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Wastewater from the soft drinks industry as a source for bioethanol production

Miguel A. Isla *, Raúl N. Comelli, Lisandro G. Seluy

Departamento de Medio Ambiente, Facultad de Ingeniería y Ciencias Hídricas (FICH) e Instituto de Desarrollo Tecnológico para la Industria Química (INTEC), Universidad Nacional del Litoral y Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Ciudad Universitaria CC 242 Paraje El Pozo, 3000 Santa Fe, Argentina

HIGHLIGHTS

- Bioethanol production from soft drink wastewater is technically feasible.
- Alcoholic fermentation was mediated by yeasts of the *Saccharomyces* genus.
- Addition of nutrients significantly reduces the fermentation time.
- Ethanol yields were close to theoretical values for this type of fermentation.

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ABSTRACT

Wastewaters from the soft drinks industry were examined as media for producing bioethanol using yeast-mediated fermentation. Fermentation assays were performed using cola-type, orange and lemon-lime soft drinks and the biomass, sugar and ethanol levels were monitored over time. The effect of the addition of yeast extract was evaluated; the results indicated that 15 g/L is a suitable value for successful fermentation. Depletion of the sugars contained in the soft drinks (10–12% w/v) was achieved in less than 12 h when the medium was inoculated with 2 g/L of *Saccharomyces cerevisiae* var. Windsor. Ethanol yields were close to the theoretical values. The performance of several kinetic models was evaluated, and their parameters were determined. A model including inhibition by ethanol enabled the best adjustment of the experimental results in all assayed media. Some soft drinks include sodium benzoate in their formulae, the effect of which on yeast metabolism is discussed.

1. Introduction

In recent years, the development of green, sustainable *biorefineries* has been the focus of scientists and industries. This concept involves the production of several compounds, such as biofuels, fine chemicals and/or energy, from renewable sources, such as biomass (Yang, 2007; Kaparaju et al., 2009; Nigam and Singh, 2011). Processes based on biomass conversion into useful biomaterials can maximize the economic value of raw materials currently discarded and reduce wastewater produced by different industries. Bioethanol is one of the most important renewable fuels and these fuels reduce the negative environmental impacts generated by the worldwide use of fossil fuels. To reduce the supply of greenhouse gases to the atmosphere, ethanol can be added to the gasoline that is used as fuel for transport (Stichnothe and Azapagic, 2011). Therefore, bioenergy technology focuses primarily on the conversion of biomass feedstock to ethanol. Ethanol production from raw agricultural materials is of global concern due to the increasing demand for limited, non-renewable energy resources in addition to the variability of oil and natural gas prices. Several materials, such as sugarcane, corn, wheat, jatropha, carrot and cassava, are used as raw materials for bioethanol production (Palmarola-Adrados et al., 2005; Kaparaju et al., 2009; Aimaretti et al., 2012; Njoku et al., 2012). Currently, lignocellulosic residues represent an attractive renewable source for bioethanol production, although the technology remains insufficiently developed and the large quantity of wastewaters produced by the fermentation processes poses a problem for large-scale production (Limayem and Ricke, 2012). At present, the high worldwide bioethanol demand exerts enormous pressure on the primary production capacity and is leading to an increase in the area of land sown with energy crops, which itself causes high demand for natural resources and competition for land that could be used for food production. In addition, the increasing demand for biofuels could raise the price of energy crops and products that compete with

* Corresponding author. Tel.: +54 3424559174x2417; fax: +54 342 45509 44.
E-mail address: misla@santafe-conicet.gov.ar (M.A. Isla).

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them for supplies, such as other crops or meat. Therefore, there is a need to identify new renewable sources for the production of bioethanol. Some effluents of the soft drinks industry can be regarded as media for yeast-mediated alcoholic fermentation. 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 in proportion to the high production of these beverages (e.g., 4500 million L/year in Argentina). They exhibit high sugar content (approximately 10–12% w/v). In this report, the ability of yeasts to ferment the sugars contained in cola, orange and lemon-lime soft drinks produced by a worldwide leading brand was evaluated. First, the effect of the addition of nutrients on fermentation times and the influence of the composition of each soft drink on yeast performance were evaluated. For the purposes of comparison, some fermentation assays were performed using selected soft drinks and a synthetic medium while monitoring the biomass, sugars and bioethanol concentration.

The main purpose of this research was to evaluate the technical feasibility of producing bioethanol via yeast fermentation from certain effluents of the soft drinks industry. The effect of added nutrients and the impact of some compounds contained in the different soft drinks were also evaluated.

2. Methods

2.1. Strain and culture media

The commercial yeast strain *Saccharomyces cerevisiae* var. Windsor (ALE yeast, British style) was used for all experiments. The strain was purchased from a handcraft beer market and recovered according to the manufacturer's instructions (Lallemand Brewing Co., Felixstowe, UK). Yeast was maintained on YPD solid medium (yeast extract 5 g/L, peptone 5 g/L and D-glucose 20 g/L that was supplemented with agar at 15 g/L, which was used as a solidifying agent) and stored at 4 °C. The culture was transferred to fresh medium monthly.

Before using it as an inoculum for fermentation, the initial yeast culture was propagated aerobically in two different media and then separated by centrifugation. To inoculate soft drinks that contained only high fructose corn syrup, the initial yeast culture was grown on YPD broth, whereas for those soft drinks that contained sucrose, the inoculum was grown on YPS broth (identical to YPD but with sucrose instead of glucose as the carbon source).

Different media were used in the fermentation assays, including selected individual soft drinks purchased in the local market (cola, orange and lemon-lime), a mix of the soft drinks and synthetic media containing sucrose and/or a mixture of glucose and fructose in the same proportion as that found in the soft drinks. Because CO_2 interferes with the quantification of ethanol, (see Section 2.3.3), the sodas were degassed using magnetic stirring (6 h at room temperature in a closed sterile vessel connected to a water trap).

