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Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates

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ABSTRACT

Significant amounts of wastes are generated by the coffee industry, among of which, coffee silverskin (CS) and spent coffee grounds (SCG) are the most abundantly generated during the beans roasting and instant coffee preparation, respectively. This study evaluated the sugars metabolism and production of ethanol by three different yeast strains (*Saccharomyces cerevisiae*, *Pichia stipitis* and *Kluyveromyces fragilis*) when cultivated in sugar rich hydrolysates produced by acid hydrolysis of CS and SCG. *S. cerevisiae* provided the best ethanol production from SCG hydrolysate (11.7 g/l, 50.2% efficiency). On the other hand, insignificant ($\leqslant$1.0 g/l) ethanol production was obtained from CS hydrolysate, for all the evaluated yeast strains, probably due to the low sugars concentration present in this medium (approx. 22 g/l). It was concluded that it is possible to reuse SCG as raw material for ethanol production, which is of great interest for the production of this biofuel, as well as to add value to this agro-industrial waste. CS hydrolysate, in the way that is produced, was not a suitable fermentation medium for ethanol production; however, the hydrolysate concentration for the sugars content increase previous the use as fermentation medium could be an alternative to overcome this problem.

1. Introduction

Significant amounts of wastes are generated by the coffee industry, among of which, coffee silverskin (CS, the tegument that cover each coffee bean) is the main waste generated during the beans roasting [1]; and spent coffee grounds (SCG, the solid residue obtained during the treatment of coffee powder with hot water or steam) is the main waste generated during the instant coffee preparation [2], with a worldwide annual production of 6,000,000 tons [3]. Although these wastes are generated in large quantities every year, few studies have been addressed to their reuse. In some countries, CS has been used as soil fertilizer or as fuel [4]; while SCG is normally released to the environment or used as fuel in industrial boilers of the same industry, due to its high calorific power (approx. 5000 kcal/kg) [5]. Some studies have evaluated the SCG use as animal feed, however, the high lignin content ($\approx$25% w/w) of this material was considered a limiting factor for this application [6]. Based on these facts and considering the large amount of coffee wastes generated every year, comes the need to find alternatives for CS and SCG reuse, both from economical and environmental view points.

The ethanol production by fermentation has received large importance in the last few years due to its increased demand as fuel and complement to gasoline [7,8]. In addition, the ethanol use as fuel is also of interest because it reduces the dependence on oil; the air pollution and climate change caused by emissions of carbon dioxide [9]. Ethanol production by fermentation of agro-industrial wastes is very attractive because of their low cost and abundance, and non-competition with foodstuffs [10]. Some recent studies report that CS and SCG wastes are rich in sugars [2,11], which could be extracted and used as substrate in fermentation processes.

There is not any published study on the production of ethanol from coffee industry wastes. For this reason, and considering the variety of sugars present in these wastes [2,11], it is of importance to find a microorganism able to consume these sugars and convert them to ethanol. *Saccharomyces cerevisiae* is the microorganism most often used for ethanol production, due to its ability to grow in media containing high sugars concentration and its high ethanol yield. However, this yeast only ferments hexoses, being unable to produce ethanol from pentoses such as xylose [12]. Xylose is usually obtained during the hydrolysis of the hemicellulose fraction of agricultural wastes, and its bioconversion is an important step in the reuse of these materials [13,14]. Yeast strains from the genera *Pichia*, *Candida*, *Schizosaccharomyces* and *Pachysolen*, the filamentous fungi *Paecilomyces*, *Mucor*, *Monilia* and *Fusarium*, and bacteria *Clostridium*, *Bacillus*, *Bacteroides*, *Thermoanaerobacter*,

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and *Erwinia*, have been reported as able to produce ethanol from pentoses [15,16]. In our recent studies on ethanol production from lignocellulosic hydrolysates, *Pichia stipitis* (NRRL-Y-7124), *S. cerevisiae* (RL-11), and *Kluyveromyces fragilis* (Kf1) were demonstrated to be good ethanol producers from different sugars sources [17,18].

