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Optimization of a low-cost defined medium for alcoholic fermentation – a case study for potential application in bioethanol production from industrial wastewaters

Research Paper

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In bioethanol production processes, the media composition has an impact on product concentration, yields and the overall process economics. The main purpose of this research was to develop a low-cost mineral-based supplement for successful alcoholic fermentation in an attempt to provide an economically feasible alternative to produce bioethanol from novel sources, for example, sugary industrial wastewaters. Statistical experimental designs were used to select essential nutrients for yeast fermentation, and its optimal concentrations were estimated by Response Surface Methodology. Fermentations were performed on synthetic media inoculated with 2.0 g L^{-1} of yeast, and the evolution of biomass, sugar, ethanol, CO_2 and glycerol were monitored over time. A mix of salts [10.6 g L^{-1} $(NH_4)_2HPO_4$; 6.4 g L^{-1} $MgSO_4{\cdot}7H_2O$ and 7.5 mg L^{-1} $ZnSO_4{\cdot}7H_2O$] was found to be optimal. It led to the complete fermentation of the sugars in less than 12 h with an average ethanol yield of 0.42 $g_{ethanol}/g_{sugar}$. A general C-balance indicated that no carbonaceous compounds different from biomass, ethanol, CO_2 or glycerol were produced in significant amounts in the fermentation process. Similar results were obtained when soft drink wastewaters were tested to evaluate the potential industrial application of this supplement. The ethanol yields were very close to those obtained when yeast extract was used as the supplement, but the optimized mineral-based medium is six times cheaper, which favorably impacts the process economics and makes this supplement more attractive from an industrial viewpoint.

Introduction

Q2 Currently, bioethanol is one of the most important renewable fuels. It is added to gasoline to reduce the negative environmental impact generated by the worldwide use of fossil fuels [1]. Several energy crops, including sugarcane, corn and jatropha, are used as raw materials for bioethanol production [2,3]. The high worldwide bioethanol demand exerts enormous pressure on primary production capacity. Thus, it is imperative that new renewable sources are identified for the production of this 'green' fuel [4]. As such, several alternatives have received increased focus, such as lignocellulosic biomass [5,6], regional agricultural discards [7] and wastewaters of the soft drink industry [8]. Although lignocellulosic residues represent an attractive renewable source for bioethanol production, the technology is not sufficiently developed, and the large quantity of wastewaters produced by the fermentation process poses a problem for large-scale production. Sugar-sweetened beverage wastewaters are generated in large quantities in proportion to the high production of these beverages (e.g., 6000 million L/year in Argentina), and some of them exhibit a high sugar content of approximately 60–180 g L^{-1}. In addition to using renewable raw materials, these alternative processes are 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.

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In biotechnology-based industrial processes, the composition of media is of critical importance due to its impact on product concentration and yield as well as on the overall process economics. To produce bioethanol at a laboratory scale, media was generally supplemented with yeast extract to provide specific nutrients such as organic nitrogen (in the form of amino acids and dipeptides), trace elements and vitamins. However, it is interesting to explore other sources of these additional nutrients in order to diminish production costs. For instance, mineral salts are generally used in industry to supplement the fermentation media and provide acceptable yields.

The main purpose of this research was to develop a low-cost mineral-based medium to replace yeast extract as the supplement for successful alcoholic fermentation. In this context, selected soft drink wastewaters were chosen as a test media to evaluate the potential industrial application of this supplement.

The use of a statistical approach has gained traction for medium optimization and for understanding the interactions among various factors using a minimum number of experiments. The combination of Full Factorial Designs (FFD) and Response Surface Methodology (RSM) is a commonly used method to assess the optimal media compositions and fermentation conditions, and it is an efficient statistical technique for the optimization of multiple variables [9]. This method has been successfully applied to optimize the composition of different media for alcoholic fermentations [10–13].

In this study, a statistically designed approach was used to optimize a low-cost mineral-based supplement for alcoholic fermentations mediated by *Saccharomyces cerevisiae*. The minerals that significantly improved the ethanol production were selected according to a series of FFD whereas the optimal concentration of key factors and parameter analysis were performed using a Central Composite Design (CCD) and RSM. Furthermore, the optimized mineral supplement was compared with the yeast extract in fermentation assays performed on a synthetic medium and on selected wastewaters. The concentrations of biomass, sugars, glycerol and ethanol, as well as the carbon dioxide production, were monitored over time in these experiences.

Material and methods

Strain, media and fermentations

The commercial yeast strain *S. cerevisiae* var. Windsor (Lallemand Brewing Co., Felixstowe, UK) was used throughout the screening and optimization experimental designs. Stock cultures were maintained on YPD (yeast extract 5 g L^{-1}, peptone 5 g L^{-1} and D-glucose 20 g L^{-1}) agar plates at 4°C. The culture was transferred to fresh medium monthly.

Fermentation assays were performed in triplicate using 500-mL glass flasks (300-mL working volume) operated in batch mode under anaerobic conditions and at a constant temperature of 30°C. Once inoculated with biomass, the reactors were closed until the end of the experiments. The gasses outlet passes through a water trap and the sampling was always outward, so that no air entry during the experiences. Despite that experiments began under a microaerobic atmosphere due to the oxygen present in the headspace of the reactor and in the initial fermentation medium, it is quickly displaced by the CO_2 produced during fermentation. Therefore, it can be assumed that the assays were carried out under anaerobic conditions. The pH was initially adjusted to 4.50 ± 0.10 and an orbital shaking (100 rpm) was maintained along the experiments to avoid the biomass precipitation. The initial concentration of yeast in each assay was 2.00 ± 0.10 g L^{-1}. The samples were collected immediately after inoculation ($t = 0$) and every 1.5 h until the end of the experiments.