2.2. Culture conditions

The soft drinks were fermented in batch mode in 500-mL glass flasks under anaerobic conditions and a constant temperature of 30 °C. The experiments were performed in triplicate. The fermentation time was measured from the time of inoculation. Samples were collected in duplicate immediately after inoculation and every 1.5 h until the end of the experiment. The fermentations were initiated under microaerophilic conditions. The initial concentration of yeast in each assay was 2.00 ± 0.20 g/L to achieve a ratio of 1/50 $g_{biomass}/g_{sugar}$. The cells had been previously grown at 30 °C for 12–18 h and were then harvested by centrifugation for 5 min at 4500 rpm, washed twice using phosphate buffer and finally resuspended in a small volume of the medium being assayed. The pH was measured at the beginning and at the end of each experiment using a sensor (ThermoOrion 105A; Thermo Fisher Scientific, Madrid, Spain). Commercial yeast extract (Britannia, Buenos Aires, Argentina) was added to the fermentation medium prior to the inoculation.

2.3. Analytical determinations

2.3.1. Biomass

The Volatile Suspended Solids concentration was chosen to measure biomass concentration. To obtain the calibration curve, yeast were grown for 12–18 h in YPD medium, harvested, washed three times using phosphate buffer and suspended in distilled water prior to spectrophotometric measurements at 600 nm using a VIS spectrophotometer (DR/2010, HACH, USA) according to the standard technique (Eaton et al., 2005). During fermentation, samples (1 mL) were taken, centrifuged and washed five times using phosphate buffer, resuspended in distilled water to the starting volume and measured using a spectrophotometer.

2.3.2. Carbohydrates

Samples (1 mL) collected during the fermentation assays were centrifuged immediately for 5 min at 4500 rpm and the supernatants were transferred to sterile 1.5-mL tubes and stored at −20 °C until sugar determination. Total sugar content was determined using the phenol–sulfuric acid colorimetric method (Dubois et al., 1956). Reducing sugar content was measured using the Miller colorimetric method (1959). The sugar concentration was calculated indirectly using a standard curve constructed from different concentrations of D-glucose (Merck, NJ, USA). Glucose was also measured using a commercial enzyme kit (Wiener lab, Rosario, Argentina) according to the manufacturer's instructions.

2.3.3. Bioethanol

The ethanol concentration during fermentation was determined using an *ad hoc* device that was based on a SnO_2 sensor (TGS Figaro 2620; Figaro Engineering Inc., Osaka, Japan). All determinations were made at 25 °C. A standard curve of different ethanol concentrations (0.05; 0.10; 0.25; 0.50; 0.75 and 1.0 g/L) was constructed each time that the device was used. There was a good linear correlation ($r^2 = 0.98$) between the sensor output signal and ethanol concentrations up to 1 g ethanol/L. Prior to measurement, the samples were centrifuged, placed in closed vessels and incubated in a water bath at 25 °C for 15 min. Samples containing greater than 1.0 g/L ethanol were diluted using distilled water. The procedure for ethanol determination was validated by gas chromatography using a Hewlett Packard HP 5890 Series II GC (Agilent Technologies Inc., Santa Clara, CA, USA) system that was equipped with a flame ionization detector (FID) with a Carbowax 20 M column at 85 °C and using helium as a gas carrier. The injector and detector temperatures were maintained at 150 °C.

2.4. Kinetic models and estimation of the fermentation parameters

Three independent experimental runs were performed for each type of soda. Mean values were used for kinetic modeling and parameter estimation. The ability of several kinetics models to fit these experimental data was tested. The minimization of the relative average quadratic deviation between the experimental biomass, ethanol and sugar concentrations and the predicted values was chosen as the objective function to obtain model parameters. Calculations were performed using MATLAB® 7.9.0 R2009b (Wiley, 2005).

3. Results and discussion

3.1. The selected effluents of the soft drinks industry show high simple sugar content that is potentially fermentable

Non-"light" soft drinks, specifically cola, lemon-lime and orange, that were produced by a leading worldwide company were used as culture media for different assays. These tastes represent the largest volumes in the market and exhibit different sugar contents that range from 10–12% w/v. Because the identity of these carbohydrates is not reported on the labels, we determined them experimentally for each type of soft drink. Different concentrations of reducing sugars and non-reducing sugars and different mixtures of these sugars were found in the different soda types as well as in individual samples of the same type. Sugars were identified using gas chromatography (GC-FID) as "sucrose", "glucose" or "fructose". Enzymatic determinations indicated that 45 ± 1% of the reducing sugars were glucose, which indicates that "high fructose corn syrup", comprising approximately 55% w/w fructose and 45% w/w glucose, was used as a sweetener in the majority of the soft drinks studied. Yeasts carry complex metabolic and enzymatic machinery that is capable of fermenting these simple sugars. High concentrations of sugars do not normally limit yeast growth. There are several reports in the literature on the ability of yeast to grow and ferment sugars under very high gravity (VHG) conditions at sugar concentrations of approximately 30% w/v (Bai et al., 2004; Hu et al., 2011; Pereira et al., 2011), i.e., up to 3-fold the sugar content of the effluents used in this research.

The measured pH values were within the 2.85–3.95 range and remained stable for each soda during fermentation. Most yeasts of the *Saccharomyces* genus tolerate a pH range of between 3.0 and 8.0, with 4.5–6.5 being optimal for yeast metabolism (Dickinson and Schweizer, 2004). Thus, pH optimization may be considered in future research. As stated on the respective labels, soft drinks include other constituents (e.g., caffeine in cola sodas) at unspecified concentrations. Of these constituents, sodium benzoate must be especially considered due to its negative effect on yeast metabolism. This preservative is used in lemon-lime and orange soft drinks but is not present in the cola-type sodas assayed in this research.

Several parameters were used to evaluate the technical feasibility of bioethanol production and to compare the performance of yeast cultured in the different soft drinks (see Fig. 1).