The objective of this work was to investigate the possibility of using coffee industry wastes (CS and SCG) as raw materials for ethanol production by fermentation. Since the hydrolysates produced from these wastes contain a mixture of hexose and pentose sugars, the ability of three different yeast strains (*P. stipitis* NRRL-Y-7124, *S. cerevisiae* RL-11, and *K. fragilis* Kf1) to metabolize their sugars and convert them to ethanol was evaluated and compared.

2. Material and methods

2.1. Raw material

Coffee silverskin (CS) and spent coffee grounds (SCG) were supplied by NovaDelta – Comércio e Indústria de Cafés, Lda (Campo Maior, Portugal). As soon as obtained, the materials were dried at 60 °C to 10% moisture content to be stored. Chemical composition of CS consisted of (g/100 g): glucan (17.8), xylan (4.7), arabinan (2.0), galactan (3.8), mannan (2.6), protein (16.2), lignin (30.2), ashes (4.7), acetyl groups (3.0), and extractives (15.0); while SCG contained glucan (8.6), arabinan (1.7), galactan (13.8), mannan (21.2), protein (13.6), lignin (32.1), ashes (1.6), acetyl groups (2.2), and extractives (5.2).

2.2. Hydrolysates preparation

The reaction conditions used for CS and SCG hydrolysis were optimized in previous studies, one of which has already been published [11]. Reaction conditions consisted in using a sulfuric acid solution (100 mg H_2SO_4/g dry matter) in a liquid/solid ratio of 10 g/g, at 163 °C during 45 min, for SCG; and in a liquid/solid ratio of 14 g/g, at 170 °C during 45 min, for CS. The reactions were performed in 200 ml stainless steel reactors, containing 70 ml of reaction medium. Under the required temperature, the dully-covered reactors (filled with CS or SCG, and the acid solution) were introduced into a silicone oil bath, were they were maintained during the desired time. At the end of the reaction, the reactors were immediately cooled in ice bath. The hydrolysates were then separated from the solid residue by centrifugation (6000 rpm, 15 min), and characterized regarding the concentration of sugars (glucose, xylose, arabinose, mannose, and galactose) and toxic compounds (furfural, hydroxymethylfurfural, acetic acid, and phenolic compounds) by HPLC. To be used as fermentation medium, the hydrolysates had their pHs adjusted to 5.5 by addition of NaOH (pellets); the remaining solid residue was removed by centrifugation (6000 rpm, 15 min). Sugar losses after this neutralization step were less than 4%.

2.3. Microorganisms

Three different yeast strains were evaluated, including *P. stipitis* (NRRL-Y-7124), *S. cerevisiae* (RL-11), and *K. fragilis* (Kf1). *S. cerevisiae* was supplied by University of Lavras (Department of Biology), Brazil. This yeast was selected among other strains due to its high capacity of producing ethanol [19]. *K. fragilis* was also supplied by University of Lavras (Department of Biology), Brazil, and was isolated from cocoa fermentation. This yeast has been demonstrated to have great ability for ethanol production from cheese whey [17].

2.4. Inocula and fermentation conditions

Cultures of the yeasts were maintained at 4 °C on Petri plates containing malt extract agar medium whose composition consisted in (g/l): yeast extract (3.0), malt extract (3.0), peptone (5.0), glucose (10.0), and agar (20.0). For the inoculum preparation, cells of the yeasts grown during 24 h in the maintenance medium were transferred to 500 ml Erlenmeyer flasks containing 100 ml of culture medium composed of (g/l): glucose (30.0), $(NH_4)_2HPO_4$ (3.0), $MgSO_4{\cdot}7H_2O$ (1.0), and yeast extract (3.0). Concentrated solutions of each compound were prepared separately and sterilized in an autoclave at 121 °C for 20 min, with the exception of glucose and yeast extract that were autoclaved at 112 °C for 15 min. The solutions were aseptically mixed in order to obtain the desired concentration of each nutrient in the culture medium.

