{sugar}$), and this value represents the upper limit to this fermentation round. The addition of ammonium salts to yeast extract (at 5%) notably reduced the Y_e, which equaled 0.41 ± 0.04 or 0.42 ± 0.02 when $(NH_4)_2SO_4$ or $(NH_4)_2HPO_4$ at 10.6 g/L was added, respectively. It is clear that simultaneous availability of organic nitrogen (amino acids) and ammonium salts (inorganic nitrogen) affected the production of ethanol by yeasts. For this reason, it would not be convenient mix organic nitrogen sources and ammonium salts when SBY will be used as inoculum in a fermentation. Additional studies must be performed to generalize this fact to other yeast industrial strains.

The results presented in this section confirm that nitrogen supplementation is necessary to maximize the conversion into ethanol of the sugars contained in wastewaters of the soft drink industry. The next section addresses the optimization of the combined use of SBY and CSW for alcoholic fermentation of SDW.

Bioethanol Production Using SDW, SBY and CSW

A three-level central composite design was performed with different combinations of SBY and CSW to maximize the ethanol yields in fermentations from SDW. All experiments were performed in triplicate, and three replications at the central point were included. The experimental data and the values predicted by the model constructed using the final ethanol yield as response variable are shown in Table 4.

Table 4 Optimization of the supplementation ratios of SBY and CSW to maximize ethanol yield

Trial	Factor (% v/v)		Response variable: Y_e (g_e/g_s)	
	SBY	CSW	Experimental	Model-predicted value
1–3	10	10	0.443 ± 0.027	0.442
4–6	2.93	10	0.087 ± 0.004	0.074
7–9	10	10	0.440 ± 0.002	0.442
10–12	17.1	10	0.377 ± 0.044	0.359
13–15	15	15	0.267 ± 0.026	0.295
16–18	5	5	0.171 ± 0.014	0.175
19–21	5	15	0.105 ± 0.020	0.129
22–24	10	17.1	0.262 ± 0.024	0.232
25–27	10	2.93	0.348 ± 0.030	0.347
28–30	15	5	0.405 ± 0.025	0.412

Fermentations were performed on a mixture of soft drinks according to a central composite design

By applying multiple regression analysis to the experimental data, the following second-order polynomial equation giving the ethanol yield (Y_e) as a function of SBY [X_1] and CSW [X_2] concentrations was obtained: $Y_e = -0.5060 + 0.1175X_1 + 0.0601X_2 - 0.0045X_1^2 - 0.0007X_1X_2 - 0.0031X_2^2$.

Increased ratios of supplementation of SBY and CSW showed a clear positive correlation with ethanol yield, except for supplementation ratios above approximately 14 and 10% v/v, respectively, which had an opposite effect (Fig. 3a). The optimal concentrations for the two factors that maximized ethanol production were estimated using the optimization function in statistical software, resulting in 12.35% v/v SBY and 8.4% v/v CSW, with a predicted value of the maximum ethanol yield of 0.472 $g_{ethanol}/g_{consumed\ sugar}$. The contours of the estimated surface response are shown in Fig. 3b.

To verify the predicted results, three independent experiments were performed on a mixture of wastewaters of the soft drink industry supplemented with 12.4% v/v SBY and 8.4% v/v CSW. The evolution of the concentrations of biomass, sugar and ethanol was monitored over time (Fig. 4) and the production of other fermentation products, such as carbon dioxide and glycerol, was also measured. An average ethanol yield of 0.495 ± 0.005 $g_{ethanol}/g_{sugar}$ was achieved, i.e., approximately 105% of the value predicted by the software. The good agreement between the predicted and experimental results confirmed the validity of the model. Concerning the rest of yields, 0.09 $g_{glycerol}/g_{sugar}$, 0.41 g_{CO_2}/g_{sugar} and 0.06 $g_{biomass}/g_{sugar}$ were obtained. These values were very similar to those obtained in previous works performed on SDW using industrial yeast and commercial yeast extract as inoculum and nutritional supplement, respectively [12–14].