3.2. Supplementation with nutrients made yeast-mediated alcoholic fermentation of soft drinks feasible

Preliminary tests of fermentation in the soft drinks inoculated with commercial yeast *S. cerevisiae* var. Windsor showed negative results: neither biomass growth nor ethanol production was detected after seven days in culture. This evidence indicates the need to supplement the sodas with additional nutrients, to adjust the pH and/or selectively remove inhibitors. To investigate this point, the effect of addition of yeast extract was evaluated because it represents an undefined but complete source of nutrients. Yeast extract provides amino acids that act as a nitrogen source and many micronutrients (vitamins, minerals and growth factors) that are essential for yeast metabolism (Albers et al., 1996; Dickinson and Schweizer, 2004). Fermentation assays were performed using soft drinks supplemented with different concentrations of yeast extract (5, 10, 15, 20 and 25 g/L) and the levels of biomass, sugars and ethanol were monitored over time. A highly favorable impact on fermentation was verified. Thus, we confirmed the hypothesis that the nutrient deficit of soft drinks must be overcome. The parameters defined in Fig. 1 were obtained from these experimental data.

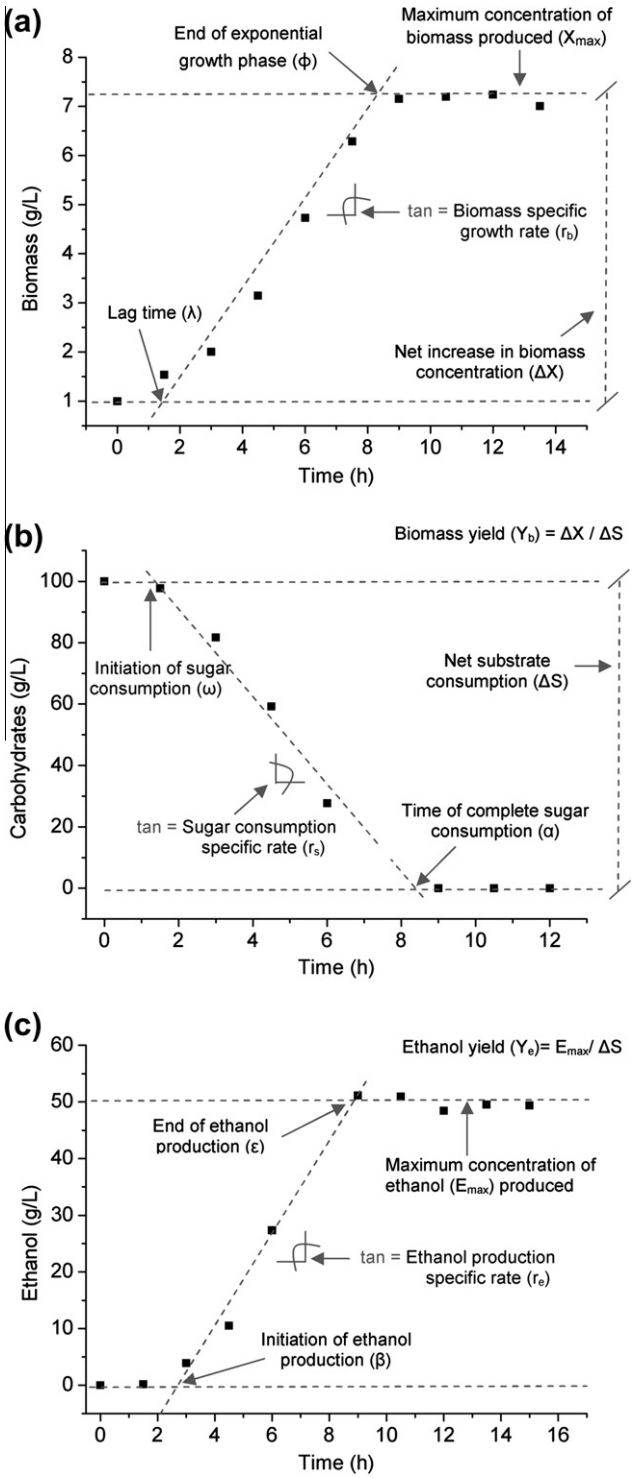

Fig. 1. Definition of the parameters used to evaluate yeast performance. Changes in the concentration of biomass (A), sugars (B) and ethanol (C) during fermentation in the synthetic medium that was used as a control for all assays.

The effect of the addition of yeast extract on the fermentation of lemon-lime soft drinks is depicted in Fig. 2. Notably, the time to acclimate to the media (lag time, λ) declined in proportion to the concentration of extract supplement.

The addition of yeast extract at concentrations greater than 20 g/L did not lead to a statistically significant reduction in the lag phase (Fig. 2). The addition of 5 g/L of yeast extract had a negligible effect on net biomass production (ΔX), whereas higher con-

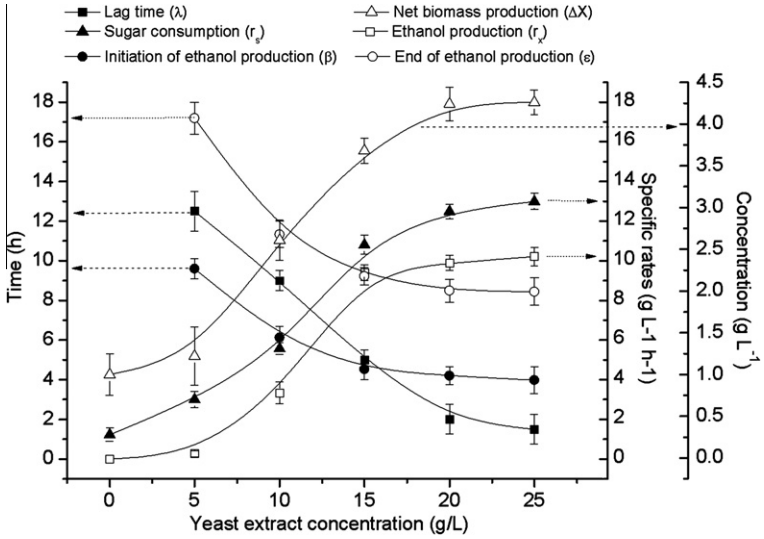

Fig. 2. Influence of the concentration of yeast extract on yeast-mediated alcoholic fermentation. The parameters λ, ΔX, r_s, r_e, ε and α were calculated as indicated in Fig. 1. The media were supplemented with commercial yeast extract (5, 10, 15, 20 and 25 g/L). The values denote the mean (±SD) of the calculated parameters from three independent experiments using lemon-lime soft drinks for each extract concentration.

