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Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans

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Abstract

Samples of cocoa beans were taken on two separate occasions during heap and tray fermentations in Ghana, West Africa. In total 496 yeast isolates were identified by conventional microbiological analyses and by amplification of their ITS1-5.8S rDNA-ITS2 regions. For important species the identifications were confirmed by sequencing of the D1/D2 domain of the 5′ end of the large subunit (26S) rDNA. Assimilations of organic acids and other carbon compounds were conducted. For dominant yeasts intraspecies variations were examined by determination of chromosome length polymorphism (CLP) using pulsed-field gel electrophoresis.

For the heap fermentations maximum yeast cell counts of 9.1×10^7 were reached, whereas maximum yeast counts of 6.0×10^6 were reached for the tray fermentations. *Candida krusei* was found to be the dominant species during heap fermentation, followed by *P. membranifaciens*, *P. kluyveri*, *Hanseniaspora guilliermondii* and *Trichosporon asahii*, whereas *Saccharomyces cerevisiae* and *P. membranifaciens* were found to be the dominant species during tray fermentation followed by low numbers of *C. krusei*, *P. kluyveri*, *H. guilliermondii* and some yeast species of minor importance. For isolates within all dominant species CLP was evident, indicating that several different strains are involved in the fermentations. Isolates of *C. krusei*, *P. membranifaciens*, *H. guilliermondii*, *T. asahii* and *Rhodotorula glutinis* could be found on the surface of the cocoa pods and in some cases on the production equipment, whereas the origin of e.g. *S. cerevisiae* was not indicated by the results obtained.

In conclusion, the results obtained show that fermentation of cocoa beans is a very inhomogeneous process with great variations in both yeast counts and species composition. The variations seem to depend especially on the processing procedure, but also the season and the post-harvest storage are likely to influence the yeast counts and the species composition.

Keywords: Cocoa bean fermentation; Microbial succession; D1/D2 sequencing; Strain typing

1. Introduction

Cocoa beans are seeds from the fruit pods of the tree *Theobroma cacao* Linné [1], which is cultivated in plantations in tropical regions throughout the world, West Africa being the major producing region accounting in year 2001–2002 for more than 66% of the world production [2]. In the pods the cocoa beans are embedded in a mass of mucilaginous pulp and after removal of both beans and pulp from the pods the first step in cocoa processing is a spontaneous fermentation [3,4]. The methods of fermentation vary considerably from country to country and even adjacent farms may differ in their processing practices [5]. The cocoa beans are either fermented in heaps, boxes, baskets or in trays. In West Africa, fermentation in heaps varying in sizes from

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20 to 1000 kg and covered with banana or plantain leaves is by far the most dominant method [1]. Another method used, which is often claimed to give a better cocoa quality, is fermentation in stacked trays (≈20–100 kg of beans per tray), giving series of thin layers of cocoa beans with air circulating between each layer [6]. Reviews on cocoa fermentation have been given previously [1,7–9].

The mucilaginous bean pulp is rich in fermentable sugars such as glucose, 2.4–4.1% (w/w), fructose, 4.2–6.2% (w/w) and sucrose, 2.1–3.2% (w/w) and has a high concentration of especially citric acid, 2.1–2.4% (w/w), but also smaller amounts of other organic acids are present such as lactic acid, 0.03% (w/w) and acetic acid, 0.04% (w/w) [4,10,11]. The pH of the pulp has been reported to be just below 4.0 [12]. During fermentation, microbial activity leads to the formation of a range of metabolic end-products such as alcohols, acetic acid and other organic acids, which diffuse into the beans and cause their death. This induces biochemical transformations within the beans that lead to formation of precursors of the characteristic aroma, flavour and colour, which are further developed during drying and finally obtained during roasting and further processing [13,14]. Earlier studies on the microbiology of cocoa bean fermentation have shown that both yeasts, filamentous fungi, lactic acid bacteria, acetic acid bacteria and *Bacillus* species might contribute to the fermentation [4,15].

Unfortunately, not many of the studies dealing with isolation and identification of yeast species involved during cocoa fermentation have been performed in West Africa, being the main producer of cocoa beans, and not many of the studies performed deal with the microbial succession of yeast species and the biodiversity within species. Further, as many of the publications date many years back, the taxonomy is not up-to-date and the use of molecular identification methods is not included. The general assumption is that many different yeast species are involved in the fermentation of cocoa beans. Thus, in Ivory Coast, Sanchez et al. [12] have found an abundant and varied yeast population, the dominant yeast species being *Kloeckera apiculata*, *K. corticis* and *Saccharomyces chevalieri*, now recognised as *S. cerevisiae* [16]. In a recent study by Ardhana and Fleet [4] in East Java, Indonesia, amongst others *K. apis*, *S. cerevisiae* and *Candida tropicalis* were found to be the most significant yeast species. In Bahia, Brazil, de Camargo et al. [3] found the most frequent yeast species to be *C. krusei*, *Geotrichum candidum* and *C. mycoderma* (now recognised as *C. vivi* [17]). Later, Schwan et al. [18] in Bahia, Brazil, identified amongst several other yeasts the dominant species to be *S. cerevisiae*, *K. apiculata*, *Kluyveromyces marxianus* and *C. rugosa.*

Several molecular methods are now well established for identification and typing of yeast species. Among such molecular techniques are the amplification of the 5.8S rDNA and the two ribosomal intergenic spacer regions (ITS1 and ITS2) used for grouping of yeast isolates e.g. in combination with restriction or sequence analysis [19–23]. Within recent years sequencing of the D1/D2 domain at the 5′ end of the large subunit (26S) rDNA has been well acknowledged for its ability to identify yeast to the species level [24–26]. Chromosome length polymorphism (CLP) determined by pulsed-field gel electrophoresis (PFGE) has been used successfully for strain typing of different yeast species such as *C. krusei*, *Debaryomyces hansenii*, *Hanseniaspora* spp., *S. cerevisiae* and *Saccharomyces pastorianus* [27–31].

The aim of the present study has been to identify dominant yeast species and to follow the microbial successions during heap and tray fermentations of cocoa beans in Ghana, West Africa. Samples of pod surfaces and process equipment were included to examine the origin of predominant yeasts. The yeast species were identified by both conventional microbiological analyses and by sequencing of the D1/D2 domain of the 5′ end of the large subunit (26S) rDNA. For dominant yeasts, intraspecies variations were examined by determination of their chromosome length polymorphism (CLP).

2. Materials and methods

2.1. Cocoa bean fermentation

Fermentations from two cocoa producers were followed; one fermenting the cocoa beans in heaps and one using trays for fermentation. Both producers were located in Ghana, West Africa, ≈150 km north of Accra. Samples were collected early December, corresponding to early season, and then repeated mid January. According to normal practice the pods for the heap fermentations were harvested over 18 days before fermentation, whereas the pods for the tray fermentations were harvested over four days. Before fermentation the harvested cocoa pods were stored on the ground. Only matured pods were used for the fermentation. For the heap fermentations 200–1000 kg of cocoa beans were piled, stacked in a heap on plantain leaves, covered with plantain leaves and left to ferment for 72 h without turning of the heap, due to local practice. For the tray fermentation ≈50–100 kg beans were placed in a tray (90 cm × 120 cm, 10 cm deep), eight trays were stacked on top of each other, the top tray covered with plantain leaves and the beans left to ferment for 72 h without mixing.

2.2. Sampling

From the heap fermentation samples were collected from both the inner (≈15 cm) and the outer part of