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Identification and quantification of the caproic acid-producing bacterium *Clostridium kluyveri* in the fermentation of pit mud used for Chinese strong-aroma type liquor production

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ABSTRACT

Chinese strong-aroma type liquor (CSAL) is a popular distilled alcoholic beverage in China. It is produced by a complex fermentation process that is conducted in pits in the ground. Ethyl caproate is a key flavor compound in CSAL and is thought to originate from caproic acid produced by Clostridia inhabiting the fermentation pit mud. However, the particular species of *Clostridium* associated with this production are poorly understood and problematic to quantify by culturing. In this study, a total of 28 closest relatives including 15 Clostridia and 8 Bacilli species in pit muds from three CSAL distilleries, were detected by culture-dependent and -independent methods. Among them, *Clostridium kluyveri* was identified as the main producer of caproic acid. One representative strain *C. kluyveri* N6 could produce caproic, butyric and octanoic acids and their corresponding ethyl esters, contributing significantly to CSAL flavor. A real time quantitative PCR assay of *C. kluyveri* in pit muds developed showed that a concentration of 1.79×10^7 16S rRNA gene copies/g pit mud in LZ-old pit was approximately six times higher than that in HLM and YH pits and sixty times higher than that in LZ-new pit respectively. This method can be used to improve the management of pit mud microbiology and its impact on CSAL quality.

1. Introduction

Chinese strong-aroma type liquor (CSAL), also named *Luzhou-flavour* liquor, is a popular distilled alcoholic beverage in China due to its unique flavor and good taste, with both production and market share around 70% in the national liquor industry (Xu et al., 2010; Zhao et al., 2012). It is produced by a unique, recycling solid-state fermentation process. In brief, the crushed raw materials (mixtures of sorghum, wheat, corn, rice, and sticky rice) are soaked in water and then mixed with about 4–5 amounts (w/w) of mash (*Zaopei*) that has already finished the fermentation process. This mixture is then steamed to distil off the liquor which is collected and stored for maturation and then diluted with water to yield an ethanol content of 40–55% (v/v) before consumption. The remaining mixture of fermented and new raw materials is cooled and then inoculated with *Daqu*, which is a traditional starter culture consisting of a mixture of filamentous fungi, yeasts and bacteria. This preparation is now placed into the fermentation vessel and stacked layer-by-layer on a proportion of residual fermenting material (called *Shuanglundi*) that is already in the pit. The fermentation vessel is a rectangular-shaped pit (2 m × 3 m × 2 m) in the ground, the inside walls of which are covered with a layer (~10 cm) of fermentation pit mud. The top of the pit is then covered with a layer of pit mud and the material in the pit (now called *Zaopei*) is allowed to ferment at 28–32 °C for 60 days. After this time, a proportion of the *Zaopei* is taken out of the pit, added to fresh raw materials, and distilled by steaming to give the liquor, as previously mentioned. In this continuous way, the pit muds are used for many years and a unique microbiological flora develops. The initial pit mud used to coat the walls of the pits is prepared separately by incubating a mixture of clay, spent grains from the pits, bean cake powder, *Daqu*, and a proportion of mud taken from an established pit under anaerobic conditions for about one year (Xu et al., 2010).

The microbiology of the process is complex. The main microorganisms responsible for fermenting *Zaopei* are fungi (genera *Aspergillus*, *Rhizopus*, *Pichia* and *Saccharomyces*) and bacteria (classes Bacilli, Clostridia and Bacteroidia) and have been investigated in recent years using both culture and culture independent, molecular methods (Wang et al., 2008; Xu et al., 2010). They conduct simultaneous saccharification and alcoholic fermentation of the raw materials. These microorganisms originate from the raw materials, *Daqu* (Wang et al., 2011; Wang and Xu, 2015) and the pit mud. Compared to the *Daqu* starter, pit mud mainly harbors a highly complex prokaryotic microbiota, with the main species belonging to the bacterial classes of Clostridia, Bacteroidia, Bacilli and two archaeal classes containing Methanobacteria and Methanomicrobia (Ding et al., 2013; Tao et al., 2014; Wang et al.,

