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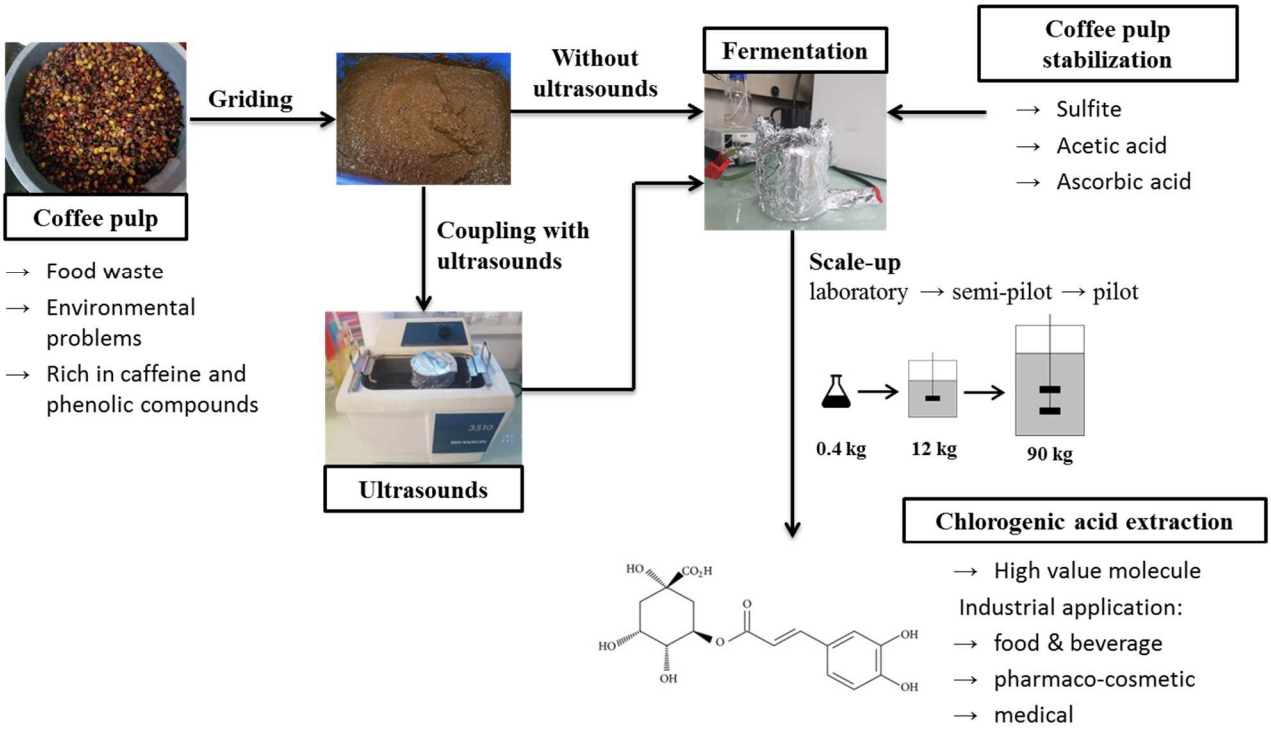
Graphical Abstract



Abstract

In this study, coffee pulp was used as carbon source in solid-state fermentation to produce a phenol-rich extract for industrial applications. Fermentations were carried out at laboratory (0.4 kg), semi-pilot (12 kg) and pilot (90 kg) scales in presence of 2.5 g kg^{-1} yeast strains and with three different coffee pulp materials. The extract stability was investigated using different stabilizing agents: SO_2, ascorbic and acetic acids. Then, a study was conducted to determine the effect of ultrasound treatment on extraction yields. Results showed that higher concentrations of chlorogenic acids were obtained with fermentation without ultrasound treatment and by adding sulfite at 0.5 wt% at 8 h of fermentation. Despite of variations in coffee pulp composition, the process was validated at semi-pilot and pilot scales, providing an extract 400% richer in chlorogenic acids (600 mg per kg of coffee pulp) and with lower sugar amounts to separate during the downstream processing.

Keywords: coffee pulp, food waste treatment, alcoholic fermentation, phenolic compounds extraction, chlorogenic acids

Highlights

- *Saccharomyces cerevisiae* was used to ferment coffee pulp
- Fermentation was investigated at laboratory scale and validated at pilot scale
- Fermentation increased chlorogenic acid content up to 400% in coffee pulp extract
- Sulfite at 0.5 wt% was selected to stabilize phenolics during the fermentation
- Use of ultrasounds was unable to enhance chlorogenic acid extraction

1. Introduction

Worldwide, population growth has accelerated the agro-industrial residues generation causing major environmental, economic and societal problems (water/soil/air pollution, toxicity, waste treatment and disposal). The coffee production sector, one of the most important commodity in the world, generates huge amounts of residues, such as coffee pulp, husk, skin, among others (Esquivel and Jiménez, 2012), which contribute about 50% of the total coffee fruit production. According to data from the International Coffee Organization ("ICO" 2017), global green coffee production in 2017 was estimated to be around 9.5 million t with over 90% of coffee production taking place in developing countries (Central and South America, Central and East Africa, Asia), whereas consumption is mainly in the industrialized economies. Coffee pulp is the main residue (40% mass of coffee fruit) obtained from the wet processing of coffee cherries and is considered as both ecotoxic and antinutritional to animals due to its polyphenols, tannins, and caffeine content (Murthy and Madhava Naidu, 2012). Nevertheless, coffee pulp is rich in sugars, proteins, minerals, amino-acids and also contains functional molecules of high industrial interest (phenolic compounds with antioxidative, anti-inflammatory, antimutagenic, antibacterial, and anticancer properties; caffeine with well-known psychotropic and diuretic properties) (Shahidi and Chandrasekara, 2010; Arellano-González et al. 2011).

Studies have shown that the data on coffee pulp composition can vary due to the characteristics of coffee fruits (cultivar, place of production, culture conditions, maturity, etc.), the postharvest management (methods of depulping, drying, storage, etc.), or even the analytical methods used (Duangjai et al., 2016; Rios et al., 2014; Rodríguez-Durán et al., 2014). Four major classes of phenolic compounds were identified in recent studies:

65 flavonols, flavan-3-ols, anthocyanidins, hydroxycinnamic acids and chlorogenic acids as
66 the predominant phenolic compound (Heeger et al., 2016). These molecules find evident
67 applications in pharmaco-cosmetic, food and medical industries (Quideau et al., 2011).
68 Moreover, hydroxycinnamic acids (HA) including chlorogenic acids (CGA) can be
69 converted into products of even higher added value (vanillin hemisynthesis as an
70 example) (Di Gioia et al., 2011). However, most of these molecules are found in coffee
71 pulp conjugated to sugars as glycosides. Compounds such as chlorogenic acids are found
72 in an ester form bonded to cell wall forming highly complex polysaccharide structures
73 (Asther et al., 2002).

74 Biotechnological processes such as solid-state fermentation can be used to
75 enhance the phenolic content in plant extracts through the breakage of ester bonds
76 between phenolics and the plant cell wall, increasing their concentration and consequently
77 functional properties (Palmieri et al., 2018; Arellano-González et al. 2011). Solid-state
78 fermentation is defined as any fermentation process performed on moist solid materials
79 in the absence of free-flowing water that acts both as physical support and source of
80 nutrients for microorganisms. Due to this low water availability, a limited number of
81 microorganisms, mainly yeasts and fungi, can be used for solid-state fermentation
82 (Thomas et al., 2013).