The inoculated flasks were incubated in a rotary shaker at 30 °C, 200 rpm, for 24 h. After this time, the cells were recovered by centrifugation (5000 rpm, 20 min) and resuspended in the fermentation medium. Fermentation assays were performed in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium (hydrolysates) inoculated with an initial cell concentration of 1 g/l. The flasks were incubated in a rotary shaker at 30 °C, 200 rpm for 48 h. During the experiments, samples were taken for sugars, ethanol and cell growth determinations. All the assays were performed at least in duplicate.

2.5. Analytical methods

Microbial growth was determined after drying the samples at 105 °C to constant weight. Quantification of total phenolic compounds was carried out using the Folin–Ciocalteu method. Total sugars were determined by the dinitrosalicylic (DNS) and anthrone methods.

Ethanol and acetic acid concentrations were determined by high performance liquid chromatography (HPLC) on a Jasco chromatograph equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H (300 × 7.8 mm) column at 60 °C, using 0.005 M sulfuric acid as eluent in a flow rate of 0.7 ml/min. Concentration of glucose, xylose, arabinose, mannose and galactose was also determined by HPLC using a refractive index detector, but with a Varian column Metacarb 87P (300 × 7.8 mm) at 80 °C, and ultrapure water as eluent in a flow rate of 0.4 ml/min. Furfural and hydroxymethylfurfural (HMF) were determined by HPLC using a UV detector (at 280 nm) and a Nucleosil 120-5 C18 5 μm (4.6 × 250 mm) column at room temperature, acetonitrile/water (1/8 with 10 g/l acetic acid) as the eluent in a flow rate of 0.8 ml/min. In all the cases, a sample volume of 20 μl was injected.

The ethanol yield factor ($Y_{P/S}$, g/g) was defined as the ratio between the maximum ethanol concentration (g/l) and total sugars consumed (g/l). Ethanol volumetric productivity (Q_P, g/l h) was calculated as the ratio between the maximum ethanol concentration (g/l) and the respective fermentation time (h). The efficiency of sugars conversion to ethanol (η, %) was determined as the ratio between the obtained $Y_{P/S}$ (g/g) and the theoretical value (0.51 g/g) of this parameter [15].

3. Results and discussion

3.1. Hydrolysates composition

The hydrolysates produced from SCG and CS contained significant total sugar amounts, correspondent to approx. 50 and 20 g/l, respectively. For both wastes, the hydrolysis process was performed with high efficiency of sugars extraction (>85%), and the different values of sugars concentration in the hydrolysates is a

consequence of the original content present in each raw material, since SCG is richer in sugars than CS (as can be seen in Section 2.1). Besides the release of sugars from the raw materials structures, the hydrolysis process also promoted the release and/or formation of some compounds, namely acetic acid, furfural, hydroxymethylfurfural (HMF) and phenolic compounds, which can be toxic for the microbial metabolism depending on the concentration that they are present in the fermentation medium [20]. Concentration of these toxic compounds in SCG and CS hydrolysates are given in Table 1. As can be observed in this table, the concentration of furfural and HMF (compounds generated from the degradation of pentose and hexose sugars, respectively) was low (<1.0 g/l) demonstrating that the hydrolysis conditions were able to promote a highly efficient sugars extraction with few degradation of the monosaccharides. Acetic acid that is released from the hemicellulose structure was also present in both hydrolysates, but in a slightly higher concentration in CS hydrolysate, that is in agreement with the chemical composition of the wastes, since CS contains more acetyl groups than SCG (3.0 and 2.2% w/w, respectively). Phenolic compounds were present in similar concentration in both hydrolysates.