centrations of yeast extract induced a marked effect on this parameter (Fig. 2). The specific sugar consumption rate (r_s) increased dramatically following the addition of yeast extract and reached a maximum at yeast extract concentrations close to 20 g/L, whereas the specific rate of ethanol production (r_e) was maximal in media supplemented with $\geqslant$15 g/L yeast extract (Fig. 2). It should be noted that in the experimental time the total consumption of sugars (105 g/L) was achieved only in the media supplemented with $\geqslant$15 g/L yeast extract, whereas the yeasts grown in media supplemented with 0, 5 or 10 g yeast extract/L consumed just 12, 25 or 40 g/L of sugar, respectively. For yeast extract concentrations of >15 g/L, there was no marked diminution in initiation of ethanol production (β) and in latency to maximal ethanol production (ε), as shown in Fig. 2. These data suggest that 15 g/L yeast extract is a suitable value for successful fermentation. A similar behavior was observed in the other soft drinks tested, which confirms a deficit in nutrients for appropriate yeast metabolism in these drinks.

These results indicate that alcoholic fermentation mediated by yeasts for bioethanol production from selected wastewaters of the soft drinks industry is a technically feasible process when the wastewaters are supplemented with appropriate nutrients. The identification of these nutrients requires additional experiments using different inorganic salts that are not addressed in this report. With the aim of eventually scaling-up of this process for industry, a concentration of 15 g/L yeast extract was adopted for the remaining assays because this concentration was the lowest that enabled an acceptable rate of ethanol production. In the future, it would be interesting to study the possibility of self-sustainability of the process, i.e., the extract added as a supplement to batch fermentation should be the biomass produced from the previous fermentation after the inoculum to be used in the next cycle has been separated.

3.3. Performance of the yeast in different soft drinks and in a mixture of soft drinks

Because soft drinks include other constituents besides sugars that differ between flavors, several assays were performed using the following media: (a) individual soft drinks, (b) a mixture of soft drinks based on the marketing volumes in Argentina (65% cola type, 28% lemon-lime and 7% orange) and c) a synthetic medium that was used as a control. Fig. 3 shows the concentrations of biomass, sugars and ethanol during the fermentations, whereas the performance parameters of yeast defined in Fig. 1 are also reported in Table 1 for comparative purposes.

Of note, the behavior of the yeast depended on the soft drink. Because the sugars are similar between the conditions, these data indicate that each flavor contains different constituents, each of which affects the fermentation performance differently.

With respect to biomass, typical growth curves for these microorganisms were obtained in all the experiments (Fig. 3A). The lag time (λ) required by the yeast grown in cola-type soft drinks was shorter than for the control, whereas for the remaining soft drinks, this time was longer (see Table 1). The yeast grown on the mixture also exhibited a lower λ value than the control, indicating that the cola soft drinks could contain an inducing compound that is capable of accelerating the acclimatization of yeast to the medium. For the other soft drinks, in particular the lemon-lime flavor, a prolonged lag phase was observed, which may be due to the presence of an inhibitor (e.g., sodium benzoate).

The biomass yields (Y_b) in the individual assayed soft drinks were less than that observed in the synthetic medium (Table 1), which indicates the presence of inhibitors in all soft drinks. This effect was reduced in the cola flavor and more pronounced in the lemon-lime soft drinks. An interesting phenomenon occurred to yeast grown in the mixture of soft drinks: the Y_b values were similar to the control, whereas the specific growth rate of biomass (r_b) was slightly higher. This finding could be due to the following aspects of the mixture: (a) the particular (and different) inhibitors present in each soft drink are diluted; (b) the aforementioned inducing effect of some substances present in the cola type confers this effect to the mixture also; (c) a subsequent pH that is more appropriate for the yeast; and (d) a relationship between the inductors/inhibitors that drives the energy generated during fermentation primarily toward biomass production, thus minimizing maintenance costs as discussed in the analysis of the kinetic model (see Section 3.4). This evidence is very important from the industrial point of view because it suggests that there would be no need to segregate flavors when applying the proposed process.

Sugar consumption was complete for all assayed media in less than 12 h (Fig. 3B). Notably, the time (α) required was lower when using cola soft drinks compared with the control; however, the times were not different for the other soft drinks or for the mixture.