Analytical procedures

During the fermentation assays, samples (1 mL) were taken in duplicate and immediately centrifuged for 5 min at 1200 × *g*. The pellet (yeasts) was washed five times 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). These measurements were correlated to biomass concentration using a calibration curve built according to the standard technique for determination of Volatile Suspended Solids (VSS). To build the calibration curve, the yeasts were grown on YPD medium at 30°C for 12–18 h and were then harvested by centrifugation for 5 minutes at 1200 × *g*, washed five times using distilled water. Several dilutions on distilled water were made by triplicate. An aliquot of each diluted sample was used for measure of turbidity (at 600 nm) using distilled water as blank. Another aliquot of the well-mixed sample (50-mL) was filtered in vacuum through a weighed standard Whatman GFC glass fiber filter (47 mm diameter and 1.2 μm nominal pore size, Biopore, Buenos Aires, Argentina) and the residue retained on the filter was dried to a constant weight at 103–105°C. The increase in weight of the filter represents the total suspended solids (TSS). The next step was the combustion of the filter at 500°C for 15 minutes and the weight lost after combustion represents the weight of Volatile Suspended Solids (VSS) in the sample [14].

The total sugar content was determined using the phenol-sulfuric acid colorimetric method [15], and the reducing sugar content was measured using the Miller colorimetric method [16]. The sugar concentration was calculated indirectly using a standard curve constructed from different concentrations of D-glucose (Merck, NJ, USA).

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 a previous work [8]. The CO_2 production was measured online using a mass flowmeter with a transducer (Matheson, East Rutherford, NJ, USA) and the total CO_2 production was estimated by integration. Glycerol was measured using an enzymatic kit (SB Lab., Santa Fe, Argentina), whereas ammonium, magnesium, zinc and inorganic phosphorus were determined by colorimetric methods (Wiener Lab., Rosario, Argentina).

Identification of the most important nutrient components

A statistical approach was performed to screen the following salts at the initial concentration recommended in the literature: $(NH_4)_2SO_4$ 10 g L^{-1}, $(NH_4)_2HPO_4$ 10 g L^{-1}, NH_4Cl 8 g L^{-1}, K_2SO_4 13.2 g L^{-1}, K_2HPO_4 13.2 g L^{-1}, KCl 11.3 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 5 g L^{-1}, $MgCl_2{\cdot}6H_2O$ 4.2 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 10 mg L^{-1}, $ZnCl_2$ 4.8 mg L^{-1}, $CaSO_4{\cdot}2H_2O$ 2.2 g L^{-1}, $CaCl_2$ 1.4 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 5 mg L^{-1}, $FeCl_3{\cdot}6H_2O$ 4.8 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 10 mg L^{-1}, $CuCl_2$ 5.3 mg L^{-1}, $CoSO_4{\cdot}7H_2O$ 5 mg L^{-1}, $CoCl_2$ 2.2 mg L^{-1}, $MnSO_4{\cdot}H_2O$

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5 mg L^{-1} and $MnCl_2{\cdot}4H_2O$ 5.8 mg L^{-1}. The effect of the inorganic salts on the performance of yeasts was evaluated using several parameters defined in a previous work [8], 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.

A set of Full Factorial Designs (FFD) was chosen as the strategy to determine which minerals had a significant effect on fermentation performance, primarily ethanol yield. First, the salts were grouped into different groups, and each one was considered a variable (factor) in the FFD. Their presence and absence levels ($k = 2$) were tested. All trials were performed in triplicate. The presence (or absence) of a 'group' of salts implies the presence (or absence) of all minerals included in this group. The ethanol yield was chosen as the response variable. The significance of the factors and their interactions were tested through the analysis of variance (ANOVA). A p-value lower than 0.05 was considered statistically significant for analyzed variables. Finally, the analysis of each group was deepened by considering each individual salt as a variable in a new FFD.

Optimization of mineral ingredients

A Central Composite Design (CCD) that included the independent variables (factors) selected as significant in the screening design was performed to determine the concentrations of these salts that maximize ethanol production. The axial distance was chosen to be 1.68 to allow a rotatable design. For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between the independent variables and the response. The selected factors were correlated by the following equation:

$$Y = b_0 + \sum b_i X_i + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2$$

where Y is the predicted response corresponding to the ethanol yield at the end of the fermentation process. X_i and X_j are the independent variables, b_0 is an offset term, b_i and b_j are linear effects and b_{ij} is an interaction term.

Data analysis

All analysis of statistical experimental designs and results was performed using Statgraphics® Centurion XV v15.2.06 (StatPoint Inc., USA). 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. The variance explained by the model is given by the multiple determination coefficients, R^2, and the parameter values should be close to 1.0 for a good statistical model, while a value above 0.75 indicates the suitability of the model [17].

Results and discussion

Screening of minerals that affect the bioethanol production of Saccharomyces cerevisiae *var. Windsor*

As reported in the literature [11,13,18–20], the following salts could exert a favorable effect on the alcoholic fermentation mediated by *S. cerevisiae*: $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2HPO_4$, K_2SO_4, KCl, K_2HPO_4, $MgSO_4$, $MgCl_2$, $ZnSO_4$, $ZnCl_2$, $CaSO_4$, $CaCl_2$, $FeSO_4$, $FeCl_3$, $CuSO_4$, $CuCl_2$, $CoSO_4$, $CoCl_2$, $MnSO_4$ and $MnCl_2$. Nevertheless, preliminary tests showed that chloride salts (at the same cation concentrations) had a less evident effect than the sulfates of the same cation on fermentation and even negative effects in some cases, probably due to the inhibitory effect of the chloride ion reported in previous works [21]. Therefore, all metals were incorporated in the form of sulfate salts in the fermentation assays.

