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Isolation, selection and evaluation of yeasts for use in fermentation of coffee beans by the wet process

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ABSTRACT

During wet processing of coffee, the ripe cherries are pulped, then fermented and dried. This study reports an experimental approach for target identification and selection of indigenous coffee yeasts and their potential use as starter cultures during the fermentation step of wet processing. A total of 144 yeast isolates originating from spontaneously fermenting coffee beans were identified by molecular approaches and screened for their capacity to grow under coffee-associated stress conditions. According to ITS-rRNA gene sequencing, *Pichia fermentans* and *Pichia kluyveri* were the most frequent isolates, followed by *Candida Candida glabrata*, *quercitrusa*, *Saccharomyces* sp., *Pichia guilliermondii*, *Pichia caribbica* and *Hanseniaspora opuntiae*. Nine stress-tolerant yeast strains were evaluated for their ability to produce aromatic compounds in a coffee pulp simulation medium and for their pectinolytic activity. *P. fermentans* YC5.2 produced the highest concentrations of flavor-active ester compounds (viz., ethyl acetate and isoamyl acetate), while *Saccharomyces* sp. YC9.15 was the best pectinase-producing strain. The potential impact of these selected yeast strains to promote flavor development in coffee beverages was investigated for inoculating coffee beans during wet fermentation trials at laboratory scale. Inoculation of a single culture of *P. fermentans* YC5.2 and co-culture of *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 enhanced significantly the formation of volatile aroma compounds during the fermentation process compared to un-inoculated control. The sensory analysis indicated that the flavor of coffee beverages was influenced by the starter cultures, being rated as having the higher sensory scores for fruity, buttery and fermented aroma. This demonstrates a complementary role of yeasts associated with coffee quality through the synthesis of yeast-specific volatile constituents. The yeast strains *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 have a great potential for use as starter cultures in wet processing of coffee and may possibly help to control and standardize the fermentation process and produce coffee beverages with novel and desirable flavor profiles.

1. Introduction

Coffee is an important plantation crop grown in more than 50 developing countries and is one of the most popular non-alcoholic beverages consumed throughout the world (Selvamurugan et al., 2010). Its annual production has reached 8.4 million metric tons, with a turnover close to US $10 billion. Brazil is the leading producer of coffee, followed by Vietnam, Colombia, Indonesia and Mexico (FAO, 2013). Post-harvest processing of coffee cherries is carried out in producing countries using two processes, referred to as wet and dry (Pandey et al., 2000). Wet processing is used mainly for arabica coffee: the ripe fruits are depulped and then submitted to 24–48 h of underwater tank fermentation and dried until a final water content of 10–12% (Avallone et al., 2001; Murthy and Naidu, 2012). The wet method is widely used in some regions, including Colombia, Central America and Hawaii (Vilela et al., 2010). In the dry processing, in contrast, entire coffee fruits are dried (in the sun) on platforms and/or on a floor without prior removal of the pulp (Silva et al., 2008). Brazil is the largest producer of coffees obtained by dry process; however, the wet process has increasingly been used as a way to improve the coffee quality (Borem, 2008; Gonçalves et al., 2008).

During wet processing, the ripe coffee fruits undergo a spontaneous fermentation, carried out by a complex microbiological process that involves the actions of microorganisms like yeasts, bacteria and filamentous fungi (Avallone et al., 2001; Silva et al., 2008). The

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fermentation is carried out to eliminate any mucilage still stuck to the beans and helps improve beverage flavor by producing microbial metabolites, which are precursors of volatile compounds formed during roasting (Mussatto et al., 2011). Yeasts are among the microorganisms most frequently isolated from fermenting coffee beans, but limited information is available regarding their effect on the development of coffee's taste characteristics (Evangelista et al., 2014). Surveys have shown that the most frequently occurring species during coffee processing are *Pichia kluyveri, Pichia anomala*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* (Masoud et al., 2004; Silva et al., 2008; Vilela et al., 2010). In addition, bacteria with pectinolytic activity belonging to the genera *Erwinia*, *Klebsiella*, *Aerobacter*, *Escherichia* and *Bacillus*, as well as a variety of filamentous fungi are often isolated (Avallone et al., 2001; Silva et al., 2012; Vaughn et al., 1958).

The use of selected yeasts is well known for other fermented foods and beverages, such as wines, to which as many as eight strains or more of *S. cerevisiae* may contribute to the one wine fermentation (Fleet, 2008). This was possible after many studies to understand the impact of individual yeast strains upon final product quality (Ciani et al., 2006; Erten, 2002; Fleet, 2003, 2008; Gao and Fleet, 1988; Mendoza et al., 2007). For coffee processing that reaches this level, further research is needed to increase the understanding of the microbial ecology, physiology and biochemistry of coffee fermentation and how this scientific knowledge contributes to the development of coffee beverage character. The exploration of the biodiversity of indigenous coffee yeast strains can be an important contribution to the understanding and selection of strains with specific phenotypes able to contribute to the final product quality (Masoud et al., 2004; Silva et al., 2012).

To the best of our knowledge, no previous studies have investigated the use of aromatic yeasts as starter cultures during the fermentation step of wet coffee processing and what impact such yeasts might have on coffee beverage flavor. Here we report an experimental approach to target identification and selection of indigenous coffee yeasts and their potential use as starter cultures with the aim of improving the flavor of coffee beverage processed by wet method. The on-farm implementation of these novel starter cultures is part of a patented process developed in our laboratory (Soccol et al., 2013).