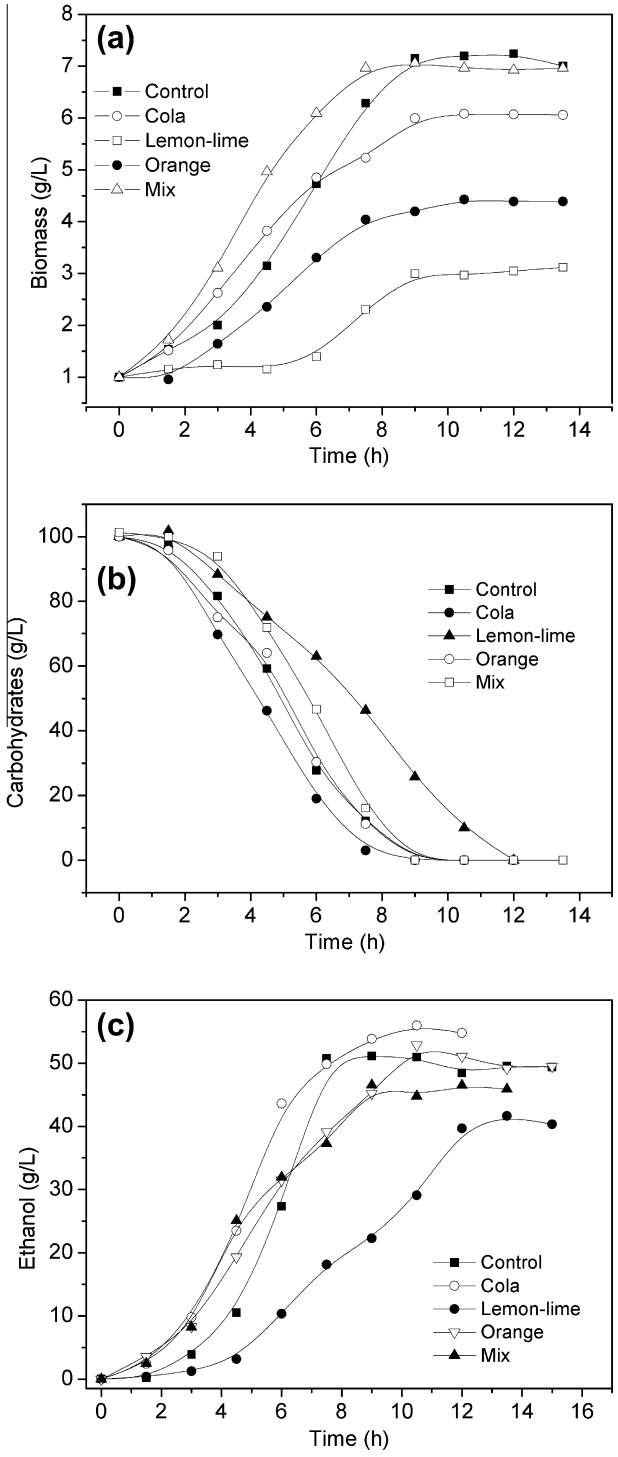

Fig. 3. Changes in concentration of biomass [A], sugar [B] and ethanol [C] during batch fermentation assays performed using individual soft drinks or a mixture (65% cola, 28% lemon-lime and 7% orange). A synthetic medium was used as a control for the fermentations. All media were supplemented with yeast extract (15 g/L). The values denote the mean of three independent experiments performed using each media.

The cola soft drink-based specific consumption rate was slightly higher when compared with the control and the lemon-lime-based rate was the lowest (Table 1). Interestingly, no statistically significant difference was detected in the length of time (approximately 6.5 h) between the complete removal of the sugars and the completion of the lag phase ($\alpha - \lambda$). This finding may be due to the latency to initiate sugar consumption (ω), which was similar among all media. Despite remaining in lag phase, the yeast grown in lemon-lime soft drinks in particular, but also in orange soft drinks, consumed sugars slowly and reached an optimal metabolic status to enter the exponential growth phase. The total time from the initiation of sugar consumption until the complete removal of sugar ($\alpha - \omega$) was higher when yeast were cultured in lemon-lime soft drinks, which is consistent with the presence of specific inhibitors in this flavor.

Table 1
A comparison of parameters of yeast performance where yeast was cultured in individual soft drinks, a mixture of soft drinks based on the marketing volumes in Argentina (65% cola type, 28% lemon-lime and 7% orange) or a synthetic medium that was used as a control. In all cases, the media were supplemented with yeast extract (15 g/L). The tabulated values denote the mean of the parameters calculated from three independent experiments.

Parameter	Control	Soft drink			
		Cola	Lemon-lime	Orange	Mix
λ (h)	1.28	0.80	5.19	1.97	0.59
ϕ (h)	8.81	7.63	9.91	8.18	7.27
ΔX (g_b)	7.03	6.10	3.52	4.43	7.06
r_b ($g_b\,L^{-1}\,h^{-1}$)	0.81	0.75	0.53	0.54	0.89
α (h)	8.01	7.46	11.84	8.34	8.21
ω (h)	1.01	0.84	1.41	0.97	2.66
r_s ($g_s\,L^{-1}\,h^{-1}$)	15.01	15.87	10.07	14.25	15.24
Y_b (g_b/g_s)	0.115	0.093	0.048	0.055	0.110
ε (h)	7.99	7.33	12.29	8.80	8.28
β (h)	2.27	1.89	3.83	1.95	1.16
r_e ($g_e\,L^{-1}\,h^{-1}$)	10.49	10.28	4.92	7.72	7.91
Y_e (g_e/g_s)	0.49	0.51	0.39	0.42	0.45

The time required to achieve maximum ethanol production (ε) depended upon the soft drink utilized. However, the elapsed time between maximum ethanol production and the end of the lag phase ($\varepsilon - \lambda$) was similar among all media. It is noteworthy that the latency to maximum ethanol production coincided with the latency to total sugar depletion, where $\varepsilon/\alpha = 1$ for all. The specific ethanol production rate (r_e) in cola soft drinks was similar to the control, whereas production rates were significantly lower in the other conditions. In contrast, the net latency to ethanol production ($\varepsilon - \beta$) was greater for the orange and lemon-lime soft drinks compared to the control and cola-based fermentation and the latter 2 conditions showed similar times (Table 1).

The time for the initiation of ethanol production (β) in lemon-lime soft drinks was twice that of the other conditions, including the control. Interestingly, the period between the initiation of ethanol production and the end of lag phase ($\beta - \lambda$) was 1 h for the control and cola soft drinks. However, in the orange soft drink, the period was null (ethanol production started practically at the completion of the lag phase). In the lemon-lime soft drinks, the period was negative (−1.36 h) (Table 1), indicating that the ethanol production began approximately 1.5 h prior to the end of the lag phase, which is consistent with the start of sugar consumption during this phase. Ethanol yields ($Y_{e/s}$) in the cola-based fermentation and in the control media were identical to the theoretical yields reported for these fermentations (Barford et al., 1995; Mwesigyeb and Barford, 1996), whereas the yields for the other individual soft drinks and their mixture were lower (Table 1). These results indicate that the yeast grown in orange or in lemon-lime or in a mixture of these soft drinks consume a fraction of the available sugars in the medium during the lag phase, which is detrimental to ethanol production. This effect was worse when using lemon-lime soft drinks. Despite initiating the experiments in a microaerophilic atmosphere, ethanol yields in the control indicated that virtually all sugars were fermented, which is indicative

of the predominance of the Crabtree effect over the Pasteur effect, a trend that occurs in media containing high concentrations of sugars (Dickinson and Schweizer, 2004).

The results obtained showed that the cola soft drinks, despite their apparent complex formulation, behaved similarly to the synthetic medium that was used as a control. These soft drinks could contain inducing compounds that would minimize the time required to acclimate to the media. Although phosphates are strong candidates, caffeine or some other compound included in the formulation of this type of soda cannot be ruled out as responsible for this effect. Some compounds present in the orange and lemon-lime soft drinks require a different analysis because they affect the yeast metabolism, prolong the lag phase and decrease biomass and ethanol yields in addition to other effects. These soft drinks include sodium benzoate in their formulae, which is a preservative that is not added to the cola-type sodas. The effect of this compound on yeast metabolism is discussed briefly in Section 3.5.