For the screening of the key minerals, the selected salts were clustered into three main groups. The 'group A' included the major macronutrients (required in the order of millimolar concentrations) for yeast metabolism: $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, K_2SO_4 and K_2HPO_4. The 'group B' included $MgSO_4$, $ZnSO_4$ and $CaSO_4$, whereas $FeSO_4$, $CuSO_4$, $CoSO_4$ and $MnSO_4$ were included in 'group C'. Full Factorial Design (FFD)-based experiments were performed to select the major minerals that enhanced the fermentation parameters of the *S. cerevisiae* var. Windsor. Fermentations were carried out on synthetic media while the evolution of biomass, sugar and ethanol concentrations was monitored over time.

The first FFD included groups A, B and C as variables and all trials were performed in triplicate (number of degrees of freedom for errors = 15), resulting in 24 independent experiments. The performance parameters of the fermentation were reported in Table 1. In all trials, the lag time (λ) was significantly higher than the observed when yeast extract was used as a nutrient. Complete

TABLE 1

Yeast performance in fermentations performed on synthetic media (100 g L^{-1} initial sugar) supplemented according to the factorial design.

Trial	Factorial design[a]			Parameter[b]						
	A	*B*	*C*	λ (h)	r_b (g_b L^{-1} h^{-1})	Y_b (g_b/g_s)	ΔS (g_s)	r_s (g_s L^{-1} h^{-1})	r_e (g_e L^{-1} h^{-1})	Y_e (g_e/g_s)
1–3	+	–	–	4.37	0.43	0.053	100	12.77	8.95	0.330
4–6	–	+	–	5.95	0.19	0.021	65.1	6.32	2.87	0.108
7–9	–	–	+	7.26	0.12	0.014	38.7	5.91	1.49	0.082
10–12	+	+	–	3.75	0.56	0.064	100	13.81	9.34	0.361
13–15	+	–	+	4.76	0.39	0.046	100	13.29	9.06	0.321
16–18	–	+	+	5.78	0.15	0.024	65.3	6.53	4.56	0.116
19–21	+	+	+	3.69	0.53	0.066	100	13.78	9.22	0.364
22–24	–	–	–	7.65	0.10	0.010	35.2	3.43	1.18	0.073
Yeast extract (control)				1.98	0.73	0.099	100	15.22	10.31	0.484

The values denote the mean of triplicate independent biological experiments. Standard deviations were intentionally excluded to simplify the reading.

[a] Groups: A, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, K_2SO_4 and K_2HPO_4; B, $MgSO_4$, $ZnSO_4$ and $CaSO_4$; C, $FeSO_4$, $CuSO_4$, $CoSO_4$ and $MnSO_4$;'+', presence; '–', absence.[b] λ, lag phase duration; r_b, biomass (b) specific growth rate; Y_b, biomass yield; ΔS, net sugar (s) consumption at the end of experiment; r_s, sugar consumption specific rate; Y_e, ethanol (e) yield and r_e, ethanol production specific rate.

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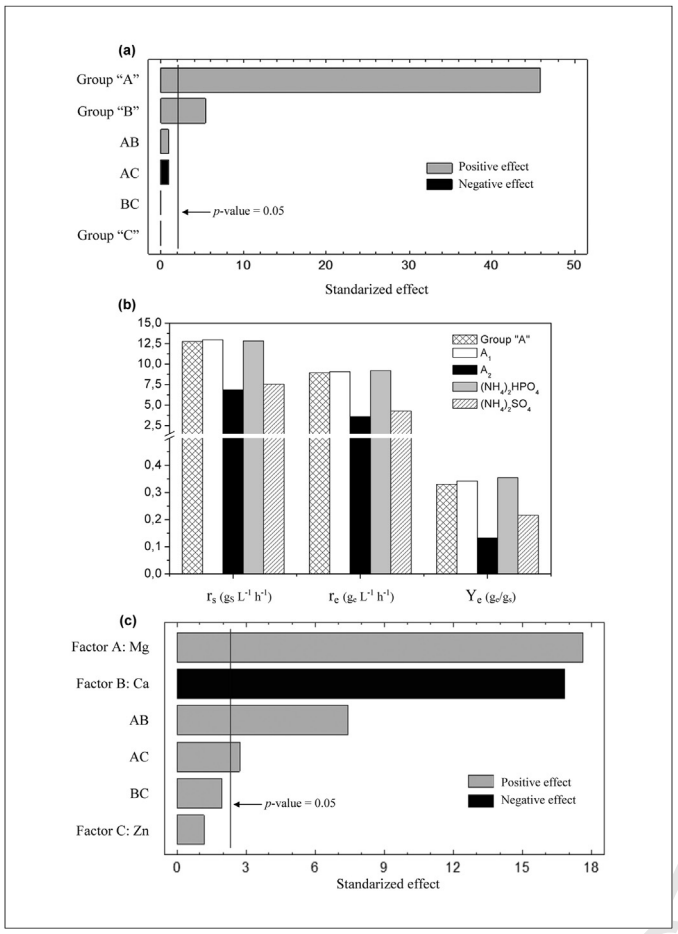

FIGURE 1

Evaluation of the effect of selected minerals on yeast performance. Screening assays were performed on synthetic media supplemented according to a sequential set of full factorial designs. **(a)** and **(c)** Standardized Pareto chart for ethanol yields (Y_e) corresponding to experiments presented in Tables 1 and 3, respectively. The vertical line was drawn at the location of the 0.05 value for the Student's *t*. Bars that extend to the right of this line indicate effects that are statistically significant (level higher than 95%). **(b)** Comparative bar graphs for some parameters reported in Table 2. *References*: 'r_s,' sugar (s) consumption specific rate; 'Y_e,' ethanol (e) yield and 'r_e,' ethanol production specific rate.

sugar consumption was achieved only in the media containing the minerals of group A (sources of N, P, S and K). The specific rates of biomass growth (r_b), sugar consumption (r_s) and ethanol production (r_e) were also clearly favorably influenced by the salts of group A, and this effect was increased in the presence of the salts of group B (Mg, Ca and Zn). Additionally, the ethanol yield (Y_e) was approximately 10% higher in trials supplemented with both salt groups in comparison to those supplemented only with group A.

