



Microorganisms during cocoa fermentation: systematic review

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Abstract:

Introduction. Cocoa (*Theobroma cacao* L.) originates from Ecuador. It is one of the oldest foods in the world. The fact that cocoa is the main component in chocolate industry makes it one of the most quoted raw materials today. The chemical, physical, microbiological, and sensory properties of cocoa determine its quality and, as a result, economic and nutritional value. The research objective was to conduct a detailed analysis of cocoa fermentation process and to study the transformations this raw material is subjected to during processing.

Study objects and methods. The present article introduces a substantial bibliographic review based on three databases: Science Direct, Scopus, and Medline. The scientific publications were selected according to several factors. First, they had to be relevant in terms of cocoa fermentation. Second, they were written in English or Spanish. Third, the papers were indexed in high-impact journals. The initial selection included 350 articles, while the final list of relevant publications featured only 50 works that met all the requirements specified above.

Results and discussion. The main characteristics of yeasts, lactic bacteria, and acetic bacteria were analyzed together with their main parameters to describe their activities during different stages of alcoholic, lactic, and acetic fermentation. A thorough analysis of the main enzyme-related processes that occur during fermentation makes it possible to optimize the use of substrates, temperature, time, pH, acidity, and nutrients. As a result, the finished product contains an optimal concentration of volatile compounds that are formed in the beans during fermentation. The study featured the main strains of fermentation-related microorganisms, their activities, main reactions, and products.

Conclusion. This study makes it possible to improve the process of fermentation to obtain beans with a better chemical composition.

Keywords: Biochemistry, fermentation, yeasts, acetic acid

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INTRODUCTION

The Latin name for cocoa, *Theobroma Cacao*, translates as “food for the gods”. The plant has its origin in the upper Amazon, where archeologists discovered theobromine alkaloid from the oldest organic cocoa matter in history at the Santa Ana archaeological site in La Florida, in the province of Zamora Chinchipe, Ecuador. From this place, cocoa beans spread throughout the rest of the continent. On the Yucatan

Peninsula, fragments of once-vast cocoa plantations have been found on the territories that were occupied by the Mayan civilization. The traces of cultivated cocoa were also discovered in Central America on the territory of the modern Mexico. Currently, cocoa is cultivated in many tropical countries of the world. It grows in the area between 20 degrees latitude north and south of the equator [1, 2].

The cocoa beans come from the *Theobroma cacao* tree. They grow in a pod that contains 30–40 beans

wrapped in a jelly-like mucilaginous substance. Raw cocoa has an unpleasant astringent taste, which means that its volatile compounds have to be generated artificially. During treatment, microorganisms modify their state and components through various processes, e.g. fermentation. After fermentation, beans are dried and exposed to the sun. Only then do the typical sensory properties develop, and the beans acquire the pleasant characteristics we associate with chocolate [3, 4].

Fermentation is considered the most critical step in the processing of cocoa, since the beans are the main raw material in chocolate industry. It is at the fermentation stage that they develop their sensory properties. These properties come from aroma precursors generated during chemical changes in the phenolic content [5]. Fermentation occurs in the pulp of the pod. The pulp is a white carbohydrate-rich mucilaginous mass that surrounds and protects the beans. The process lasts several days and depends on several groups of microorganisms [6].

The microbial activity during cocoa fermentation has complex biochemical implications. In fact, cocoa is one of few foods where so many changes and processes occur during the same process. It includes the successive growth of several species of yeasts, lactic acid bacteria, acetic acid bacteria, and, to greater or lesser extent, species of *Bacillus* and filamentous fungi [7]. Yeasts are active at the earliest stage of fermentation. The most common strains include *Saccharomyces spp*, *Candida spp*, and *Pichia spp*. The yeast stage is followed by lactic acid bacteria, which are represented by *Lactobacillus plantarum* and *Lactobacillus fermentum*. Acid bacteria acetics belong to the genus of *Acetobacter*, *Acetobacter pasteurianus* being the most common.

What exactly determines the quality of the cocoa beans still remains a mystery: groups of microorganisms or individual species? Most likely, all the strains are essential for the fermentation process, since new genera and new species are constantly being discovered [8, 9]. Therefore, the first objective of this review was to specify the characteristics and reactions of microorganisms during fermentation. The second objective was to formulate some recommendations on improving fermentation conditions or maintaining the optimal ones to achieve the best transformation in the chemical compounds.

STUDY OBJECTS AND METHODS

The bibliographic review was conducted according to three databases, namely Science Direct, Scopus, and Medline. The descriptors included the following key words: cocoa fermentation, microbiology of cocoa fermentation, and phenolic compounds in cocoa fermentation. The articles were in English or Spanish and indexed in high impact journals. They were selected according to their relevance in terms of cocoa fermentation. Of 350 initially selected articles, 149 were

excluded as irrelevant, and 101 did not fit the language criterion. Out of 100 English and Spanish articles, only 50 were selected as corresponding with all the specified requirements.

RESULTS AND DISCUSSION

Cocoa fermentation. Cocoa fermentation is a post-harvest process which includes several stages. The first step after cultivation is to open the pods and remove the beans. They are covered with white pulp, or mucilage, which is mainly sugars and water [10]. The initial pH of the pulp is 3.6. It is a nutrient-rich medium that encourages microbial growth. The pulp has the following composition: about 85% of water, 10–15% of sugars (the concentration of glucose, fructose, and sucrose depending on the age and maturation), 2–3% of pentoses, 1–3% of citric acid, and 1.5% of pectin, proteins, amino acids, vitamins, and minerals. Vitamin C and potassium are the most common representatives of vitamins and minerals. They are minor but very important components [10–12].