83 *Saccharomyces cerevisiae* is an important microorganism used worldwide for
84 producing food and beverages. This yeast has several advantages for ethanol production
85 from lignocellulosic biomass: efficient ethanol production from simple sugars, it does not
86 require oxygenation, has a relatively high tolerance to ethanol and inhibitors, has low pH
87 optimum and is generally recognized as safe (GRAS) as a food additive for human
88 consumption. The production of ethanol during alcoholic fermentation also presents an

advantage for the recovery of phenolic compounds from agri-food solid wastes, as mixtures of water/ethanol have been shown to enhance the solubilization of phenols (Benmeziane et al. 2014).

Solid-state fermentation offers numerous advantages over other techniques such as lower energy requirements and the absence of sophisticated and complex machinery and control systems, providing a low-cost process in a sustainable framework. The main drawbacks of this method concern the process scale-up (heat transfer and culture homogeneity) and production yields. Several researchers have studied the application of ultrasound-assisted extraction to increase the conversion of starch materials to glucose as well as overall ethanol yield during fermentation processes by destroying plant cell wall and making it easier to microorganisms to access sugars from plant matrix (Chemat et al., 2017; Nikolić et al., 2010). Ultrasonication has been applied widely in various biological and chemical processes. However, the use of ultrasound-assisted extraction coupled to solid-state fermentation to treat food waste has not been widely investigated. The use of ultrasonics has the potential to break the pulp cell wall and to release sugars due to acoustic cavitation, enhancing ethanol production and phenolic compounds recovery.

A major concern expressed with regards to the extraction of polyphenols has been in relation to their stability. Phenolic compounds may undergo degradation due to temperature, light, oxygen, enzymes, and pH. Light and oxygen in the air are the two most important factors that facilitate degradation reactions. Enzymes (mainly oxidative enzymes) already present in the plant material can be released during the extraction process and promote such degradation reactions (Mäkilä et al., 2016). Therefore, to build an efficient extraction method, it is crucial to keep the stability of phenolic compounds. One way to avoid phenol degradation during extraction is by adding a stabilizing agent

to the solution. In addition to presenting antifungal and antibacterial properties, sulfur dioxide (SO_2) is commonly used as a reducing agent and as an inhibitor of endogenous oxidases in winemaking (Blouin, 2014; Ribereau-Gayon et al., 2006). Especially under its bisulfite form (HSO_3^-), it can efficiently protect phenolics from chemical and enzymatic oxidations. Another well-known food preservative is ascorbic acid, a water-soluble antioxidant naturally found in many fruits and vegetables. In industry, ascorbic acid can be added to plant-based products to preserve its antioxidant capacity and provide chemical stability. Furthermore, in the presence of oxygen, ascorbic acid tends to oxidize, removing the environmental resources of oxygen (Varvara et al., 2016).

The acidification of the solution presents an alternative to prevent phenol degradation by adjusting the pH with acidity regulators. The pH optimum of enzymes, such as polyphenol oxidase (PPO), ranges around pH 6-7 and becomes inactive below pH 4. Hence, the role of acidity regulators is to maintain the pH well below that necessary for optimal catalytic activity. Moreover, it is well-known that hydroxycinnamic acids are stable at lower pH (3-5), unlike basic conditions where isomerization and oxidation reactions can occur (pH≥7.0) (Friedman and Jürgens, 2000; Ma et al., 2011; Narita and Inouye, 2013). A typical acidity regulator is acetic acid, an organic acid that has traditionally been used to improve the shelf-life and microbiological safety of food products. Glacial acetic acid can potentially be used during fermentation processes to enhance phenolic compounds extraction and stability by acidification of the medium, but can cause yeast death at certain concentrations (Leão et al., 2001).

Based on these premises, the aim of this study was to investigate the extraction and stabilization of chlorogenic acids from coffee pulp during solid-state fermentation using commercial yeast strains. First, the process optimization was investigated at

laboratory scale under well-controlled conditions. Then, the fermentation process was applied to larger scales in real condition of production in order to evaluate its interest for industrial application. The outcomes of this study are expected to contribute to the development of a sustainable and competitive method for coffee pulp treatment, applicable to all regions where coffee is produced by the wet processing method (around 50% of the worldwide production).

2. Materials and Methods

2.1. Raw material

Coffee pulp from the wet depulping and demucilaging process of coffee beans (*Coffea arabica*) was supplied by the Beneficio Coopeunión, a coffee-producer cooperative located in Trés Rios (Costa Rica), during the 2015 and 2017 harvests for assays at laboratory and semi-pilot scale, respectively. As soon as obtained, raw coffee pulp was split into lots of 3 kg, frozen at -20°C, and shipped to France by aircraft in a controlled temperature system. Upon arrival, the bags containing frozen coffee pulp were stored at -20°C. Prior to experiments, batches of 1 kg of coffee pulp were thawed at room temperature and ground using a mixer Thermomix TM31 (Vorwerk, Wuppertal, Germany) for 1 min at maximum power (level 10) and without heating. The coffee pulp was then split into hermetically sealed flasks and stored at -20°C.

For the fermentation at pilot scale, coffee pulp was supplied by the Beneficio San Diego, a coffee-producer cooperative located in San José (Costa Rica), during the 2018 harvest. The night before the experiment, coffee pulp was collected and stored at room temperature. Prior to the experiment, batches of 10 kg of coffee pulp were ground using a Bowl Cutter SM 45 (K+G Wetter, Biedenkopf, Germany) for 7 min at maximum power

(level 2) and without heating. All batches of coffee pulp were gathered in a sterilized container and placed in a room at 28°C.

2.2. Chemicals

Methanol, sulfuric acid, and phosphoric acid were all of analytical grade from Honeywell (Seelze, Germany). Standard of chlorogenic acids containing a mix of 3-Caffeoylquinic acid (3-CQA), 5-Caffeoylquinic acid (5-CQA), 4-Caffeoylquinic acid (4-CQA), 4-Feruloylquinic acid (4-FQA), 5-Feruloylquinic acid (5-FQA), 3.4-Dicaffeoylquinic acid (3.4-diCQA), 3.5-Dicaffeoylquinic acid (3.5-diCQA), and 4.5-Dicaffeoylquinic acid (4.5-diCQA), was purchased from International Development and Manufacturing (New Jersey, USA). Standard of glucose, fructose and ethanol were purchased from Sigma Aldrich (Steinheim, Germany). L(+)-ascorbic acid, glacial acetic acid, and sodium metabisulfite, used to stabilize the chlorogenic acids during the assays, were all of analytical grade purchased from Sigma Aldrich (Steinheim, Germany).

2.3. Solid-state fermentation

Two *Saccharomyces cerevisiae* strains used for solid-state fermentation, with a population of 10^7 CFU g^{-1}, were kindly provided by Lallemand (Toulouse, France). The yeast strains were dried and stored at 4°C.

Fermentations at laboratory scale were carried out using a double-walled glass reactor (Legallais, Montferrier-sur-Lez, France) with a working volume of 400 mL. The day prior to experiments, samples of 400 g of coffee pulp were thawed at room temperature under darkness. The yeast strain was re-activated and multiplied using deionized water at 35°C for 30 min, at a ratio of 10 mL of water per g dried yeast. During

this time, the coffee pulp was maintained at 28°C in the fermentation vessels. The temperature of the outer jacket was controlled with a thermostatic water bath. Coffee pulp was inoculated at a ratio of 2.5 g yeast/kg coffee pulp, and solid-state fermentation was carried out at a fixed temperature of 28°C, in darkness and without stirring to maintain anaerobic conditions during the fermentation. Prior to sampling, coffee pulp was gently mixed using a sterilized laboratory tool to ensure homogeneity of the mixture. Samples were clarified by centrifugation at 8603 x g for 15 min.