The influence of the principal effect of each factor using the ethanol yield as the response variable was determined through analysis of variance. These effects are depicted in Fig. 1a as a standardized Pareto chart. Regression analysis showed that the model is adequate ($R^2 = 0.96$ and adjusted $R^2 = 0.94$). As expected, groups A and B showed a positive effect and contributed significantly in enhancing the ethanol production. The probability values (*p*-value) were below 0.01 (significance level higher than 99%), and the effect of group A was the most critical.

Although several trace elements were reported as being important to yeast metabolism [20,22,23], the contribution from group C was not significant (*p*-value >0.05). Therefore, these minerals were disregarded for future tests.

As seen in Fig. 1a, there was no significant effect for the interactions; therefore, the aforementioned increase in ethanol yield when the B-salts were added to a medium containing A-salts could be due to the sum of some individual effects. The next step was to deeply study groups A and B.

Ammonium phosphate exerts the main effect on yeast metabolism

The effect of the salts present in group A was evaluated as follows. First, the salts were regrouped into two subgroups: A_1 [$(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$] and A_2 [K_2SO_4 and K_2HPO_4] to differentiate the effects of ammonium and potassium ions on yeast performance. These new groups were considered as variables in a FFD ($k = 2$). Next, as a major positive effect from the A_1 subgroup and a negative interaction between the two subgroups on ethanol yield was observed (see below), the individual effect of each ammonium salt present in subgroup A_1 was tested. In all these fermentations, the salts of groups 'B' and 'C' were not added to the medium, and all assays were performed in triplicate.

Fermentations were carried out on synthetic media, while the evolution of biomass, sugar and ethanol concentrations was monitored over time. The performance parameters were reported in Table 2. To better illustrate the differences, several bar graphs are shown in Fig. 1b.

The results showed that among the salts of group A, ammonium phosphate had the most significant positive effect on yeast metabolism. In trials only supplemented with this salt, sugar consumption was complete and higher ethanol yields and ethanol production specific rates were obtained, confirming that nitrogen and phosphorus sources are critical macronutrients for yeast fermentation. *S. cerevisiae* is a non-diazotrophic and non-proteolytic organism (i.e., it cannot fix nitrogen and it is incapable of using proteins as a nitrogen source). Therefore, the fermentation media should contain readily utilizable sources of nitrogen [20], which can be provided by ammonium salts [24,25]. From Table 2, it can be noticed that the addition of ammonium sulfate as the sole source of nitrogen was not enough to achieve complete sugar consumption, reinforcing that phosphate is essential for uptake of carbon sources and for the energy metabolism of yeasts. Inorganic phosphate is one of the most abundant anions in cells and plays an essential role in the synthesis of nucleic acids, phospholipids, amino acids and several key cellular metabolites [26,27].

Concerning potassium, the most abundant cation in yeast (1–2% of cell dry weight), it is an essential electrolyte for osmoregulation, charge-balancing of macromolecules, protein biosynthesis, carbohydrate catabolism and the regulation of phosphate and divalent cation uptake, among others functions [20,28]. Nevertheless, in our assays, it was observed that the inclusion of potassium salts favors biomass growth in detriment to the ethanol production. This should be attributed to the role played by potassium in the activation of enzymes that could redirect carbonaceous intermediates of anaerobic sugar metabolism toward anabolic pathways, such as amino acid synthesis [20,22].

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TABLE 2

Effect of minerals included in group 'A' [$(NH_4)_2SO_4$; $(NH_4)_2HPO_4$; K_2SO_4 and K_2HPO_4] on yeast performance.

Trial	Factorial design[a]		$(NH_4)_2SO_4$	$(NH_4)_2HPO_4$	Parameter[b]						
	A_1	A_2			λ (h)	r_b (g_b L^{-1} h^{-1})	Y_b (g_b/g_s)	ΔS (g_s)	r_s (g_S L^{-1} h^{-1})	r_e (g_e L^{-1} h^{-1})	Y_e (g_e/g_s)
1–3	+	–	–	–	4.36	0.38	0.035	100	12.97	9.07	0.342
4–6	–	+	–	–	3.83	0.24	0.028	68.9	6.88	3.61	0.133
7–9	+	+	–	–	4.17	0.44	0.055	100	12.85	8.99	0.321
10–12	–	–	–	–	7.65	0.10	0.010	35	3.43	1.18	0.078
13–15	–	–	+	–	5.11	0.34	0.036	58.2	7.56	4.29	0.216
16–18	–	–	–	+	4.16	0.45	0.037	100	12.84	9.22	0.354
Group 'A' (A_1 + A_2)					4.37	0.44	0.053	100	12.77	8.95	0.330

The values denote the mean of triplicate independent biological experiments. Standard deviations were intentionally excluded to simplify the reading.
[a] Subgroups: A_1, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$; A_2, K_2SO_4 and K_2HPO_4. [b] λ, lag phase duration; r_b, biomass (b) specific growth rate; Y_b, biomass yield; ΔS, net sugar (s) consumption at the end of experiment; r_s, sugar consumption specific rate; Y_e, ethanol (e) yield and r_e, ethanol production specific rate.

TABLE 3

Effect of minerals included in group 'B' ($MgSO_4$, $CaSO_4$ and $ZnSO_4$) on yeast performance in fermentations performed on synthetic media with $(NH_4)_2HPO_4$ 10 g L^{-1} (in all cases) supplemented according to the factorial design.