It is expected that furfural, HMF and acetic acid, in the concentrations present in SCG and CS hydrolysates, do not cause inhibition in the yeasts metabolism, since several studies report inhibition of the microbial metabolism by concentration values higher than those here found. For example, ethanol production by *P. stipitis* CECT 1922 was not affected by furfural concentrations up to 2 g/l, neither by acetic acid concentrations up to 6 g/l; and the simultaneous presence of acetic acid (1.5 g/l), formic acid (0.5 g/l), and furfural (1 g/l) did not affect also the yeast performance in converting xylose and glucose to ethanol [21]. On the other hand, phenolic compounds have been considered the most toxic compounds present in lignocellulosic hydrolysates, being toxic for the microorganism even when present at low concentrations [20]. Several works report the use of detoxification processes previous the hydrolysate use as fermentation medium, to decrease the concentration of toxic compounds to levels not able to affect the microbial metabolism. In the present study, CS and SCG hydrolysates were used as fermentation medium as obtained. The inclusion of an additional step of hydrolysate detoxification was avoided to not increase the costs of ethanol production.

3.2. Fermentation of SCG hydrolysate

The total sugars consumption from SCG hydrolysate varied to each evaluated yeast (Fig. 1A). *S. cerevisiae* consumed faster these compounds than the other strains, with almost total depletion after 24 h fermentation. *P. stipitis* also consumed practically all the sugars present in the medium, but required a longer fermentation time (36 h). On the other hand, *K. fragilis* consumed only 22% of the sugars present in SCG hydrolysate. The difference observed for the three yeasts is related to the variety of sugars present in this medium. SCG hydrolysate contains glucose, arabinose, galactose and mannose sugars; galactose and mannose being the most abundant, accounting for about 77% of total sugars present [11]. *S. cerevisiae* is a yeast strain with ability to utilize and metabolize hexose sugars such as galactose, mannose and glucose [22]. *P. stipitis* has been reported as able to consume both kinds of sugars: hexoses and pentoses like xylose, for example [23]. *K. fragilis* is a strain mainly reported as able to consume lactose [24], sugar that was not present in SCG hydrolysate. Glucose was present in low concentration (0.81 g/l) in this medium, and was totally consumed by the three yeasts. On the other hand, galactose and mannose