3.4. Kinetic modeling and parameter determinations

Ethanol is a product of the primary metabolism of yeasts and is the final product of the primary pathway to obtain energy under anaerobic conditions. This energy is intended for cell growth or maintenance. The basic equations describing the rates of biomass growth, substrate consumption and ethanol production are:

$$\frac{dx}{dt} = \mu(s,e)x \tag{1}$$

$$\frac{ds}{dt} = -\frac{\mu(s,e)}{Y_{x/s}}x \tag{2}$$

$$\frac{de}{dt} = Y_{e/x}\mu(s,e)x + \gamma x \tag{3}$$

where μ is the biomass specific growth rate and x, s and e are the concentrations of biomass, sugars and ethanol, respectively, all expressed in g/L. $Y_{x/s}$ is the biomass yield coefficient ($g_{biomass}/g_{sugar}$), $Y_{e/x}$ is the ethanol/biomass yield coefficient ($g_{ethanol}/g_{biomass}$) and γ the kinetic constant of ethanol production by maintenance ($g_{ethanol}/g_{biomass}$).

The performance of the following models of the biomass specific growth rate was evaluated:

$$\mu(s) = \mu_{max}\frac{s}{s+K_s} \tag{4}$$

$$\mu(s,e) = \mu_{max}\frac{s}{s+K_s}\left(1-\frac{e}{K_e}\right) \tag{5}$$

$$\mu(s,e) = \mu_{max}\frac{s}{s+K_s+K_e e^2} \tag{6}$$

where μ_{max} is the maximum specific growth rate of the biomass, K_S is the saturation constant and K_e is the inhibition constant by ethanol. The function selected to obtain the parameters was the mean squared deviation between the experimental values and those predicted by the model (Wiley, 2005).

As shown in Fig. 4, the model described by Eqs. (1)–(3), (and) (6) enabled the best adjustment of the experimental results in all assayed media. Table 2 summarizes the optimal values of the kinetic parameters for the different media. Notably, K_S values were low for the yeast strain used in all assays. This finding is consistent with the values reported in the literature for *S. cerevisiae* grown in simple sugars, such as fructose and glucose (Birol et al., 1998; Jones and Kompala, 1999).For the orange soft drinks, μ_{max} was similar to that obtained in the cola-type drinks. However, the value of the ethanol production rate constant not associated with growth (γ) of the orange drinks was almost twice that of cola-type soft drinks, whereas the biomass yield of the orange drinks was almost

Table 2
Parameters of the kinetic models provided by Eqs. (1)–(3), (and) (6) for the individual soft drinks or the mixture. *Abbreviations*: RMS, root mean square deviation.

Parameter	Cola	Lemon-lime	Orange	Mix
μ_{max} (h^{-1})	0.333	0.192	0.333	0.320
$Y_{x/s}$ ($g_{b,prod}/g_{az,cons}$)	0.101	0.049	0.054	0.114
$Y_{e/x}$ ($g_{e,prod}/g_{b,prod}$)	3.605	6.562	3.817	3.873
K_e (Lg_{az}/g_e^2)	0.026	0.036	0.048	0.011
K_s (g_{az}/L)	0.207	0.006	0.032	0.001
γ (g_e/g_b h)	0.176	0.420	0.338	0.024
RMS	0.0266	0.0220	0.0250	0.0270

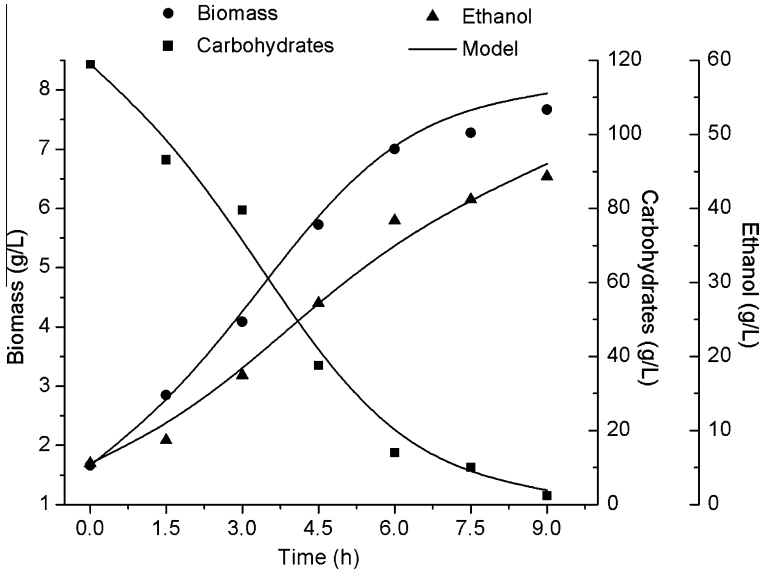

Fig. 4. Experimental results versus predicted values provided by the model given by Eqs. (1)–(3), (and) (6) for biomass, sugars and ethanol. Curves denote the fermentation assays performed using orange soft drinks.

half that of the cola-type soft drinks. This finding confirms the effect of inhibitory substances that generate a more active maintenance metabolism, direct energy to this path and affect biomass yields but not the specific growth rate. Therefore, ethanol production is largely associated with cell maintenance. In the case of lemon-lime soft drinks, μ_{max} and the biomass yield were significantly lower than the cola-type soft drinks. In addition, γ was higher in lemon-line soft drinks than in other soft drink conditions. Again, this finding confirms the presence of inhibitory substances that affect both the specific growth rate constant and the biomass yield. In regard to orange soft drinks, a fraction of the ethanol produced is not associated with growth.

3.5. Sodium benzoate, which is present in some soft drinks, affects the metabolic activity of yeast

Sodium benzoate is used as a food additive due its ability to inhibit the proliferation of spoilage microorganisms, primarily filamentous fungi and yeast, that contaminate food. This preservative affects the homeostasis and metabolism of yeasts by modifying the internal cellular pH, by generating a loss in membrane integrity and a greater permeability to ions and metabolites and by acting directly on key enzymes of carbon metabolism, such as hexokinase and phosphofructokinase (Krebs et al., 1983; Arroyo-Lopez et al., 2008; Mira et al., 2010).