Semi-pilot and pilot scale fermentations were carried out respectively in France in a 20 L stainless steel tank (Artame, Baguim do Monte, Portugal) containing 12 kg of coffee pulp and in Costa Rica in a 200 L sterilized polypropylene drum (Lacoplast S.A., Guatemala) containing 90 kg of coffee pulp. The tanks were placed in climate rooms set and preheated at 28°C for temperature control. Fermentations were carried out at 28°C, without stirring and under darkness. Coffee pulp was inoculated, sampled and clarified as described for laboratory scale. The coffee pulp extract was separated from the mash by pressing using a hydraulic press (Stossier LI P MO, Simaco, Bouzonville, France or TC Y125, Owatonna Tool Company, Minnesota, USA) at 50-60 bar for 30 min.

2.4. Stabilization procedures for chlorogenic acid stability

In order to prevent chlorogenic acid (CGA) degradation during the fermentation process, the performance of different stabilizing agents was studied at laboratory scale. Sulfur dioxide was used as sodium metabisulfite at 3 concentrations: 30 mg kg^{-1} according to data from the yeast supplier as the limit sulfite concentration supported by their *Saccharomyces cerevisiae* strains, 500 mg kg^{-1} a concentration used for sulfitic maceration of musts in winemaking that favors polyphenol extraction rate (Blouin, 2014),

209 and 0.5 wt% a very high concentration which guarantees total protection against
210 oxidation. Glacial acetic acid was chosen as an acidity regulator at two concentrations:
211 1 wt% to stabilize CGA and allow yeast growth, and at 10 wt% to stabilize CGA and
212 inhibit yeast activity. Finally, ascorbic acid at 1 wt% was used as an antioxidant (Narita
213 and Inouye, 2013). The stabilizing agent was directly mixed to the coffee pulp before
214 inoculation or at a certain time during the fermentation to study its effect on the
215 stabilization of CGA and on yeast activity.
216

217 **2.5. Ultrasound-assisted extraction**

218 For particular experiments, the fermentation at laboratory scale was coupled to
219 ultrasound-assisted extraction in order to evaluate the possible enhancement of
220 chlorogenic acid extraction. The ultrasound treatment was carried out using a Bransonic
221 model 3510E-MT 100 W ultrasound equipment (Danbury, USA) using 42 kHz as
222 frequency. Ultrasounds were applied during 10 min (1) prior to inoculation or (2) at a
223 selected time during fermentation where the maximum amount of CGA was extracted.
224 Total power used was 250 W/kg of coffee pulp.
225

226 **2.6. Analytical methods**

227 The moisture of the raw coffee pulp was determined by gravimetric method in
228 triplicate at 100°C after 48 h. The pH and temperature were obtained using an ALMEMO
229 measuring instrument (Ahlborn, Ilmenau, Germany). The concentration of sugars and
230 ethanol was determined by High-Performance Liquid Chromatography (HPLC) using a
231 Dionex Ultimate 3000 chromatograph (Thermo Scientific) that was equipped with a
232 refractive index detector (RID-10A Schimadzu), a UV detector (210 nm), and an Aminex

HPX87H column that was operated at a temperature of 35°C using 5 mM sulfuric acid as the eluent at a flow rate of de 0.6 mL min^{-1}.

The concentration of chlorogenic acid isomers was determined by HPLC on a system consisting of Shimadzu (Kyoto, Japan) Model LC 20AD pumping units, an automated sample injector (Shimadzu SIL 20 AXR), a variable-wavelength UV detector (Shimadzu SPD20A), column Uptisphere type ODB 5 (5 µm particle size, 250 x 4.6 mm), with identical pre-column, thermostatically controlled at 30°C. The elution program used two solvents, A and B. Solvent A was 4 mM phosphoric acid and solvent B was methanol. The following elution program was used: A—B mixture 95/5 v/v from min 0 to 35, to A—B mixture 25/75 v/v from min 35 to 40, then pure solvent B from min 40 to 50, to A—B mixture 95/5 v/v from min 50 to 55. Flow rate was 1 mL min^{-1}. UV detection was at 327 nm, which corresponds to maximum CGA absorption.

Samples were filtered through 0.45 µm pore size filter before HPLC injection. The compounds (sugars, ethanol, CGA) were identified by comparison of their retention times with the retention times of certified standards. The quantification of compounds was performed using calibration curves with 5 different concentrations of standard solutions ($R^2 > 0.99$).

3. Results and Discussion

3.1. Coffee pulp

This study explored the fermentation of coffee pulp at three different scales: laboratory, semi-pilot, and pilot. Different batches of coffee pulp with different pretreatment conditions were used during the experiments, which resulted in a variability of the raw material composition. A chemical characterization of coffee pulp was carried

out to determine its initial composition in terms of fermentable sugars, ethanol and chlorogenic acid 5-CQA (Table 1).

Table 1

Characterization of raw coffee pulp from different batches in terms of origin, pretreatment steps, local and year of assays, pH, moisture, sugar, ethanol and 5-CQA content.

Laboratory scale					
Harvest year	2015	**Supplier**	Coopéunion	**Pretreatment steps**	Freezing, grinding and freezing
Year of assay(s)	2016-2017	**Local of assay(s)**	France		
		Moisture [%]	85 ± 0.6		
		pH	4.4		
	Glucose [g L^{-1}]	**Fructose [g L^{-1}]**	**Ethanol [g L^{-1}]**	**5-CQA [mg L^{-1}]**	
Upper value	35.16	48.35	0.85	12.7	
Lower value	28.93	37.13	0.36	4.3	
Average(σ)$_n$	31.73(1.84)$_8$	42.97(4.17)$_8$	0.65(0.15)$_8$	7.93(3.22)$_8$	
Semi-pilot scale					
Harvest year	2017	**Supplier**	Coopéunion	**Pretreatment steps**	Freezing, grinding and freezing
Year of assay(s)	2018	**Local of assay(s)**	France		
		Moisture [%]	85 ± 0.8		
		pH	4.3		
	Glucose [g L^{-1}]	**Fructose [g L^{-1}]**	**Ethanol [g L^{-1}]**	**5-CQA [mg L^{-1}]**	
	14.39	21.42	0.6	54.91	
Pilot scale					
Harvest year	2018	**Supplier**	Beneficio San Diego	**Pretreatment steps**	Grinding
Year of assay(s)	2018	**Local of assay(s)**	Costa Rica		
		Moisture [%]	85 ± 0.8		
		pH	4.3		
	Glucose [g L^{-1}]	**Fructose [g L^{-1}]**	**Ethanol [g L^{-1}]**	**5-CQA [mg L^{-1}]**	
	19.47	28.44	0.65	198.66	