2. Materials and methods

2.1. Spontaneous coffee fermentation and yeast isolation

A total of 150 kg of coffee cherries (*Coffea arabica* var. Mundo Novo) were manually harvested at the mature stage from a farm located in the city of Lavras, Minas Gerais State, Brazil, and mechanically depulped using a BDSV-04 Pinhalense depulper (Pinhalense, Sao Paulo, Brazil). Approximately 75 kg of depulped beans were then conveyed in a clear water stream to tanks and left to ferment for 48 h in accordance with local wet processing method. The environmental temperature was 23–30 °C (day-time temperature) and 11–15 °C (night-time temperature). Every 8 h, liquid fraction samples were withdrawn in triplicate from the middle depth of the tank fermentation, placed aseptically in sterile plastic bags and transferred to the laboratory in ice boxes. Ten milliliters of each sample was added to 90 ml sterile saline-peptone water, followed by serial dilutions. Yeasts were enumerated by surface inoculation on YEPG agar [1% yeast extract (Merck, São Paulo, Brazil), 2% peptone (Himedia, São Paulo, Brazil), 2% glucose (Merck) and 2.5% agar (Difco, São Paulo, Brazil); pH = 5.6] containing 100 mg/l chloramphenicol (Sigma, São Paulo, Brazil) and 50 mg/l chlortetracycline (Sigma) to inhibit bacterial growth. Plating was performed, in triplicate, with 100 μl of each dilution. Cultures were incubated at 30 °C for 4 days. According to the macroscopic indications (texture, surface, margin, elevation, and color), colonies of different types on YEPG medium were counted separately, and representatives isolated from different fermentation times were purified by repetitive streaking on YEPG agar. The purified isolates were stored at −80 °C in YEPG broth containing 20% (v/v) glycerol (Difco).

2.2. Identification of yeast isolates

The yeast DNA was extracted from the pure cultures according to the method described by Pereira et al. (2013). The 5.8S ITS rRNA gene region of yeast isolates was amplified using the primers ITS1 and ITS4 (Masoud et al., 2004). The obtained ITS-rRNA gene region of yeast isolates was digested by restriction endonucleases *Hae*III and *Msp*I, according to the manufacturer's instructions (Invitrogen, São Paulo, Brazil). The PCR products and restriction fragments were separated by gel electrophoresis on 0.7% (w/v) agarose gel, and stained with ethidium bromide (Sigma). The bands were then visualized by UV transilluminator and photographed. A size marker (Gene Ruler of 100 bp DNA Ladder Plus, Fermentans) was used as a reference. The patterns of Amplified rRNA gene Restriction Analysis (ARDRA) were clustered using BioNumerics Version 6.50 (Applied Maths, Sint-Martens-Latem, Belgium). Representative isolates were selected on the basis of genotypic groupings, and the 5.8S ITS rRNA gene region was sequenced using an ABI3730 XL automatic DNA sequencer. The sequences obtained were compared with sequences available in the GenBank database through a basic local alignment search tool (BLAST). The nucleotide sequences of representative isolates were deposited in the GenBank database under access numbers KF747750 to KF747757.

2.3. Pre-selection of isolates: evaluation of individual stress factors

All yeast species were evaluated for their ability to grow under stress conditions that occur during the wet fermentation of coffee beans according to the procedure described by Pereira et al. (2012). The isolates were transferred from YEPG plates to pre-culture 10 ml YEPG broth and incubated at 30 °C for 24 h, 120 rpm. Subsequently, 1 ml of the resulting yeast cultures were transferred to 50-ml Erlenmeyer flasks containing 10 ml YEPG broth and grown for 3 h at 120 rpm (until early exponential phase). At this point, cells were harvested and diluted in sterile water to Abs600 of 0.2. Spots of 3 μL were placed onto stress plates, which were incubated for at least 48 h at 30 °C.

The test medium used was composed of basal medium [0.05% yeast extract (Sigma), 0.3% (w/v) vitamin-free Casamino Acids (Difco) and 2.5% agar (Difco)] and 5% glucose (Merck). The basal medium without an added carbon source was used as a negative control. The heat stress plates were incubated at 25, 30, 37 or 43 °C. Plates with different glucose or fructose concentration were prepared by adjusting the sugar concentration of the basal medium to 15, 30 or 50% (w/w) hexose-equivalent; the sugar being added by sterile filtration. Ethanol, acetic acid and lactic acid stress plates were composed of basal medium with glucose supplemented with 6, 8 or 10% (v/w) ethanol (Sigma); 1, 2 or 3% (v/w) lactic acid (Sigma); 1, 2 or 3% (v/w) acetic acid (Sigma) (added aseptically). Acidic stress plates were composed of basal medium with glucose in the pH 2.0, 4.0, 6.0 or 8.0; pH adjustments were made with sterile 1 M H_2SO_4 or 1 M NaOH.

2.4. Formulation of coffee pulp simulation medium and micro-fermentation trials with pre-selected yeasts

A coffee pulp simulation medium was formulated containing 50% (v/v) fresh coffee pulp extract plus 2.0 g/l citric pectin (Sigma), 15 g/l fructose (Merck), 15 g/l glucose (Merck), 5.0 g/l yeast extract (Merk) and 5.0 g/l soya peptone (Oxoid); pH = 5.5. Citric pectin, which is present in coffee pulp, was added as energy sources for yeast, plus glucose and fructose, while fresh coffee pulp was added to ensure the availability of nitrogen, trace elements and growth factors naturally present during the wet fermentation of coffee beans. For the preparation of coffee pulp extract, 200 g of the coffee pulp and coffee peel from *C. arabica* var. Mundo