The effect of sodium benzoate was studied by conducting fermentations in synthetic medium supplemented with yeast extract (15 g/L) and sodium benzoate at initial concentrations of 0.5 g/L and 1.0 g/L. The first value is the maximum allowed by the Argentine Food Code (Código Alimentario Argentino, 2012), whereas the second was used to emphasize the inhibitory effect because the concentration of yeast in the experiments is far above the contamination expected in soft drinks. As shown in Fig. 5, benzoate affected the growth and metabolism of yeast. The effect of benzoate on the various parameters of yeast performance (defined in Fig. 1) can be summarized as follows (see Table 3): (a) increasing the duration of the lag phase, (b) decreasing the biomass yield, (c) decreasing the latency to sugar consumption, (d) lowering sugar consumption and ethanol production rates, and (e) decreasing ethanol yields. Sugar consumption was complete in less than 12 h for medium containing 0.5 g/L benzoate whereas by that time, approximately 10 g/L sugar remained in the medium containing 1.0 g/L benzoate. These results suggest that a portion of the biomass would suffer metabolic inhibition with the possible accumulation of intermediate products of the glycolytic pathway without production of ethanol. This fraction would decrease the effective concentration of sodium benzoate in the media and the remaining biomass would produce ethanol. Additional studies of the concentration of benzoate during the experiments and the use of a more concentrated inoculum are required to support this hypothesis.

Interestingly, the behavior of the yeast in the medium supplemented with 0.5 g/L benzoate was similar to those cultured in the orange soft drinks. In particular, the ethanol yields, the net biomass increase, the initiation of ethanol production at the end of the lag phase ($\beta - \lambda = 0$), the period of ethanol production ($\varepsilon - \beta$), the elapsed time between maximum ethanol production and the end of the lag phase ($\varepsilon - \lambda$) were similar. These results suggest that sodium benzoate could be the main inhibitory compound in the orange soft drinks.

In regard to the lemon-lime soft drinks, the yeast showed greater difficulty in adapting to this medium than the medium containing benzoate, with a more prolonged lag phase and lower maximum biomass value; specific rates of sugar consumption and ethanol production were similar to the medium containing 1.0 g/L benzoate. However, sugar consumption was completed and the ethanol yield was higher, indicating that the lemon-lime could contain some other components that affect yeast growth. This flavor includes concentrated lemon juice in its formula. The biostatic activity of compounds in this additive, such as some flavonoids (Cushnie and Lamb, 2005; Tripoli et al., 2007) and other preservatives added during the manufacturing process (e.g., potassium sorbate or sodium metabisulphite), have been reported in the literature. Additional studies are required to investigate this issue.

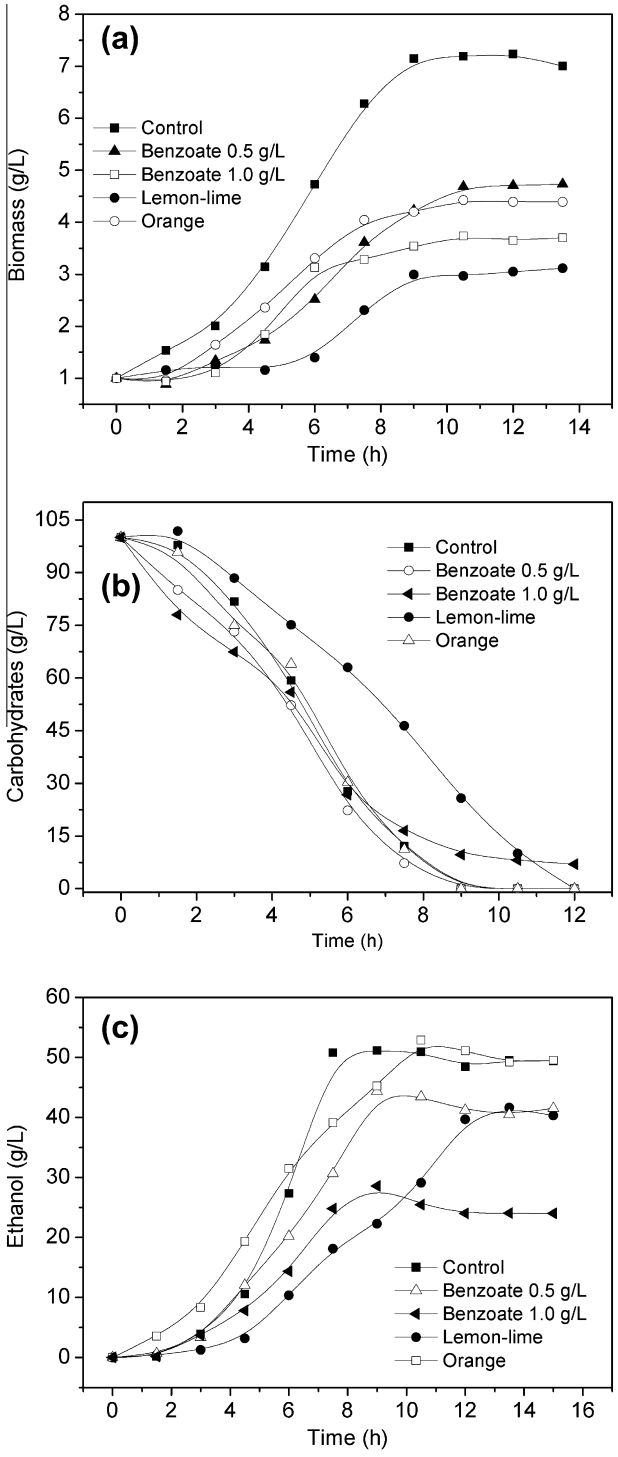

Fig. 5. Effects of sodium benzoate on yeast metabolism. Changes in concentration of biomass [A], sugar [B] and ethanol [C] during fermentation assays performed in synthetic medium (control) supplemented with sodium benzoate (0.50 and 1.0 g/L). The values denote the mean of three independent experiments. The results obtained in orange and lemon-lime soft drinks are shown for comparison. *Abbreviations*: Bz, sodium benzoate.