s

The chlorogenic acid 5-CQA was reported as being the most abundant simple polyphenol present in fresh coffee pulp (Martínez and Clifford, 2000) and its concentration was followed in all assays of this work. For laboratory scale experiments, eight samples of coffee pulp from the 2015 batch were used. Fermentation at semi-pilot and pilot scales was carried out only once due to limitations in coffee pulp supply. According to results, we observed a difference in coffee pulp composition between samples of the same batch (2015) as well as significant differences between the three batches. Glucose and fructose contents varied by less than 10 % within the laboratory scale samples whereas changed by 41% (glucose) and 36% (fructose) between batches at different scales. Traces of ethanol were found in coffee pulp in concentrations inferior to 1 g L^{-1}, probably due to a natural (but very limited) fermentation of coffee pulp during storage. The biggest difference was found in terms of the amount of 5-CQA. We observed that the highest concentration of 5-CQA, 198 mg L^{-1}, was found in the 2018 batch that was only stored for one night prior to the experiment. Much lower concentrations were found in the coffee pulp batches of 2015 that were milled, frozen and stored for several months. An intermediate value was obtained for the batch of 2017 that was stored for a shorter period than the 2015 batch. This can be explained by the high sensitivity of phenolic compounds, such as chlorogenic acid 5-CQA, to isomerization and/or oxidation by external factors (temperature, light, oxygen) (Narita and Inouye, 2013; Xie et al., 2011) that can occur during the pretreatment and storage steps, leading to CGA degradation.

This finding illustrates the importance of studying the initial coffee pulp composition. Even though the same biomass is used as a substrate for fermentation, quality of the raw material may lead to different ethanol production and chlorogenic acid

289 extraction during fermentation, and this can be a challenge for industrial scale
290 productions.

291

292 **3.2. Study of the fermentation process at laboratory-scale**

293 **3.2.1. Selection of yeast strains and kinetics of CGA extraction**

294 A preliminary study was carried out to select a *Saccharomyces cerevisiae* strain
295 for coffee pulp fermentation with the view to enhance chlorogenic acid extraction. Two
296 strains were tested, Yeast A and Yeast B, which differed in terms of sulfite production
297 during fermentation (Yeast A presenting lower SO_2 production). Table 2 presents the
298 relative concentration (ratio between the final and initial concentrations of a compound)
299 for the glucose, fructose, ethanol and 5-CQA after 24 h of fermentation.

300 **Table 2**
301 Relative concentration C/C_0 (ratio between the final and initial concentrations) of glucose,
302 fructose and ethanol after 24 h of fermentation and final concentration of 5-CQA obtained at
303 laboratory scale for both yeast strains tested (Yeast A and Yeast B) and for a control of coffee
304 pulp non-inoculated.

305

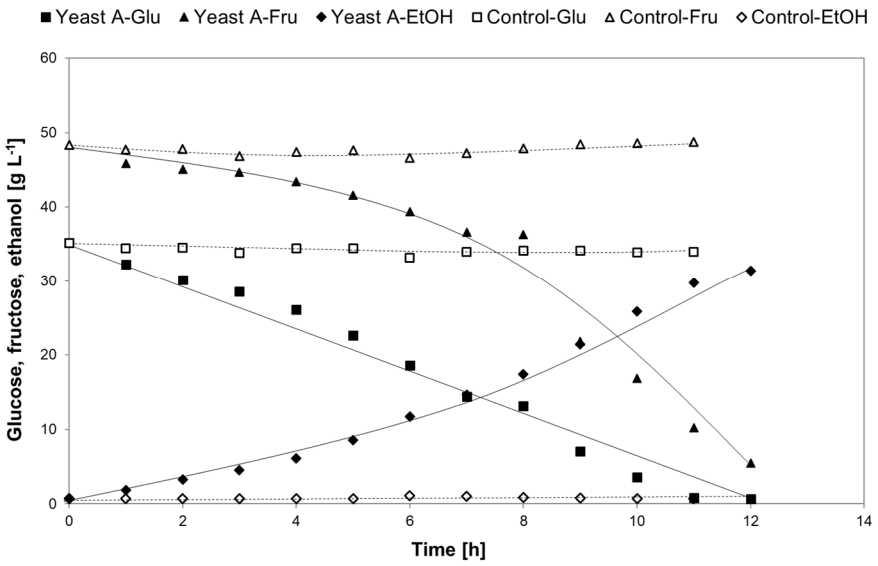
	Relative concentration (C/C_0) after 24 h			Final concentration of 5-CQA after 24 h [mg L^{-1}]
	Glucose	Fructose	Ethanol	
Control	1.00	1.00	0.98	8
Yeast A	0.01	0.11	42.80	30
Yeast B	0.01	0.11	42.60	4

306

307 Results from the control (non-inoculated coffee pulp) show that no alcoholic
308 fermentation occurred within the 24 h period and that the final coffee pulp extract
309 contained 8.0 mg L^{-1} of 5-CQA. Stability of the raw coffee pulp even with its endogenous
310 flora is probably correlated with its high phenolic content that inhibits microorganism
311 growth. Both yeasts were able to ferment coffee pulp and lead to very similar

312 performances in terms of sugar consumption and ethanol production. Nevertheless, only
313 one strain was capable of increasing the concentration of 5-CQA in the extract from
314 coffee pulp. While the fermentation using Yeast A increased by almost 4 times the
315 concentration of 5-CQA compared to the control, results with Yeast B showed the
316 degradation of this molecule during the fermentation process. This result is probably
317 correlated to a difference in the production of pectinases, β-glucanases and other
318 hydrolytic enzymes that are able to facilitate the release of the 5-CQA out of plant cells
319 (Alimardani-Theuil et al., 2011; Pinelo et al., 2006). This shows that only specific strains
320 can be used for the extraction of chlorogenic acids from coffee pulp. The Yeast A was
321 then selected for all the experiments presented in this work.

322 To better understand the fermentation and extraction processes, a kinetic study
323 was carried out with coffee pulp inoculated with Yeast A and the results were compared
324 to a control experiment (non-inoculated coffee pulp). Figure 1 presents the evolution of
325 glucose, fructose and ethanol concentrations during the solid-state fermentation for both
326 samples.
327



328

329 **Fig. 1.** Substrate consumption (glucose, fructose) and ethanol production during solid-state
330 fermentation of coffee pulp carried out at lab scale with Yeast A, compared to a control non-
331 inoculated.

332

333 Results showed that glucose was the preferred carbon source, followed by fructose
334 as usual. Both sugars were almost completely consumed after 12 h of fermentation. The
335 production of ethanol was correlated to the consumption of sugars, increasing during
336 fermentation and levelling off to a final concentration of 31.4 g L^{-1} (around 4% vol.) at t
337 = 12 h, when sugar stocks reached a very low level (glucose < 1 g L^{-1}; fructose < 6 g L^{-1}
338). No fermentation occurred in the control.

339 Figure 2 presents the evolution of chlorogenic acids (CGA) extraction in the
340 inoculated coffee pulp compared to the control. The CGA identified were: 5-CQA, 4-
341 CQA, 4-FQA, 5-FQA, and 4.5-diCQA. The evolution of the total amount of these five
342 chlorogenic acids is also presented (total CGA).