Trial	Factorial design			Parameter[a]						
	$MgSO_4$	$CaSO_4$	$ZnSO_4$	λ (h)	r_b (g_b L^{-1} h^{-1})	Y_b (g_b/g_s)	ΔS (g_s)	r_s (g_S L^{-1} h^{-1})	r_e (g_e L^{-1} h^{-1})	Y_e (g_e/g_s)
1–3	–	–	–	4.23	0.47	0.036	100	12.82	9.33	0.342
4–6	+	–	–	3.75	0.50	0.038	100	13.21	9.47	0.387
7–9	–	+	–	3.66	0.57	0.062	100	13.48	4.53	0.171
10–12	–	–	+	4.29	0.46	0.035	100	12.78	9.26	0.324
13–15	+	+	–	3.71	0.54	0.048	100	13.44	8.86	0.322
16–18	+	–	+	3.78	0.51	0.038	100	13.25	9.56	0.410
19–21	–	+	+	3.64	0.55	0.061	100	13.51	4.46	0.183
22–24	+	+	+	3.89	0.53	0.056	100	13.49	9.29	0.355

The values denote the mean of triplicate independent biological experiments. Standard deviations were intentionally excluded to simplify the reading.
[a] λ, lag phase duration; r_b, biomass (b) specific growth rate; Y_b, biomass yield; ΔS, net sugar (s) consumption at the end of experiment; r_s, sugar consumption specific rate; Y_e, ethanol (e) yield and r_e, ethanol production specific rate.

Following the same stepwise explanation so far, ammonium phosphate was selected for the following trials, and both the individual and combined effects of each mineral included in group B were evaluated.

The addition of magnesium and zinc salts increased the effect of ammonium phosphate

Magnesium, calcium and zinc have been reported to favorably influence the rate of sugar conversion, and these cations are required as cofactors for several metabolic pathways [11,19,20]. Furthermore, the protective effects of magnesium and calcium against ethanol stress have been extensively studied [13,20,29]. Accordingly, the last set of FFD-based experiments was performed using the Mg, Zn and Ca salts as variables in an attempt to establish their individual effect on yeast metabolism. All assayed media included ammonium phosphate as a supplement, and all trials were performed in triplicate (number of degrees of freedom for errors = 15), resulting in 24 independent experiments.

Table 3 shows the experimental data, while the corresponding standardized Pareto charts constructed using ethanol yield as the response variable are shown in Fig. 1c. Regression analysis showed that the model is adequate (R^2 = 0.98 and adjusted R^2 = 0.96). Note that results obtained in assays performed on control media (supplemented only with ammonium phosphate, trials 1–3) were similar to those reported for trials 16–18 in Table 2. This underlies the consistency of the obtained data. Similar results were observed for trials 22–24 (supplemented with all minerals) and the data reported in Table 1 (AB, trials 10–12); the slight differences can be attributed to the presence of potassium salts in latter.

The ANOVA of the results of the screening experiments shows that the addition of $MgSO_4$ had a significant positive effect on ethanol production (p-value < 0.01), while the supplementation with $ZnSO_4$ alone also showed a positive – but not significant ($p > 0.05$) – effect. Conversely, the addition of $CaSO_4$ had an unexpected strong negative effect on ethanol production. Regarding the interactions between these minerals, it was noted that the addition of $ZnSO_4$ enhanced the positive effect induced by $MgSO_4$. Despite the fact that media containing salts of both Mg and Ca showed an improved ethanol yield, competition between Mg and Ca salts was evident, as seen in Fig. 1c and Table 3.

In addition to increasing the ethanol yields (Y_e) obtained in control media (supplemented just with ammonium phosphate) by approximately 10%, the presence of magnesium ions reduced lag time and increased the specific rates of biomass growth, sugar

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TABLE 4

Optimization of the concentrations of the minerals (factors) selected by previous screenings. Fermentations were performed on synthetic media supplemented according to a Central Composite Design.

Trial	Factor (g L^{-1})		Response variable: Y_e (g_e/g_s)		
	$(NH_4)_2HPO_4$	$MgSO_4$	$ZnSO_4$	Experimental[a]	Model[b]
1–2	15	2.5	15	0.267 ± 0.005	0.242
3–4	5	7.5	5	0.374 ± 0.008	0.380
5–6	10	9.2	10	0.428 ± 0.002	0.443
7–8	5	7.5	15	0.307± 0.007	0.271
9–10	10	5	10	0.440 ± 0.004	0.428
11–12	10	5	1.6	0.390 ± 0.002	0.401
13–14	15	7.5	5	0.381 ± 0.003	0.357
15–16	15	7.5	15	0.324 ± 0.005	0.338
17–18	1.6	5	10	0.142 ± 0.006	0.167
19–20	10	0.8	10	0.277 ± 0.009	0.289
21–22	5	2.5	5	0.326 ± 0.005	0.293
23–24	10	5	10	0.421 ± 0.003	0.428
25–26	5	2.5	15	0.031 ± 0.007	0.037
27–28	18.4	5	10	0.320 ± 0.004	0.321
29–30	10	5	18.4	0.155 ± 0.008	0.170
31–32	15	2.5	5	0.390 ± 0.005	0.408

[a] The values denote the mean (±SD) of duplicate independent experiments.
[b] Model-predicted value.

consumption and ethanol production. This metal is essential for yeast metabolism because it acts as a cofactor for many key enzymes of sugar metabolism, among which are hexokinase (E.C. 2.7.1.1), phosphofructokinase (E.C. 2.7.1.11), enolase (E.C. 4.2.1.11) and pyruvate decarboxylase (E.C. 4.1.1.1), in addition to being indispensable for cell division and growth, organelle structure and protection against osmotic stress [20,22,28–30].