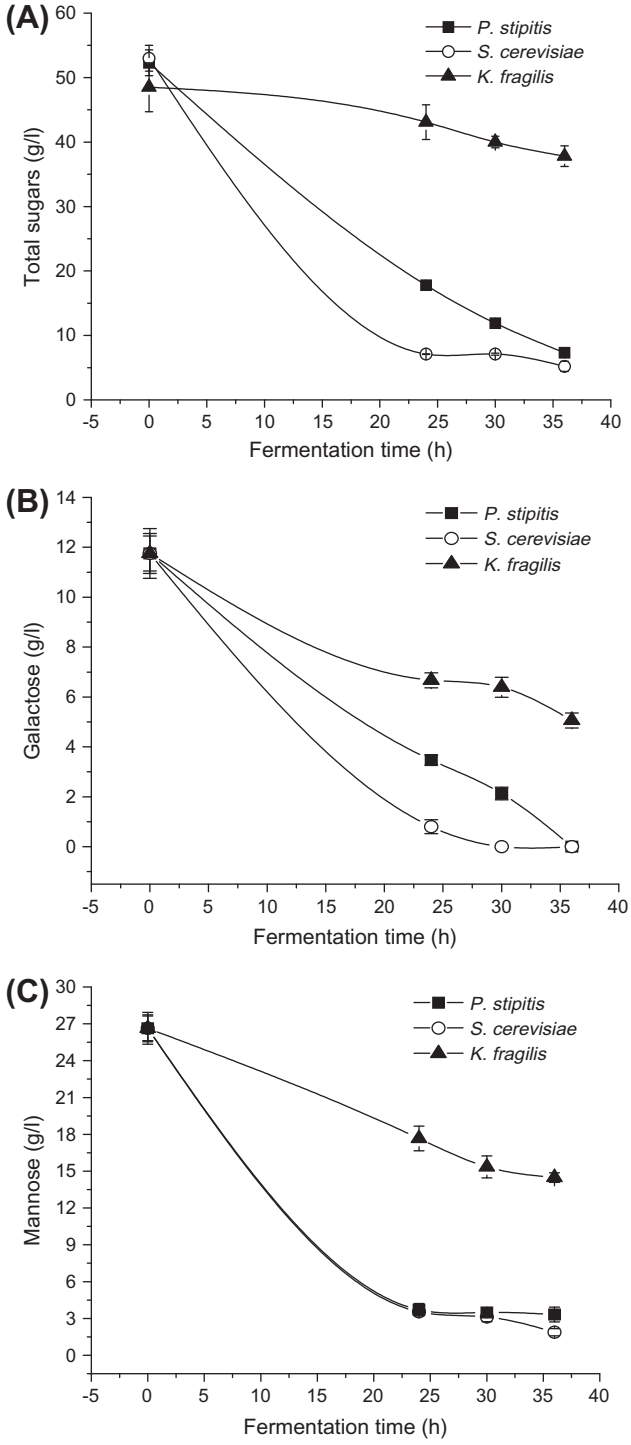

Fig. 1. Consumption of total sugars (A), galactose (B) and mannose (C) in spent coffee grounds hydrolysate, during the fermentation for ethanol production by different yeast strains.

Table 1
Concentration of toxic compounds in coffee silverskin (CS) and spent coffee grounds (SCG) hydrolysates.

Toxic compounds	Concentration in the hydrolysate (g/l)	
	CS	SCG
Acetic acid	1.10 ± 0.10	0.80 ± 0.05
Furfural	0.20 ± 0.06	0.70 ± 0.03
HMF	0.30 ± 0.04	0.10 ± 0.01
Phenolic compounds	3.70 ± 0.20	3.50 ± 0.12

consumption was clearly different for each yeast strain (Fig. 1B and C). Galactose and mannose consumption by *K. fragilis* was really small, especially when compared with the results observed for the other two yeasts. Mannose consumption was similar for *S. cerevisiae* and *P. stipitis*; however, *S. cerevisiae* consumed galactose faster than *P. stipitis*, although both yeasts consumed totally this hexose. Arabinose was not consumed by any of the yeasts, during the considered fermentation time.

As a consequence of the differences in sugars consumption, biomass formation and ethanol production varied to each yeast strain (Fig. 2). All the yeasts strains were able to grow in SCG hydrolysate, but *S. cerevisiae* exhibited the highest and fastest growth results, as result of its higher and faster sugars consumption compared to the other strains. Otherwise, *K. fragilis* had the worst growth results, since it consumed few sugars amount (Fig. 2A). In terms of ethanol production, fermentation with *S. cerevisiae* gave the best results (11.7 g/l) (Fig. 2B), followed by the fermentation with. *P. stipitis*, which produced ethanol concentrations similar to those of *S. cerevisiae*, but needed a longer time to achieve the maximum concentration of this compound. Ethanol production by *K. fragilis* was low throughout the fermentation time. It is evident by these results that this yeast strain had difficulties in metabolizing the main sugars present in SCG hydrolysate (mannose and galactose), and convert them to ethanol.

Table 2 presents the fermentation parameters obtained during the conversion of SCG hydrolysate by the three yeasts. Note that the ethanol yield factors ($Y_{P/S}$) were similar for the fermentations performed with *P. stipitis* and *S. cerevisiae*, which shows that both yeasts had the same ability to convert the sugars to ethanol. As a consequence, both yeasts proportioned similar results of efficiency (η). However, ethanol productivity (Q_P) was higher for *S. cerevisiae* than for *P. stipitis*, suggesting that *S. cerevisiae* had greater ability for ethanol production from sugars present in SCG hydrolysate. *K. fragilis* yielded an $Y_{P/S}$ value correspondent to half the value obtained for the other yeasts, and a very low Q_P, as a result of the low ethanol concentration formed during the fermentation (about 1.1 g/l).

Table 2
Fermentation parameters obtained during the conversion of spent coffee grounds hydrolysate to ethanol by different yeast strains.