343 Results from the inoculated pulp (Yeast A) show that 5-CQA was the only isomer
344 that was significantly extracted during the fermentation process. Its concentration
345 increased over time reaching a maximum at t = 8 h and decreasing afterward, probably

346 due to a higher degradation rate of this compound compared to the extraction. The
347 concentration of 4-CQA, 5-FQA, and 4.5-diCQA varied very little over time, while the
348 concentration of 4-FQA slowly decreased. The control showed similar behavior for the
349 4-CQA, 5-FQA, and 4.5-diCQA, with their concentrations staying relatively stable over
350 time. On the contrary the concentrations of 5-CQA and 4-FQA decreased over time.
351 During fermentation, different behaviors were observed. Surprisingly for 5-CQA the
352 concentration increased over time reaching a maximum at 8 h and then decreased
353 drastically. It was already reported that the hydroxycinnamic acids are mostly present in
354 the coffee pulp under linked-form with cell walls whereas the soluble compounds could
355 be mainly located inside the plant vacuoles (Rodríguez-Durán et al., 2014). The
356 enzymatic pool of the yeast which is responsible for the cell wall weakening could favor
357 the wall-bonded 5-CQA release as well as the increasing ethanol concentration could
358 favor the solubility of this compound and its extraction. Losses of 5-CQA become
359 predominant after 8 h of fermentation through oxidation reactions by chemical, enzymatic
360 and even fermentation pathways or through adsorption by the yeasts itselves (Mazauric
361 and Salmon, 2005). Furthermore, with lower sugar stocks in the media, chlorogenic acids
362 could become the carbon source in its turn. Figure 3 summarizes the possible pathways
363 of extraction and degradation of CGA during coffee pulp fermentation.

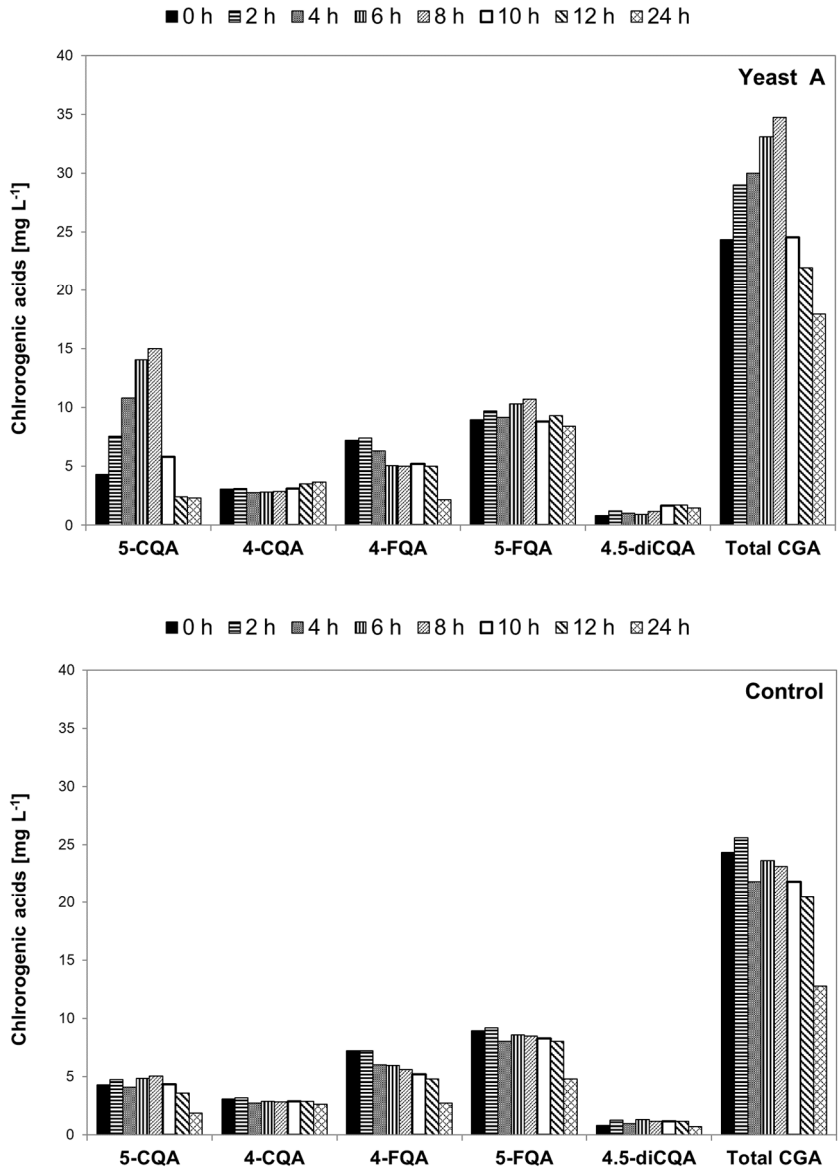
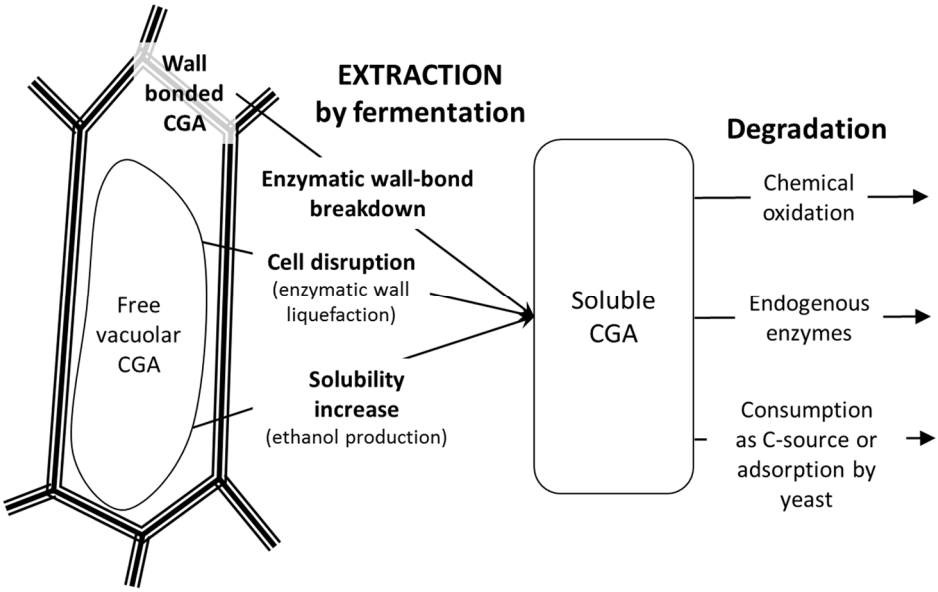


Fig. 2. Extraction of chlorogenic acids from coffee pulp during fermentation at lab scale with yeast A compared to control non-inoculated.



369

370 **Fig. 3.** Suggested competing pathways of the extraction and degradation of chlorogenic acids
371 (CGA) during fermentation of coffee pulp.

372

373 Finally, chlorogenic acids may also undergo various isomerization reactions
374 (Liang and Kitts, 2015; Moon et al., 2009) and could explain the surprising stability
375 observed in Figure 2 for 4-CQA, 5-FQA and 4.5-diCQA, compared to 5-CQA and 4-
376 CQA, during fermentation (as an example, 5-CQA could be partially converted into 4-
377 CQA and 5-FQA into 4-FQA).