The addition of $ZnSO_4$ to media containing $(NH_4)_2HPO_4$ and $MgSO_4$ led to an approximately 8% increase in the ethanol yield. This is consistent with the role played by zinc as an activator of ethanol dehydrogenase (E.C. 1.1.1.1), a metalloenzyme that catalyzes the conversion of acetaldehyde into ethanol that is coupled to NAD^+ reduction [19,22,31].

Because the results showed an unexpected negative influence of $CaSO_4$ on the fermentation metabolism of yeast, the effect of this ion required a particular analysis. When calcium was added to media, a significant decrease in both ethanol yield and the specific rate of ethanol production was observed, while the growth of yeasts and biomass yield were notably favored when compared with media supplemented with $(NH_4)_2HPO_4$. Although the experiments were initiated in a microaerobic atmosphere, the sugars were metabolized down the fermentative pathway due to the predominance of the Crabtree effect over the Pasteur Effect, a trend that occurs in media containing high concentrations of sugars [22]. The magnesium availability is one of the reported reasons to explain this phenomena. In essence, this hypothesis proposes that pyruvate decarboxylase (which channels carbon down the fermentative pathway) and pyruvate dehydrogenase (which channels carbon down the respiratory pathway) possess low and high affinities for free intracellular magnesium ions, respectively [20]. Calcium antagonizes magnesium uptake and can suppress magnesium-dependent enzymes, thus promoting respiration and maximizing yeast biomass [20]. Based on these results, calcium must be avoided when the Windsor strain of *S. cerevisiae* is used if maximum ethanol yields are desired. Additional studies must be performed to generalize this fact to other industrial strains, mainly on hybrid strains containing the genetic background of *S. cerevisiae*.

Optimization of the mineral concentrations

A three-level Central Composite Design was performed with different combinations of the minerals that were selected as significant by previous screenings to enhance the final ethanol yields, for example, $(NH_4)_2HPO_4$, $MgSO_4$ and $ZnSO_4$. All experiments were performed in duplicate and two replications at the central point were included (number of degrees of freedom for errors = 21), leading to a total number of 32 trials. Table 4 shows the experimental data and the values predicted by the model constructed using the final ethanol yield as the response variable. 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 $(NH_4)_2HPO_4$ [X_1], $MgSO_4$ [X_2] and $ZnSO_4$ [X_3] concentrations was obtained:

$$Y_e = -0.0677 + 0.0659X_1 + 0.0519X_2 + 0.0028X_3 - 0.0026X_1^2 - 0.0027X_1X_2 + 0.0009X_1X_3 - 0.0035X_2^2 + 0.0029X_2X_3 - 0.0020X_3^2$$

The ANOVA of the experimental results showed that the ethanol yield was statistically influenced by all assayed factors and their interactions. The regression analysis of the data showed that the polynomial equation was a suitable model to describe the

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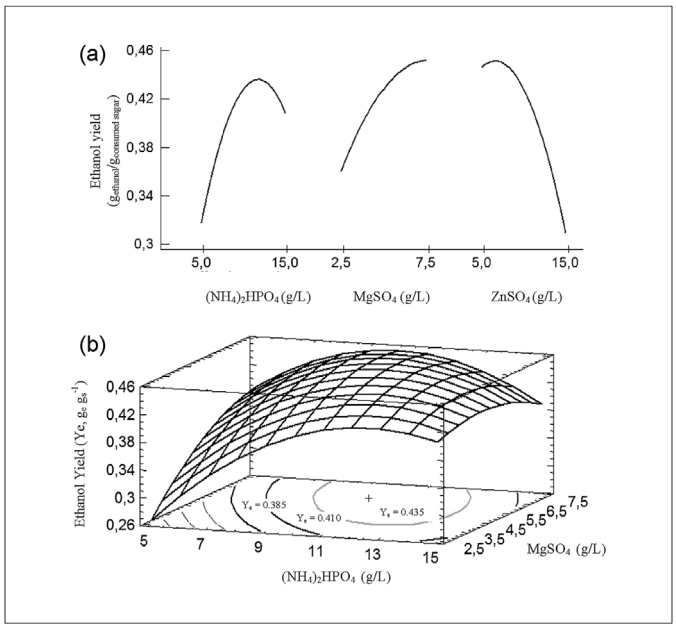

FIGURE 2

Optimization of $(NH_4)_2HPO_4$, $MgSO_4$ and $ZnSO_4$ concentrations using surface response methodology. Fermentations were performed on synthetic media supplemented according to the three-level central composite design, whereas the ethanol yield (Y_e, $g_{ethanol}/g_{consumed\ sugar}$) at the end of the experiments was used as the response variable. **[a]** Main effects graph of each mineral on Y_e. **[b]** Estimated surface response and contours showing the effect on Y_e of $(NH_4)_2HPO_4$ and $MgSO_4$ at a fixed level of $ZnSO_4$ (7.5 mg L^{-1}). The predicted optimal point ('+') corresponds to the following concentrations: $(NH_4)_2HPO_4$ 10.6 g L^{-1}, $MgSO_4$ 6.4 g L^{-1} and $ZnSO_4$ 7.5 mg L^{-1}.

response of the experiment to ethanol production (R^2 value of 0.97 and adjusted R^2 value of 0.92).