Yeast	$Y_{P/S}$ (g/g)	Q_P (g/l h)	η (%)	Time (h)
Pichia stipitis	0.26 ± 0.01	0.35 ± 0.01	51.9 ± 2.3	30
Saccharomyces cerevisiae	0.26 ± 0.02	0.49 ± 0.01	50.2 ± 3.1	24
Kluyveromyces fragilis	0.13 ± 0.02	0.05 ± 0.01	25.5 ± 3.3	24

$Y_{P/S}$ = ethanol yield factor; Q_P = ethanol productivity; η = ethanol efficiency.

The maximum values of $Y_{P/S}$ (0.26 g/g) and Q_P (0.49 g/l h) obtained in this study are comparable or even better than others found during the ethanol production from hemicellulosic hydrolysates obtained from different raw materials. For example, bioconversion of brewer's spent grain hemicellulosic hydrolysate to ethanol by *P. stipitis* NRRL-Y-7124 yielded an $Y_{P/S}$ of 0.34 g/g [25]; the ethanol production by fermentation of sunflower seed hull hydrolysate, also using *P. stipitis* NRRL-Y-7124, gave $Y_{P/S}$ and Q_P values of 0.32 g/g and 0.065 g/l h, respectively [26]; and the fermentation of sugarcane bagasse hemicellulosic hydrolysate by *Scheffersomyces stipitis* UFMG-IMH 43.2 yielded $Y_{P/S}$ of 0.19 g/g and Q_P of 0.13 g/l h [27]. When compared to the fermentation results obtained from these raw materials, it can be concluded that SCG hydrolysate has great potential for use as fermentation medium for ethanol production. Additionally, the sugars content present in the original raw material is also an important factor to support this conclusion, because high ethanol production may be achieved if high sugars content is available for fermentation. In this sense, sugars composition in SCG (45.3% w/w) [11], is well comparable to the sugars composition of other raw materials, such as brewer's spent grain [28], corn fiber [29], and corn stover [30], for example.

It is worth mentioning that this is a first study on ethanol production from SCG hydrolysate, and the results here obtained may be improved if the best operational conditions to perform this process (for example: initial substrate concentration, pH, addition of nutrients, oxygen availability, and others) are established. Additionally, studies about the influence of the inhibitory compounds (mainly the phenolic compounds) present in this hydrolysate on yeast metabolism could be useful to verify if the yeast performance is improved when using the detoxified hydrolysate as fermentation medium.

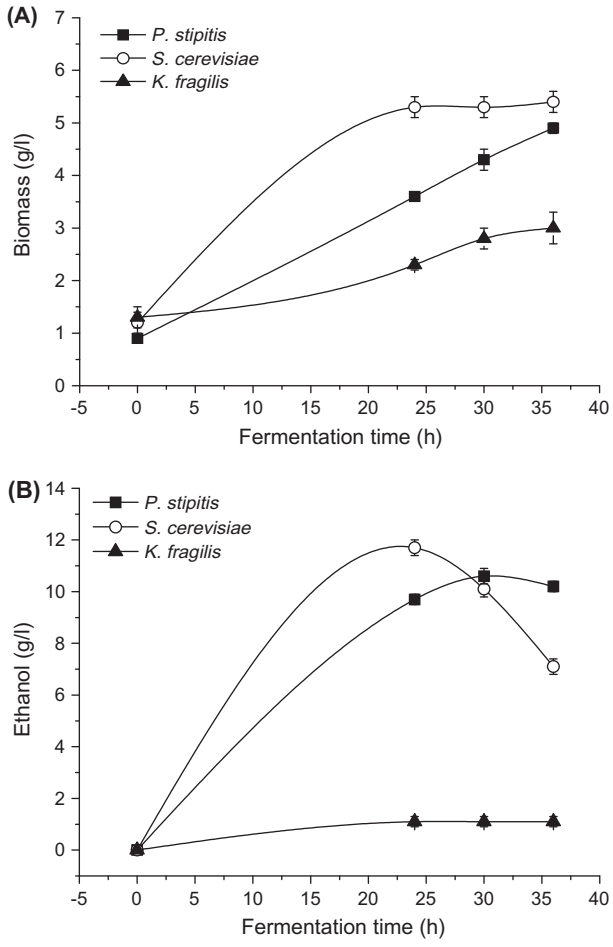

Fig. 2. Biomass and ethanol production during the fermentation of spent coffee grounds hydrolysate by different yeast strains.