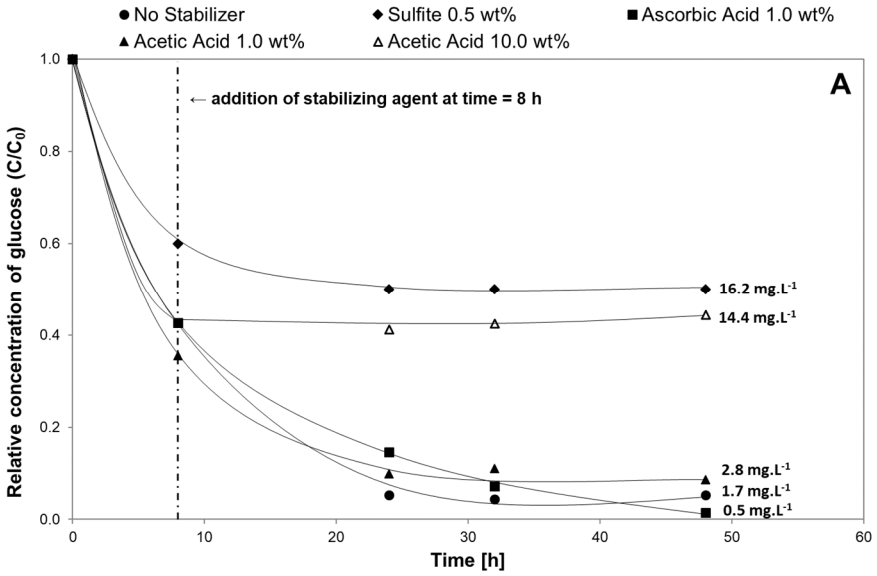
378 To conclude this part of the study, these results showed that it was possible to
379 significantly extract the 5-CQA using solid-state fermentation. In fact, alcoholic
380 fermentation with Yeast A allowed an increase of 3.5 times of 5-CQA initial
381 concentration, from 4.3 mg L^{-1} to 15 mg L^{-1}, at only 8 h of fermentation. However, in
382 order to preserve the 5-CQA extracted during the fermentation process, it is important to
383 develop a stabilization method for chlorogenic acids.

384

3.2.2. Stabilization of chlorogenic acids

A study was carried out to compare different strategies for the stabilization of chlorogenic acids in the coffee pulp. Initially, two concentrations of sulfite were chosen to stabilize coffee pulp prior to inoculation: 30 and 500 mg kg^{-1}. It was found that when adding this agent at the beginning of the fermentation, there were either complete yeast inactivation resulting in no chlorogenic acid extraction (500 mg kg^{-1} of SO_2) or coffee pulp fermentation but no chlorogenic acid stabilization (30 mg kg^{-1} of SO_2). So, under those tested conditions, the stabilization step applied to the coffee pulp before the fermentation process was not suitable. A complementary study was then performed comparing 3 different stabilizing agents (sulfite 0.5 wt%, ascorbic acid 1 wt%, and acetic acid 1 wt% and 10 wt%) added into the culture medium at the optimal time of 5-CQA extraction, i.e. at 8 h of fermentation (as previously shown in Figure 2). This new strategy aimed to stabilize the 5-CQA at its highest concentration in the extract and to study the effect of the addition of stabilizing agent on the yeast activity.

Figure 4 presents the evolution of glucose and 5-CQA concentrations for the different assays. Since 5 coffee pulp samples from batch 2015 were used during the experiments, the concentrations were expressed in terms of the relative concentration of each compound (C/C_0) due to raw material variability. Indeed, it is worth noting that, irrespective to the fermentation trials, fructose consumption and ethanol production showed the same evolution as previously described that confirmed the good reproducibility of the fermentation of the pulp by yeast. For this raison, the evolution of glucose concentration was used as the fermentation indicator in all the following results.



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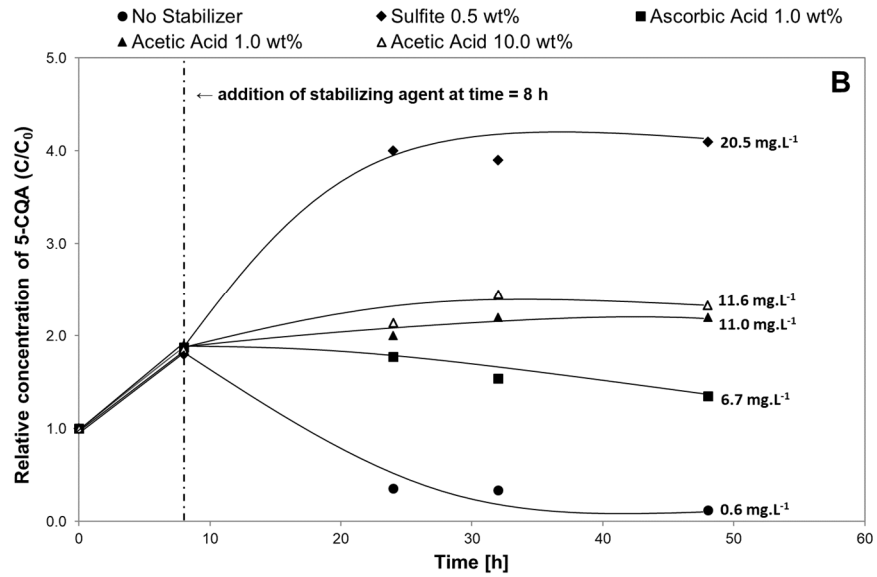

408

409 **Fig. 4.** Effect of different stabilizing agents on the (A) glucose consumption and (B) 5-CQA
410 extraction during solid-state fermentation at lab scale with yeast A (glucose: $C_0 = 32.5 \pm 1.5$ g L^{-1}; 5-CQA: $C_0 = 5 \pm 2$ mg L^{-1}).

412

413 From Fig.4, the addition of ascorbic acid at 1 wt% or acetic acid at 1 wt% didn't
414 affect yeast activity; the concentration of glucose continued to decrease over time as it
415 was observed in the experiment done without any stabilizing agent. As expected, the
416 addition of sulfite at 0.5 wt% or acetic acid at 10 wt% stopped the fermentation process,
417 which indicates that, at the given concentration, sulfite and acetic acid inhibited yeast

418 activity. Both of these stabilizing agents are known for acting through a strong
419 acidification of intracellular content and, also by an intrinsic toxic activity like in the case
420 of HSO_3^-, by blocking many carbonyl functions that disrupt the cell metabolism (Blouin,
421 2014). Concerning the extraction of 5-CQA, the addition of ascorbic acid tended to
422 stabilize the 5-CQA until 24 h of fermentation. Afterward, due to a gradually oxidation
423 of ascorbic acid, the 5-CQA started to degrade faster. The acetic acid, in both
424 concentrations tested, was successful to stabilize the 5-CQA. Surprisingly, the addition
425 of sulfite not only stabilized the 5-CQA, but also increased its concentration over time,
426 reaching a maximum at 24 h of fermentation and representing an enhancement of 400%
427 of 5-CQA concentration compared to its initial concentration. This can be explained first
428 by the fact that 5-CQA can undergo reversible electrochemical reactions (Tomac and
429 Šeruga, 2016), that is to say, oxidized forms of 5-CQA can be reversibly reduced by the
430 redox action of sulfite, unlike the other stabilizing agents tested in the study. Second, this
431 result could also be due to a faster destruction of the plant cells resulting in higher
432 amounts of 5-CQA being released. This well-known phenomenon in winemaking is often
433 called the "dissolvent activity" of sulfites (Ribereau-Gayon et al., 2006).