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2014; Zheng et al., 2013). The microorganisms inhabiting the pit mud, especially the obligate anaerobic clostridia, synthesize important flavor compounds such as caproic acid and ethyl caproate that determine the quality of CSAL (Ding et al., 2013; Fan and Qian, 2006; Wang et al., 2014; Zheng et al., 2013). In particular, ethyl caproate, with an extremely low odor threshold value (ppb level) (Guth, 1997) and pineapple-like aroma, has been identified as a key flavor substance and is positively correlated with CSAL quality (Fan and Qian, 2006; Tao et al., 2014). Thus, the caproic acid-producing bacteria inhabiting pit mud, identified as species of *Clostridium*, are considered to be important functional microbes and are used as a microbial indicator for evaluating the pit mud quality, which determines the CSAL quality to a large extent (Wu et al., 1991; Wu and Yi, 1986; Xie, 2011; Xu et al., 2010; Xue et al., 1988).

To our knowledge, several *Clostridium* species or strains have been reported to produce caproic acid. High caproic acid-producing species include *Clostridium kluyveri*: 12.8 g/L (Weimer and Stevenson, 2012) and *C.* sp. BS-1 :2.99 g/L (Jeon et al., 2010), while *C. scatologenes* produces trace amounts of caproic acid (Küsel et al., 2000) but further research is needed on these bacteria.

Obligate anaerobic, spore-forming *Clostridium* spp., are very difficult and problematic to cultivate, isolate and quantify using culture methods due to their strict anaerobic requirements and unclear culture conditions (Doyle et al., 2014; Raskin et al., 1995; Tracy et al., 2012). For instance, although culture-independent methods indicate that *Clostridium* spp. dominate in pit mud (Ding et al., 2013; Zheng et al., 2013), very few (~1% of total isolates) clostridia can be isolated from pit muds by cultural methods (Yue et al., 2007). Mostly, the caproic acid-producing clostridia in pit muds have been quantified only by counting the number of anaerobic spore-forming bacterial colony forming units on plates of agar media (Lei et al., 2012; Wu et al., 1991), and have not been sufficiently precise to discriminate between the weak and non-caproic acid-producing species, such as *Bacillus licheniformis*, *Clostridium celerecrescens* and *Clostridium tyrobutyricum* (Wang et al., 2014). Moreover, such culture methods may not detect all of the true viable microbes (Davey, 2011). For these reasons, more reliable and rapid methods for the quantification of caproic acid-producing clostridia in pit muds are needed. Real time quantitative PCR (qPCR), may offer a faster, more convenient and accurate quantitative approach than culture methods (Heid et al., 1996). qPCR is now widely applied to quantify some known species or groups of microorganisms based on their rRNA genes or functional genes in various environments and fermentation processes (Chen et al., 2014; Kim et al., 2013). In the case of pit muds, it is necessary to first identify the caproic acid-producing clostridia and then develop an accurate method for quantifying them using qPCR. Such technology would then be useful to investigate their population dynamics in the fermentation process of CSAL.

Therefore, this study was aimed at identifying the caproic acid-producing clostridia inhabiting pit muds using combined culture-dependent and -independent methods and then developing a method for quantifying their content using qPCR.

2. Materials and methods

2.1. Sample collection and storage

Samples of fermented pit muds were collected from three different distilleries in China, namely, the Luzhou Laojiao Co., Ltd (LZ), the Jiangsu Yanghe Brewery Joint-Stock Co., Ltd. (YH) and the Yibin Hongloumeng distillery Co., Ltd. (HLM). For each distillery, three different samples of pit muds were randomly selected from which 100 g of mud were taken and combined to give one representative sample for that distillery. For the LZ distillery, two representative samples were taken: one from pits that were one year old (LZ-new), and one from pits that were 300 years old (LZ-old). The samples were transferred immediately to sterile anaerobic bags (MGC, Japan), marked and stored at 4 °C for further bacteria isolation and consortium construction.