Table 3
Comparison of the effects of sodium benzoate on yeast metabolism. In all cases the media were supplemented with yeast extract (15 g/L). The tabulated values denote the mean of the parameters calculated from three independent experiments. *Abbreviations*: BZ, benzoate.

Parameter	Synthetic medium			Orange soft drink
	BZ 0.5 g/L	BZ 1.0 g/L	Control	
λ (h)	2.70	2.98	1.28	1.97
ΔX ($g_b\ L^{-1}$)	4.71	3.73	7.03	4.43
r_b ($g_b\ L^{-1}\ h^{-1}$)	0.51	0.52	0.81	0.54
α (h)	8.52	–	8.01	8.34
ω (h)	0.37	0.27	1.01	0.97
r_s ($g_s\ L^{-1}\ h^{-1}$)	12.82	10.34	15.01	14.25
Y_b (g_b/g_s)	0.071	0.055	0.115	0.055
ε (h)	9.31	8.68	7.99	8.80
β (h)	2.70	2.52	2.27	1.95
r_e ($g_e\ L^{-1}\ h^{-1}$)	6.71	4.65	10.49	7.72
Y_e (g_e/g_s)	0.42	0.30	0.49	0.42

4. Conclusions

A process for bioethanol production from wastewaters of the soft drink industry via yeast fermentation was developed. Aspects that will be studied in future research include the possibility of achieving a self-sustained process using the biomass produced within a fermentation as the nutrient for the subsequent fermentation, the adjustment of the initial medium pH, the increase in initial sugar concentration by evaporation, the optimization of the inoculum/sugar ratio and the effect removing inhibitors, among others.

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References

Aimaretti, N.R., Ybalo, C.V., Rojas, M.L., Plou, F.J., Yori, J.C., 2012. Production of bioethanol from carrot discards. Bioresour. Technol. 123, 727–732.

Albers, E., Larsson, C., Liden, G., Niklasson, C., Gustafsson, L., 1996. Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. Appl. Environ. Microbiol. 62 (9), 3187–3195.

Arroyo-Lopez, F.N., Bautista-Gallego, J., Duran-Quintana, M.C., Garrido-Fernandez, A., 2008. Modelling the inhibition of sorbic and benzoic acids on a native yeast cocktail from table olives. Food Microbiol. 25, 566–574.

Bai, F.W., Chen, L.J., Zhang, Z., Anderson, W.A., Moo-Young, M., 2004. Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium conditions. J. Biotechnol. 110 (3), 287–293.

Barford, J.P., Johnston, J.H., Mwesigye, P.K., 1995. Continuous culture study of transient behaviour of *Saccharomyces cerevisiae* growing on glucose and fructose. J. Ferment. Bioeng. 79 (2), 158–162.

Birol, G., Doruker, P., Kirdar, B., Ilsen-Önsan, Z., Ülgen, K., 1998. Mathematical description of ethanol fermentation by immobilized *Saccharomyces cerevisiae*. Proc. Biochem. 33 (7), 763–771.

Código Alimentario Argentino, 2012. Cap. XII: Bebidas Hídricas, Agua y Agua Gasificadas. Ley 18284, Decreto No 2126/71, Rep. Argentina.

Cushnie, T., Lamb, A.J., 2005. Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents 26 (5), 343–356.

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

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28 (3), 350–356.

Eaton, A.D., Clescerl, L.S., Greenberg, A.E., 2005. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, USA.

Hu, C., Qin, Q., Gao, P., 2011. Medium optimization for improved ethanol production in very high gravity fermentation. Chin. J. Chem. Eng. 19 (6), 1017–1022.

Jones, K.D., Kompala, D.S., 1999. Cybernetic model of the growth dynamics of *Saccharomyces cerevisiae* in batch and continuous cultures. J. Biotechnol. 71, 105–131.

Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P., Angelidaki, I., 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour. Technol. 100 (9), 2562–2568.

Krebs, H.A., Wigginst, D., Stubbs, M., 1983. Studies on the mechanism of the antifungal action of benzoate. Biochem. J. 214, 657–663.

Limayem, A., Ricke, S.C., 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog. Energy Combust. Sci. 38, 449–467.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Mira, N.P., Teixeira, M.C., Sá-Correia, I., 2010. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. J. Integr. Biol. 14 (5), 525–540.

Mwesigyeb, P.K., Barford, J.P., 1996. Batch growth and transport kinetics of utilization of mixtures of sucrose and maltose by *Saccharomyces cerevisiae*. J. Ferment. Bioeng. 82 (2), 101–108.

Nigam, S.P., Singh, A., 2011. Production of liquid biofuels from renewable resources. Prog. Energy Combust. Sci. 37, 52–68.

Njoku, S.A., Xahring, B.K., Uellendahl, H., 2012. Pretreatment as the crucial step for a cellulosic ethanol biorefinery: testing the efficiency of wet explosion on different types of biomass. Bioresour. Technol. 124, 105–110.

Palmarola-Adrados, B., Choteborska, P., Galbe, M., Zacchi, G., 2005. Ethanol production from non-starch carbohydrates of wheat bran. Bioresour. Technol. 96, 843–850.

Pereira, F.B., Guimarães, P.M., Teixeira, J.A., Domingues, L., 2011. Robust industrial *Saccharomyces cerevisiae* strains for very high gravity bioethanol fermentations. J. Biosci. Bioeng. 112 (2), 130–136.

Stichnothe, H., Azapagic, A., 2011. Bioethanol from waste: life cycle estimation of the greenhouse gas saving potential. Resour. Conserv. Recycling 53 (11), 624–630.

Tripoli, E., La Guardia, M., Giammanco, S., Di Majo, D., Giammanco, M., 2007. Citrus flavonoids: molecular structure, biological activity and nutritional properties: A review. Food Chem. 104 (2), 466–479.

Yang, S.T., 2007. Bioprocessing for Value-added Products from Renewable Resources, first ed. Elsevier, Ohio, USA.

Wiley, J., 2005. Applied Linear Regression, third ed. John Wiley & Sons, New Jersey, USA.