434 Combining the results in terms of glucose consumption and 5-CQA extraction,
435 two stabilizing agents could be used for the fermentation of coffee pulp: acetic acid at
436 1 wt% that allows yeast growth while stabilizing the 5-CQA gradually extracted, and the
437 sulfite at 0.5 wt% that stops fermentation, stabilizes and enhances 5-CQA extraction. For
438 acetic acid, the advantage is a complete consumption of sugars during the fermentation.
439 So, the low sugar content of the final extract should considerably simplify the further
440 purification steps during downstream processing. The use of sulfite will provide an
441 extract richer in chlorogenic acids but less pure (with more sugars), and the use of acetic

442 acid at 1wt% will provide a purer extract, but with lower antioxidant capacity. Therefore,
443 a compromise must be found when choosing a stabilizing agent for this process.
444
445 **3.2.3. Study of the process optimization using ultrasound-assisted extraction**
446 Two strategies were chosen to combine ultrasound-assisted extraction to the
447 fermentation process: prior to fermentation (i.e. before addition of yeasts) or after 8 h of
448 fermentation when the highest concentration of 5-CQA was achieved (according to
449 results in Section 3.2.2). Even if ultrasounds can disrupt also the yeast cells causing yeast
450 death and thus stop the fermentation as it was recently reported (Chemat et al., 2017), the
451 fermentation during the first hours could potentiate ultrasound effects and thus favor
452 CGAs' extraction. Since the main purpose of our process was to enhance the amount of
453 CGA in the coffee pulp extract, the use of ultrasounds during the fermentation was an
454 interesting strategy to explore. Figure 5 presents the results for glucose consumption and
455 5-CQA extraction during the fermentation process. In that case, coffee pulp was stabilized
456 using acetic acid at 1 wt% in order to preserve the 5-CQA while allowing the fermentation
457 process to continue.
458 Results showed that glucose consumption was similar for both systems.
459 Surprisingly, the glucose was not completely consumed even after 48 h of fermentation,
460 compared to previous results that showed the glucose being mainly consumed at 24 h of
461 fermentation (Figure 4). A moderate 5-CQA extraction optimization could be obtained
462 with ultrasound-assisted extraction, as the content of 5-CQA at 8 h of fermentation was
463 only increased by around 280% (US at 8 h) or 220% (US prior to fermentation) compared
464 to 200% (fermentation without ultrasounds). Moreover, 5-CQA became unstable after
465 24 h of fermentation, which could be explained by the possible release of endogenous

466 oxidative enzymes due to the ultrasound treatment. Another proposed explanation for the
467 5-CQA instability relies on the fact that the ultrasound treatment could release other
468 compounds that could interact with the 5-CQA throughout chemical reactions causing its
469 degradation. Additionally, ultrasound treatment could enhance the extraction of many
470 phenolic compounds from the coffee pulp that in turn could create an inhibitory
471 environment for yeast growth, leading to the decrease of fermentation activity. Under the
472 experimental conditions applied in this work, the coupling of ultrasounds prior or during
473 the fermentation process is not an interesting method to improve the extraction yields of
474 5-CQA from coffee pulp. It didn't improve the 5-CQA extraction yield as expected, and
475 even slowed down the fermentation process. Therefore, no ultrasound treatment was
476 applied on further studies.

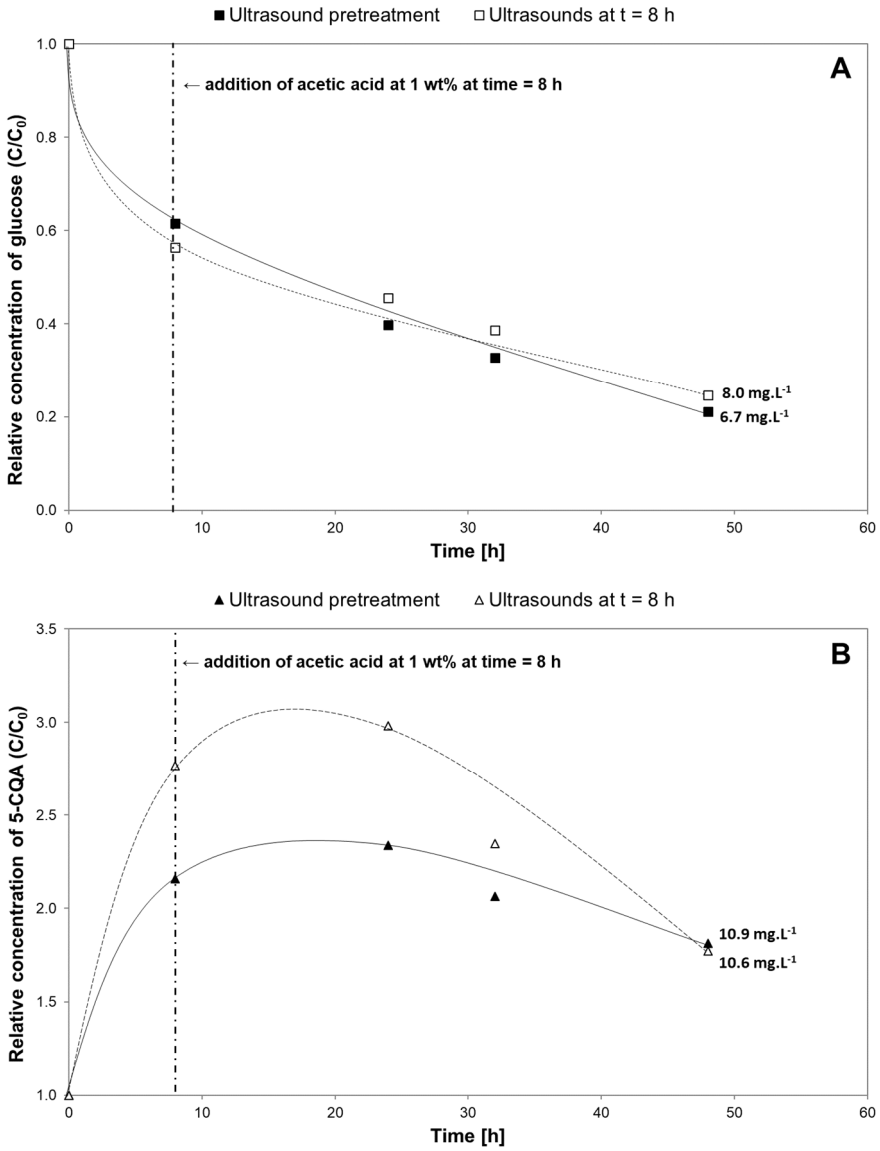

Fig. 5. Effect of ultrasounds on glucose consumption (A) and 5-CQA extraction (B) during fermentation at laboratory-scale. (Glucose: $C_0 = 31.9 \pm 1.8$ g L^{-1}; 5-CQA: ($C_0 = 6 \pm 2$ mg L^{-1}).

3.3. Application of the fermentation process at larger scales

To evaluate the validity of the results obtained at laboratory scale, fermentation at semi-pilot scale was carried out and monitored during 24 h. Sulfite at 0.5 wt% was chosen as the best stabilizing agent for the coffee pulp stabilization after a first step of fermentation. As mentioned in Section 3.1, a different batch of coffee pulp was used for this assay (batch of 2017), which resulted in differences on the initial concentrations of

sugars and chlorogenic acids compared to the coffee pulp used for laboratory scale experiments (batch of 2015). The batch of 2017, used for this study, contained lower concentrations of sugars and a higher initial amount of 5-CQA, as presented in the Table 1 and discussed in Section 3.1. Figure 6 presents the results for the glucose consumption and 5-CQA extraction during the fermentation process.