Before sample mixing, the genomic DNA of each subsample was extracted according to the protocols of E.Z.N.A.™ soil DNA kit (D5625-01, Omega, USA), and stored at −20 °C for qPCR assay.

2.2. Isolation and identification of anaerobic spore-forming bacteria from pit muds

Ten grams of each representative sample were suspended in 100 mL sterile Peptone Physiological Salt (PPS) solution, containing 5 g of sterile glass beads (3 mm). The mixture was homogenized for about 5 min by shaking constantly, and then heated at 80 °C for 10 min to kill vegetative bacterial cells.

To isolate clostridial species, liquid reinforced clostridial medium (RCM, 3.0 g/L yeast extract, 10.0 g/L "Lab-Lemco" powder, 10.0 g/L peptone, 1.0 g/L soluble starch, 5.0 g/L glucose, 0.5 g/L cysteine hydrochloride, 5.0 g/L sodium chloride, 3.0 g/L sodium acetate) and ethanol/sodium acetate (ES) medium were used. ES medium consisted of the following (in 1000 mL of deionized water): 5 g sodium acetate, 0.5 g ammonium sulfate, 0.4 g di-potassium hydrogen phosphate trihydrate, 0.2 g magnesium sulfate, 1 g yeast extract, 10 g calcium carbonate, pH 7.0, sterilized at 121 °C for 20 min, 20 mL ethanol (added after sterilization) (Wu and Yi, 1986). Simultaneously, direct plating and enrichment isolation methods were applied. In the direct method, 0.2 mL of the above heated mixture was spread inoculated onto RCM and ES agar plates. For enrichment, 5 mL heated suspension was inoculated into 95 mL liquid RCM or ES medium and incubated for 3 days at 37 °C, after which 0.2 mL were spread on RCM or ES agar plates. Plates were incubated for 7 days at 37 °C under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2, Jinghong Gas, Suzhou, China).

Pure cultures were prepared from colonies picked from each plate and were re-cultured in liquid RCM or ES medium under the above conditions to provide biomass for DNA extraction. Genomic DNA from all isolates was extracted by a TIANamp Bacteria DNA Kit (DP302) (Tiangen, Beijing, China), and amplified using thermal cyclers (Cycler C1000, Bio-Rad) with primer set 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) targeting the bacterial 16S rRNA gene (Zhang et al., 2014). The purified PCR products were sent to Sangon (Shanghai, China) for sequencing. For strain identification, 16S rRNA gene sequences of isolates were blasted against NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Identification of caproic acid-producing species in pit muds by PCR-DGGE analysis

Samples (10 g in 100 mL of diluent) of pit mud from each distillery were heat treated as previously described. Then 5 mL of the heated suspension of each pit-mud sample was inoculated into (i) 95 mL fresh ES medium and incubated for 6 days at 37 °C followed by five successive transfers (5 mL/95 mL fresh ES medium, made at 6-day intervals) and (ii), 5 mL of above enrichment culture was inoculated into 95 mL fresh RCM medium and incubated for one day at 37 °C followed by 5 successive transfers (5 mL/95 mL fresh RCM medium, made at one day intervals). At the end of successive culture, for each medium and each distillery, a portion of the culture was tested for the presence and concentration of caproic acid. In addition, the biomass from these cultures was sedimented by centrifugation and used for DNA extraction and PCR-DGGE. Genomic DNA was extracted by a TIANamp Bacteria DNA Kit (DP302) (Tiangen, Beijing, China) and amplified using the Clostridial universal primer set GC-SJ-F (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCC CGGTGAAATGCGTAGAKATTA-3′) and SJ-R (5′-TCGAATTAAACCACATGCTCCG-3′) (Hu et al., 2014).

For the PCR amplifications, the reaction system and conditions were performed as described in our previous study (Hu et al., 2014). PCR products were further analyzed by DGGE using the Bio-Rad DCode apparatus (USA). Each concentrated amplicons (10 μL) were applied in lanes on 8% (w/v) polyacrylamide gels in 1 × TAE buffer with a denaturing