The concentrations evaluated for each factor were 5.0, 10.0 and 15.0 g L^{-1} for $(NH_4)_2HPO_4$; 2.5, 5.0 and 7.5 g L^{-1} for $MgSO_4$ and 5.0, 10.0 and 15.0 mg L^{-1} for $ZnSO_4$. These values were defined by taking the concentration used in the screenings as central values ±50% for the upper and lower limits. Fig. 2a shows the effects of each factor on the response variable. Increased concentrations of $(NH_4)_2HPO_4$ and $MgSO_4$ showed a positive correlation with ethanol yields, except for concentrations above approximately 13 g L^{-1} for the ammonium salt, values from which an opposite effect was detected. Conversely, increased $ZnSO_4$ concentrations had a negative effect on maximum ethanol yields, possibly due to the toxic effects of zinc (and other heavy metals like copper) on fungi when threshold concentrations were overcome [22,32]. The optimal concentrations for the three factors that maximize ethanol production were estimated using the optimization function in the statistical software, resulting in $(NH_4)_2HPO_4$ 10.6 g L^{-1}, $MgSO_4$ 6.4 g L^{-1} and $ZnSO_4$ 7.5 mg L^{-1}, with a predicted value of the maximum ethanol yield of 0.435 $g_{ethanol}/g_{consumed\ sugar}$. The contours of the estimated surface response showing the effect of $(NH_4)_2HPO_4$ and $MgSO_4$ on ethanol yield at a fixed optimal zinc concentration are shown in Fig. 2b.

To verify the predicted results, three independent experiments were performed on synthetic media with the aforementioned optimized conditions. The evolution of concentrations of biomass, sugar and ethanol were monitored over time as well as the carbon dioxide production. Because glycerol is the main non-volatile by-product of alcoholic fermentations [22,33], its concentration was also determined at the beginning and the end of the experiments. An average ethanol yield of 0.418 ± 0.005 g L^{-1} was achieved, approximately 96% of the value predicted by the software. The good agreement between the predicted and experimental results confirmed the validity of the model. The complete set of yeast performance parameters is shown in Table 5 while the parameters of additional fermentations performed using yeast extract as a nutrient were also reported. The latter tests were performed (a) to obtain additional information to that reported in a previous work [8] in which neither CO_2 production nor the final glycerol concentrations were determined and (b) to ensure the fidelity of comparison between salts and yeast extract as nutritional supplements, avoiding differences in the performance of yeast that could be due to variability in the yeast extract batch, some operating variables (temperature, stirring speed, etc.) or inoculum features (e.g., 'age', genetic variations, etc.).

Unlike media supplemented with yeast extract, vitamins, trace elements and/or growth factors are absent in media supplemented with salts. Therefore, a higher lag time in yeast growth is observed in the latter case. Nevertheless, the fermentative metabolism of the yeasts was not affected; sugars were completely consumed in less than 12 h, and the specific rates of sugar consumption and ethanol production were slightly lower that the observed when yeast extract was used as a supplement. However, it is noteworthy that the glycerol and CO_2 yields were similar for both supplements, while differences in the biomass and ethanol yields were evident.

To check the consistency of the experimental data, a balance of C was performed for each assayed medium. 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,

TABLE 5

Comparison of parameters of yeast performance in assays performed on synthetic medium.

Parameter[a]	Synthetic media	
	Yeast extract (15 g L^{-1})	Optimized minerals
Initial sugar content (g L^{-1})	100	
λ (h)	1.73	3.75
r_b (g_b L^{-1} h^{-1})	0.77	0.52
r_s (g_s L^{-1} h^{-1})	14.89	13.23
r_e (g_e L^{-1} h^{-1})	10.36	9.64
α (h)	8.22	10.17
Y_e (g_e/g_s)	0.482	0.418
Y_b (g_b/g_s)	0.108	0.041
Y_{CO_2} (g_{CO_2}/g_s)	0.409	0.420
$Y_{glycerol}$ (g_g/g_s)	0.120	0.101

The values denote the mean of triplicate independent biological experiments. Standard deviations were intentionally excluded to simplify the reading.

[a] λ, lag phase duration; r_b, biomass (b) specific growth rate; r_s, sugar (s) consumption specific rate; r_e, ethanol (e) production specific rate; α, time of complete sugar consumption; Y_b, biomass yield; Y_e, ethanol yield; Y_g, glycerol yield; Y_{CO_2}, carbon dioxide yield.

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CO_2 and glycerol, respectively, while the yeast 'formula' considered was $CH_{1.613}O_{0.557}N_{0.158}$ [34], with 0.48 g C/g compound being its corresponding carbon fraction. For the assays performed with minerals as a supplement, the sum of the yields reported in Table 5 multiplied by the respective carbon fractions in grams of carbon per grams of sugar was 0.390; this value successfully closed the C balance (the theoretical value is 0.40 g carbon/g sugar). 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. Moreover, the balance of C for assays using yeast extract as a supplement was 0.460 g carbon/g sugar, that is, a bit higher than the theoretical value, suggesting that yeast extract incorporates additional carbon sources to the media, such as the carbon skeletons of amino acids.

An example of a potential industrial application of the developed inorganic supplement

To explore the technical feasibility of applying the mineral supplement to the production of bioethanol using yeast-mediated fermentation from sugar-sweetened beverage industry wastewaters, we selected soft drink wastewater as a case of study. These effluents, 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 generated in large quantities (close to 250 million L/year in Argentina) and exhibit high sugar content (approximately 10–12%, w/v). In a previous work, the technical feasibility of the fermentation process has already been demonstrated and the effect of adding yeast extract was evaluated, resulting in 15 g L^{-1} 1 as a suitable value for successful fermentation [8]. Then, in similar fashion, several experiments were performed using individual soft drinks (cola type, lemon-lime and orange) and a mixture of them, but they were supplemented with mineral salts at their optimal concentrations. In addition, the same media but supplemented with yeast extract in the aforementioned concentration (15 g L^{-1}) [8] was also assayed for comparative purposes and to avoid differences in the performance of yeast that could be due to variability in the soft drink composition or operating variables.