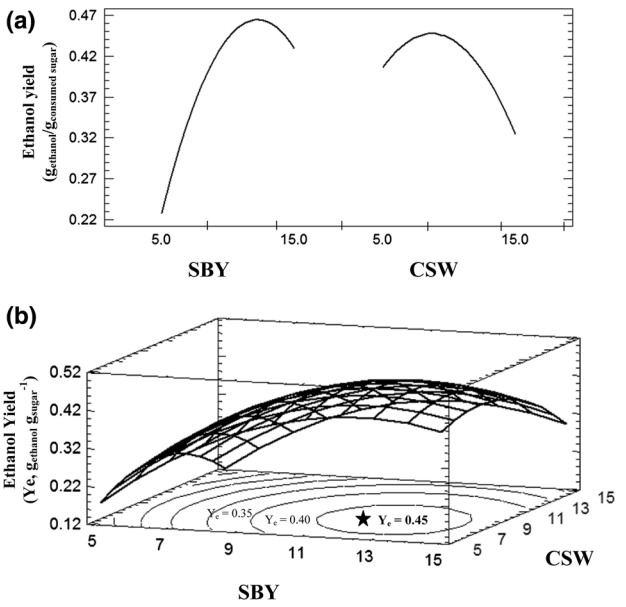


Fig. 3 Optimization of SBY and CSW supplementation ratios using surface-response methodology. Fermentations were performed on a mixture of soft drinks supplemented according to the three-level central composite design, whereas the ethanol yield (Y_e, $g_{ethanol}/g_{sugar}$) at the end of the experiment (12 h) was used as the response variable. **a** Main-effects graph of each factor on Y_e. **b** Estimated surface response and contours showing the effect on Y_e of SBY and CSW. The predicted optimal point (black star) corresponds to the following supplementation ratio: CSW 12.4% v/v and SBY 8.4% v/v

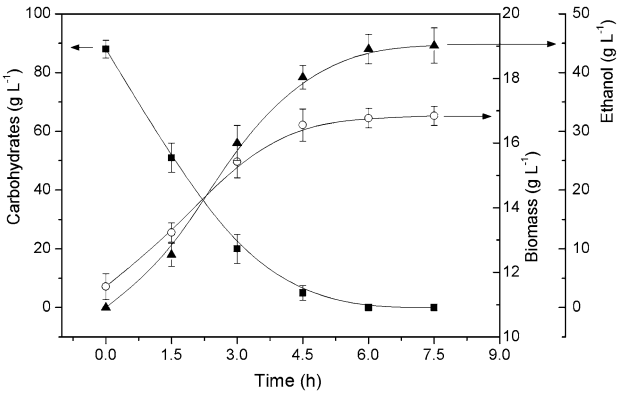


Fig. 4 Evolution of concentrations of biomass, sugar and ethanol during batch fermentation assays performed in triplicate on a mixture of soft drinks (65% cola, 28% lemon-lime and 7% orange) supplemented with 12.4% v/v of SBY and 8.4% v/v of CSW

To check the consistency of the experimental data, a balance of C was performed. The carbon fractions (g C/g compound) of the carbonaceous compounds involved in the fermentation were 0.40, 0.52, 0.27 and 0.39 for sugar, ethanol, CO_2 and glycerol, respectively, while the yeast 'formula' considered was $CH_{1.613}O_{0.557}N_{0.158}$ [29], with 0.48 g C/g compound being its corresponding carbon fraction. The sum of the yields multiplied by the respective carbon fractions in grams of carbon per grams of sugar was 0.430 g carbon/g sugar; that is, slightly higher than the theoretical value (0.40 g carbon/g sugar) suggesting that SBY and/or CSW could incorporate additional carbon sources to the media, such as the carbon skeletons of amino acids. It is of note that a similar event was reported in a previous work: the balance of C in assays performed on SDW using yeast extract as a supplement was 0.460 g carbon/g sugar [14]. This confirms the reliability and consistency of the experimental data as well as the fact that no carbonaceous compounds different from biomass, ethanol, carbon dioxide and glycerol were produced in significant amounts in the fermentation process.

Concerning the evaluation of the fermentation performance, some useful parameters were calculated following a previous method [12]. Notably, there was no latency time (lag phase) to the beginning of growth on SDW. Despite the presence of some preservatives and inhibitors on the SDW [12, 13], the main yeast composing the SBY needed no acclimatization, nor did its growth slow after inoculation in a fresh medium. In consonance with this growth,

no latency time to the initiation of sugar consumption was observed. When axenic cultures of the industrial strain *Saccharomyces cerevisiae* var. Windsor were used in fermentations performed on SDW, the lag phase duration depended on the nutritional supplement used. The yeast was previously aerobically grown in a rich medium and then harvested prior to inoculation in the fermentation medium. In these experiments, the yeast exhibit a lag phase of 2 or 4 h, depending on whether yeast extract or inorganic salt-based supplement, respectively, was used [14]. The absence of a lag phase in the experiments combining SDW, SBY and CSW may have been due to several reasons: (a) the SBY contained yeasts adapted to stress after several fermentation rounds; (b) the yeast displayed an active adaptive response to weak acids [30], and brewery medium contains some similar inhibitors; (c) the dead biomass and proteins or debris remaining from barley acted like filters stopping the entrance of preservatives inside the active yeasts; (d) all of the above.