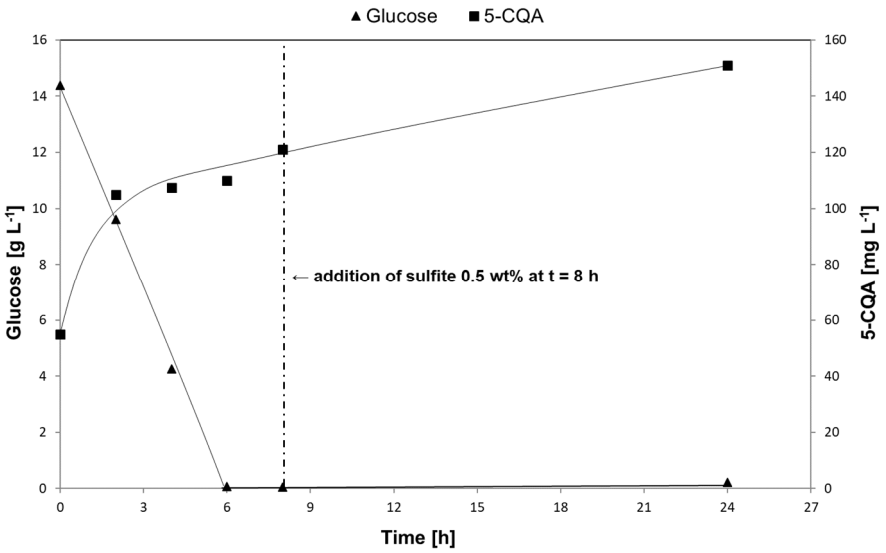

Fig. 6. Glucose consumption and 5-CQA extraction during fermentation at semi-pilot scale.

The glucose was completely consumed after only 6 h of fermentation, which can be explained by the lower initial amount of sugars in the coffee pulp from batch 2017 (14.39 g L^{-1} of glucose and 21.42 g L^{-1} of fructose) compared to the coffee pulp from batch 2015 (31.73 g L^{-1} of glucose and 42.97 g L^{-1} of fructose). With most of the fermentable sugars being consumed before 8 h of fermentation, the addition of sulfite at t = 8 h did not disturb the yeast activity since the fermentation was already completed at this time. The concentration of 5-CQA increased over time as previously observed at laboratory scale, even after the addition of the sulfite (as explained in Section 3.3), up to

505 a final concentration of 151 mg L^{-1} of 5-CQA at t 24 h, 275% higher than the initial
506 concentration (55 mg L^{-1} of 5-CQA). When the fermentation was carried out at laboratory
507 scale, using sulfite at 0.5 wt% at t = 8 h as the stabilizing agent, the content of 5-CQA in
508 the coffee pulp extract was increased by 400% at t 24h, against 275% at semi-pilot scale.
509 Nevertheless, it was shown that the fermentation of coffee pulp and the extraction of
510 chlorogenic acids were successful at semi-pilot scale, despite of the differences in coffee
511 pulp composition (Table 1) and of the limitations encountered (reduced mass and heat
512 transfers during the process scale-up).

513 Finally, to evaluate the process in a coffee producing country, fermentation was
514 carried out at pilot-scale in Costa Rica with 90 kg of fresh coffee pulp nearby the green
515 coffee production area. In order to compare the results from the semi-pilot fermentation,
516 sulfite at a concentration of 0.5 wt% was used to stabilize the chlorogenic acids extracted
517 during the fermentation. Table 3 summarizes the results for the fermentation performed
518 at different scales. As mentioned in Section 3.1, the coffee pulp composition varied due
519 to the year of harvest and the storage time, so the relative concentration (C/C_0) of each
520 compound (glucose, fructose, ethanol and CGA) was chosen instead of direct
521 concentrations (C) values.

Table 3

Overview of the year of coffee pulp batches, the average temperature during the fermentation process (T) and the relative concentration (ratio between the final C and initial C_0 concentrations) of glucose, fructose, ethanol and 5-CQA after 24 h of fermentation at laboratory, semi-pilot and pilot scales.

Scale	Batch	T [°C]	Relative concentration (C/C_0) after 24 h			
			Glucose	Fructose	Ethanol	5-CQA
Laboratory	2015	28	0.50	0.72	11.67	4.00
Semi-pilot	2017	26	0.02	0.10	24.00	2.75
Pilot	2018	22	0.14	0.22	22.86	3.09

For all three scales, sulfite at 0.5 wt% was used to stabilize the 5-CQA extracted, added at 8 h of fermentation. At laboratory scale, the fermentation was immediately stopped when sulfite was mixed to coffee pulp (Figure 4), which explains the higher relative concentrations of glucose and fructose in the product after 24 h. This was not the case for the 2017 and 2018 batches, which had lower initial concentrations of sugars, allowing the almost complete consumption of glucose and fructose prior to the addition of sulfite. In that cases, ethanol production yields reached a quite high level between 0.37 and 0.41 g ethanol / g sugar which corresponds to 72 and 80% of the maximal theoretical conversion yield (0.51 g ethanol / g sugar). Nevertheless, the extraction of 5-CQA was very similar for all three scales, with an augmentation of around 300% of its initial concentration in less than 1 day of fermentation.

Although the operating temperature was set at 28 °C, the average temperature of coffee pulp varied with the scale of the process. Obviously the bigger the amount of pulp used as substrate, the harder the temperature control of the coffee pulp. Since no stirring was applied during the fermentation, heat transfer became an important problem for the process. However, even at lower temperatures, solid-state fermentation using a

commercial yeast (Yeast A) was able to ferment coffee pulp and to produce a rich phenol-extract containing more than 600 mg of 5-CQA per kg of coffee pulp and purer in terms of the lower concentration of sugars (5 g L^{-1} of glucose and 12 g L^{-1} of fructose). We showed that the fermentation of coffee pulp at a pre-industrial (pilot) scale with very simple equipment can be used to obtain a valuable product. It was important to develop a process that could be easily implemented in the coffee producing countries, without big financial investment and operating costs, lower environmental impact and that could generate a new source of income for the small coffee producers.

Conclusions

Solid-state fermentation was used to produce a valuable product from coffee pulp at laboratory, semi-pilot and pilot scales. *Saccharomyces cerevisiae* consumed the sugars releasing chlorogenic acids that are found linked to the plant cell wall probably as glycosides. Investigation of the extract stability and the effect of ultrasounds revealed that higher extraction yields were obtained when fermentation was carried out without ultrasound treatment and by using sulfite at 0.5 wt% as the stabilizing agent. Irrespective of the scales, a phenol-rich extract was obtained containing 300-400% more chlorogenic acids than its initial concentration, within less than 24 h of fermentation. After fermentation and solid/liquid separation steps, the quantity of the remaining solid residue was considerably reduced. This final by-product will contain much less caffeine and phenolics (compounds considered as anti-nutritional and eco-toxic), which could facilitate its reuse as animal feeding, fertilizer or composting substrate.

For further studies, it could be of great interest to test other yeast strains and to optimize fermentation conditions for even increasing the extraction yield. In order to find the best stabilizing method, in terms of (1) CGA preservation, (2) effect on the fermentation activity and (3) cost and environmental impact, new stabilizing agents must be tested at larger scales. To obtain a ready-to-market product, the concentration and the purification of the extract need to be performed and investigated using sustainable technologies. Finally, the characterization of the final product will have to be carried out, e.g., in terms of antioxidant activity, to better determine its market added value and to enlighten about the economic feasibility of the process.

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