3.3. Fermentation of CS hydrolysate

Unlike the observed in the fermentation of SCG hydrolysate, where *S. cerevisiae* consumed the highest sugar amount and faster than the other yeast strains, *P. stipitis* was the yeast responsible for the quickest and highest sugar consumption from CS hydrolysate (Fig. 3A). This fact is also related to the chemical composition of this medium. CS hydrolysate contains xylose, pentose sugar that was not present in SCG hydrolysate. Xylose account for 20% of total sugars present in this medium and is metabolized by *P. stipitis* but not by *S. cerevisiae* and *K. fragilis* [22–24], explaining the lower intake of sugars observed for these other two yeasts.

In terms of cell growth (Fig. 3B) *K. fragilis* exhibited the highest values of biomass from CS hydrolysate, while the lowest values were obtained for *S. cerevisiae*. Note that initially, the biomass

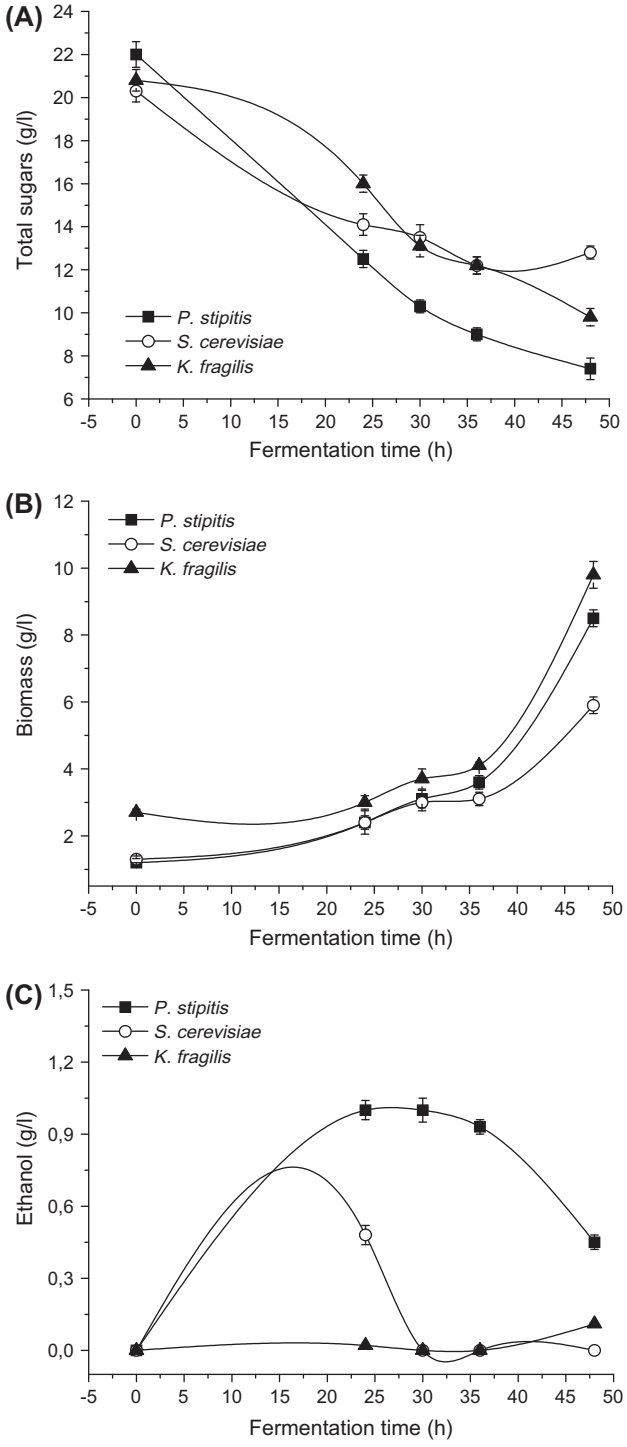

Fig. 3. Total sugars consumption (A), biomass (B) and ethanol production (C) during the fermentation of coffee silverskin hydrolysate by different yeast strains.