The specific rates of biomass growth and sugar consumption were 1.72 ± 0.05 $g_{biomass}$/L/h and 24.0 ± 2.5 g_{sugar}/L/h, respectively, i.e., notably higher than the 0.77 ± 0.04 $g_{biomass}$/L/h and 15.0 ± 1.5 g_{sugar}/L/h obtained in fermentations performed on SDW inoculated at 2 g/L with industrial *S. cerevisiae* and commercial yeast extract as supplement [14]. Regarding the specific rate of ethanol production, the obtained value (10.3 ± 0.04 $g_{ethanol}$/L/h) was identical to the one reported for *S. cerevisiae* var. Windsor in fermentations performed on SDW supplemented with yeast extract (10.4 ± 0.03 $g_{ethanol}$/L/h; [13]). This fact suggests that the ethanol production rate depends on the yeast's enzymatic activities rather than sugar availability or biomass growth rate. The sugar consumption was completed in less than 6 h, whereas more than 8 h was necessary for sugar depletion in previous assays [13, 14]. So, the proposed process exhibits performance parameters compatible with industrial uses and can compete with current bioethanol production processes.

Finally, additional fermentations on SDW supplemented with 8.4% of CSW were performed, and the reactors were inoculated with 2.0 g/L of fresh yeast. The yeast employed is the same that was previously isolated from the SBY. This yeast was propagated aerobically prior to use as an inoculum in the mentioned fermentations. As expected, the fresh yeast led to the complete fermentation of the sugars in less than 8 h with an average ethanol yield of 0.504 ± 0.004 $g_{ethanol}$/g_{sugar} (not shown). Although this value is slightly higher than that obtained in fermentations directly using SBY, it is worth deepening the analysis from an overall perspective. A common practice at the industrial scale is to inoculate from an active yeast culture. In the case of the corn-based ethanol industry, the use of liquid yeast demands high volume handling, and the process involves yeast proliferation inside the reactor using the same feedstock (corn-starch hydrolyzed by enzymes). In breweries, sequential sub-cultures to high volumes are necessary to obtain the proper inocula. These procedures are cost and time-consuming. On the other hand, the configuration proposed in our work minimizes the cost and time associated with yeast manipulation. The direct use of SBY eliminates the requirement of aerobic proliferation reactors, media for yeast growth, energy for agitation and air supply. In addition, a laboratory for storing the strains and initiating the starters is not necessary. Therefore, the direct use of SBY could compensate the loss in ethanol yields compared to the use of a fresh culture.

Last but not least, the impact on fermentation of the "microbiological quality" of the CSW was evaluated. This waste contained large amounts of bacteria, mainly lactic acid bacteria and sporulated bacilli. This living biomass may compete with yeasts for fermentable sugars, so the fermentative performance of the SBY in medium supplemented with "crude" CSW or "sterile" CSW was compared. The crude CSW was conditioned by two treatments: (a) thermal shock (20 min at 80 °C) to eliminate vegetative (non-sporulated) forms and (b) autoclaving to eliminate all microorganisms (20 min at 121 °C). The fermentations were performed in triplicate, and the ethanol yields (Y_e) were calculated. The medium supplemented with CSW conditioned by thermal shock showed a Y_e slightly higher than control supplemented with crude CSW. Because fewer of bacteria were present at the beginning of the former fermentation, the yeasts might have had more available sugar without competition. In the medium supplemented with sterilized CSW, the average Y_e was not significantly different from control. Although competition by bacteria was completely eliminated, the high-temperature conditioning may have slightly reduced the sugar content, e.g., by the formation of condensation products such as 5-hydroxymethylfurfural. No additional experiments were performed at this point. The value of a thermal shock procedure on CSW should be analyzed from an economical standpoint. If the improvement in Y_e compensates for the cost associated with CSW conditioning, this step could be adopted in a potential bioethanol production process from sugar-sweetened wastewaters.

Conclusions

The combined utilization of agro-industrial wastewaters/by-products for non-lignocellulosic bioethanol production is technically feasible. The highly available carbohydrates present in *wastewaters of the sugar-sweetened beverage industry* could be fermented into ethanol by the yeast remaining in the *purges of fermentation tanks of the brewery industry*. The *corn steep water* from the corn starch processing industry was an efficient nitrogen and vitamin source for alcoholic fermentation. By application of RSM, an optimal relationship between the selected wastes was obtained. Fermentation

performed on SDW supplemented with 12.4% v/v SBY and 8.4% v/v CSW resulted in an average ethanol yield of 0.495 $g_{ethanol}/g_{sugar}$, with complete sugar consumption in less than 8 h. The process developed in this study could become cost- and time-efficient alternative bioprocess for industrial production of second-generation bioethanol.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest and no competing financial interest.

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