The content of total nitrogen (N), magnesium (Mg), zinc (Zn) and inorganic phosphorus (P_i) were determined for each assayed soft drink. No significant amounts of N, Mg or Zn were detected, whereas P_i was only detected in cola-type at a concentration of approximately 0.175 g L^{-1} due to the H_3PO_4 included in the formulation of this soft drink. Because N, Mg and Zn were not detected and the measured P_i concentration was negligible when compared with the 2.54 g L^{-1} of P_i supplied by the $(NH_4)_2HPO_4$ at its aforementioned optimal value (10.6 g L^{-1}), adjustments to the concentrations of added minerals were not necessary.

The concentrations of sugar, biomass, ethanol and glycerol, as well as the CO_2 production, were monitored over time during the fermentation assays. The most relevant parameters of the performance of the yeasts are summarized in Table 6. The agreement with the values reported in previous works in which yeast extract was used as a supplement [8] confirms the consistency of the obtained results.

For mineral-based tests on orange and lemon-lime soft drinks, the carbon balances were very close to the theoretical value (0.40 g carbon/g sugar): 0.384 and 0.387 g carbon/g sugar, respectively. This indicates that there were not significant amounts of different carbon sources from sugar in these beverages and that other non-volatile by-products of alcoholic fermentation such as acetic, pyruvic and succinic acids [22,33] are produced in negligible amounts. For cola-type soft drinks, the C balance value (0.411 g carbon/g sugar) result was slightly higher than the theoretical, which is consistent with the presence of small amounts of additional carbonaceous sources (e.g., plant extracts) used in the formulation of these beverages.

TABLE 6

Comparison of yeast performance in fermentation performed on wastewaters selected as a case-study.

Parameter[a]	Soft drink[b]							
	Cola		Lemon-lime		Orange		Mix[c]	
	Ext	Min	Ext	Min	Ext	Min	Ext	Min
Initial sugar content (g L^{-1})	105		105		120		106	
λ (h)	0.85	2.12	5.00	5.26	2.13	2.85	0.67	1.79
r_b (g_b L^{-1} h^{-1})	0.75	0.72	0.53	0.49	0.55	0.52	0.84	0.78
r_s (g_s L^{-1} h^{-1})	15.79	11.95	10.21	9.29	13.89	11.18	15.07	13.81
r_e (g_e L^{-1} h^{-1})	10.36	9.43	5.23	4.65	7.57	6.82	8.14	7.65
α (h)	7.62	9.88	12.11	13.64	8.95	11.46	8.74	10.32
Y_e (g_e/g_s)	0.494	0.437	0.383	0.364	0.428	0.400	0.458	0.421
Y_b (g_b/g_s)	0.099	0.052	0.051	0.034	0.057	0.047	0.113	0.055
Y_{CO_2} (g_{CO_2}/g_s)	0.442	0.419	0.394	0.453	0.428	0.421	0.433	0.402
$Y_{glycerol}$ (g_g/g_s)	0.149	0.118	0.124	0.152	0.127	0.102	0.127	0.119

The values denote the mean of triplicate independent biological experiments. Standard deviations were intentionally excluded to simplify the reading.
[a] λ, lag phase duration; r_b, biomass (b) specific growth rate; r_s, sugar (s) consumption specific rate; r_e, ethanol (e) production specific rate; α, time of complete sugar consumption; Y_b, biomass yield; Y_e, ethanol yield; Y_g, glycerol yield; Y_{CO_2}, carbon dioxide yield.[b] Media were supplemented with the optimized minerals concentrations (Min) or with 15 g L^{-1} of commercial yeast extract (Ext).[c] Mixture of soft drinks based on the marketing volumes in Argentina: 65% cola type, 28% lemon-lime and 7% orange.

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The most important parameters of fermentation (r_s, r_e and Y_e) follow the same trend in media supplemented with yeast extract and with salts: cola type > mix > orange > lemon-lime.

The yield of ethanol was higher (approximately 8%) in media supplemented with yeast extract, which is consistent with the contribution of additional carbon sources by this supplement. However, this higher yield does not compete with the economic benefits of using salts. In fact, with the mean costs based on direct price quotes from international manufacturers for $(NH_4)_2HPO_4$, $MgSO_4{\cdot}7H_2O$ and $ZnSO_4{\cdot}7H_2O$ (450, 100 and 500 USD ton^{-1}, respectively), the cost of supplementation of the media with salts results in 5.4 USD m^{-3} of wastewater. This value is far below the 34.5 USD m^{-3} obtained if yeast extract (2300 USD ton^{-1}) is used as a supplement and represents a specific cost of approximately 0.1 USD L^{-1} of produced ethanol from the mixture of soft drinks, which makes the proposed process more economically attractive.

Finally, the demands on nitrogen and metal sources depend on the yeast strain and the conditions of fermentation [20,24,25]. Therefore, if other industrial yeast strains (e.g., *S. bayanus* or *S. pastorianus*) different from the *S. cerevisiae* var. Windsor are assayed to identify the minimal nutrient requirements, a study similar to the one described in this work could be useful to identify the salts and to optimize their concentrations.

Conclusions

It was demonstrated in this work that the replacement of yeast extract by an optimized mixture of low-cost mineral salts in the fermentation of sugars present in synthetic media is feasible and economically convenient. This mineral mixture was successfully employed as a nutritive supplement for bioethanol production via yeast fermentation from selected wastewaters of the soft drink industry. Then, it could be applied to effluents from other beverage industries (e.g., fruit juices, functional drinks, ciders) and even other food industries (e.g., candy and jams), thereby expanding the spectrum of potential users in the industrial application of this supplement.

Conflict of interest

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

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GLOSSARY

CCD Central composite design.
FFD Full factorial designs.
RSM Response surface methodology.
USD United States dollar.
YPD Yeast extract, peptone and dextrose liquid media.

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