formation was similar for *P. stipitis* and *S. cerevisiae*, however, after 30 h of fermentation *P. stipitis* increased its biomass in larger extends than *S. cerevisiae*, probably due to the consumption of pentose sugars, which are not metabolized by *S. cerevisiae*, as mentioned before. The highest values of biomass obtained with *K. fragilis* are related to the no production of ethanol by this microorganism (Fig. 3C), as a consequence, the yeast would have used the entire consumed carbon source only for the cell growth.

Among the studied strains, *P. stipitis* produced the highest ethanol amount from CS hydrolysate. However, the ethanol concentration values obtained in all these fermentations were very low

Table 3
Fermentation parameters obtained during the conversion of coffee silverskin hydrolysate to ethanol by different yeast strains.

Yeast	$Y_{P/S}$ (g/g)	Q_P (g/l h)	η (%)	Time (h)
Pichia stipitis	0.11 ± 0.02	0.04 ± 0.01	21.0 ± 3.0	24
Saccharomyces cerevisiae	0.13 ± 0.03	0.02 ± 0.01	24.9 ± 5.1	24
Kluyveromyces fragilis	0.01 ± 0.00	0.00 ± 0.00	2.2 ± 0.3	48

$Y_{P/S}$ = ethanol yield factor; Q_P = ethanol productivity; η = ethanol efficiency.

($\leqslant$1.0 g/l) due to the low initial sugars concentration in CS hydrolysate ($\approx$22 g/l). Since part of the carbon source is used by the microorganism for the cell growth, few amounts would have been available for use on product formation. Table 3 shows the fermentation parameters obtained during the conversion of CS hydrolysate by the three yeast strains. Note that the values of ethanol yield factor ($Y_{P/S}$) and productivity (Q_P) were low, even for *P. stipitis* that provided the highest ethanol concentration. In fact, the initial substrate concentration in the fermentation medium is directly related to the product formation, and influences the $Y_{P/S}$ and Q_P values, as a consequence [18,31,32]. The initial sugars concentration in SCG hydrolysate ($\approx$54 g/l) was twice the initial concentration in CS hydrolysate, which certainly had a positive effect on the fermentation results obtained. It has been demonstrated in other bioconversion studies that the yield and productivity of the process are improved when the initial sugars concentration is increased, of course, up to a certain limit [18,31,32]. Establishing the initial substrate concentration to be used in a fermentation process is important to attain elevated product formation [18,31,32]. Therefore, a possible alternative to improve the low ethanol production results obtained from CS hydrolysate could be to submit it to a concentration process previous the use as fermentation medium, to increase the sugars concentration. This will be the focus of our future studies.

4. Conclusions

SCG is an agro-industrial waste of large potential for use as raw material for ethanol production, since interesting results of ethanol yield ($Y_{P/S}$ = 0.26 g/g), productivity (Q_P = 0.49 g/l h) and efficiency (η = 50.2%) were obtained when the sugar-rich hydrolysate produced from this material was used as fermentation medium (without any detoxification procedure) by *S. cerevisiae*. Such results could be even improved by establishing the operational conditions that maximize the product formation. On the other hand, fermentation of CS hydrolysate did not yield significant ethanol amounts, probably due to the low concentration of sugars in this hydrolysate, which were used primarily for the microorganisms' growth. However, this hydrolysate acted as an efficient medium for the yeasts' growth, suggesting that it may also be of interest for use in fermentation processes. Concentrating the CS hydrolysate previous its use as fermentation medium could be an alternative to improve the ethanol production results.

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