The microbiological changes during fermentation are obvious. First, yeasts ferment pulp carbohydrates and transform them into ethanol and carbon dioxide. The secretions of their pectinolytic enzymes generate anaerobic environment. The yeast stage takes approximately 36 h. The next stage involves lactic acid bacteria that appear between 16 and 48 h of fermentation. They generate lactic and citric acid, increase the acidity of the medium, and change the composition of the pulp.

As fermentation continues, oxygen begins to come in. As a result, the temperature rises above 37°C, which boosts the growth of acetic acid bacteria. Their population reaches its peak in 88 h. Between 48 and 112 h of fermentation, one can even feel the smell emanating from acetic acid. After alcohol and lactic acid turned to acetic acid, the temperature rises up to 50°C. The heat finally inhibits the microorganisms that have a life span of 120 h. After fermentation, several filamentous fungi have been registered in the surface areas and in the excess fermentation mass [13–16].

Basically, the fermentation of cocoa beans begins with the initial acidity of the mucilage and the low levels of oxygen, which are the optimal conditions for yeasts. As these factors decrease, the lactic acid bacteria reach their maximum growth point. As their amount gradually decreases, it is replaced by acetic bacteria, which prefer ethanol, good aeration, and heat. Aerobic spore-forming bacteria and filamentous fungi often appear at the final stage of fermentation. They are responsible for unpleasant flavors of fermented cocoa beans [17, 18]. On the other hand, prolonged fermentation leads to an increase in bacilli and filamentous fungi, which can also cause unpleasant flavors. The physiological functions of the predominant microorganisms have been the subject of countless studies, which established the crucial

role of microbial succession in the development of the characteristic cocoa aroma [16].

The sensory properties of cocoa beans can be developed by basic conditions or by external factors, especially those connected with fermentation. The cocoa flavor increases as the fermentation time elapses, which means a negative correlation with astringency. In other words, the astringency of the beans decreases during fermentation [19].

Stages and changes of cocoa beans. Fermentation of raw cocoa beans occurs in two stages, which, in turn, are divided into four steps. The first stage involves microbial reactions that take place in the pulp and on the surface of the beans. The second phase involves several hydrolytic reactions that occur within cotyledons [20].

The system formed to ferment the mucilage that covers the cocoa beans is metabolized by a succession of microorganisms. When cocoa beans are harvested and extracted from the pod, they are exposed to natural biodiverse microflora that comes from the contact with environment, crop handling personnel, transport containers, tools, pod surfaces, etc. [21, 22].

Reactions initiated during fermentation continue at the drying and roasting stage. Thus, oxidation reactions reduce acidity and the amount of phenolic compounds responsible for bitterness and astringency. Cocoa beans can be dried in the sun or in special dryers. However, the latter method often implies extra high temperatures, which can harden the cotyledons and decrease the quality of the finished product. The quality of cocoa beans directly depends on the genotype, harvest, fermentation, drying, and roasting. For instance, beans of different cocoa genotypes should not be fermented together, as it can spoil their sensory properties [23, 24].

Microorganisms present in cocoa fermentation. Traditionally, cocoa fermentation is an uncontrolled process initiated by microorganisms that naturally appear in fermentation sites. These fermenting organisms use pulp as the main substrate. At the onset of fermentation, pulp reduces the diffusion of oxygen within the mass of the fermented bean, thus creating anaerobic conditions [25]. As it was already mentioned, there are five main groups of microorganisms that participate in cocoa fermentation: yeasts, lactic acid bacteria, acetic acid bacteria, and various species of bacilli and fungi. Unlike other fermented raw materials, endogenous enzymes play a crucial role in the development of the flavor of cocoa beans: without fermentation, cocoa beans have no flavor. During fermentation, microorganisms eliminate pulp and produce indispensable metabolites [26, 27].

Yeasts. Yeasts are eukaryotic microorganisms with a high biotechnological potential for food industry. Their properties are completely different from prokaryotic bacteria. Yeasts are resistant to antibiotics, sulfa drugs, and other antibacterial agents. This resistance is genetic, i.e. natural: it cannot be modified or transmitted to

other microorganisms [28]. Yeast particles are $5 \times 10 \mu\text{m}$ in size, which is significantly bigger than the size of bacteria ($0.5 \times 5 \mu\text{m}$) [29].

The yeast species that have been identified as the main colonizers during cocoa fermentation are the *Saccharomyces cerevisiae*, *Candida pelliculosa*, *Candida tropicalis*, *Candida zeylanoides*, *Torulopsis candida*, *Torulopsis castelli*, *Torulopsis holmii*, *Kloeckera apiculata*, *Kloeckera apis*, *Schizosaccharomyces*, *Kluyveromyces marxianus*, *Pichia membranifaciens*, *Pichia kudriavzevii*, and *Pichia membranaefaciens*. The *Saccharomyces cerevisiae* is the most common strain reported in all cocoa plantations. The exact reason why a certain strain of yeast enters fermentation process still remains unknown. However, the *Kloeckera apiculata* does not survive 24 h of fermentation as it is inhibited by the concentration of ethanol produced in the medium. As for the *Kluyveromyces marxianus*, it grows slowly and degrades gradually. Yeasts are active for approximately 48 h and reach the peak of their activity in 24 h. By that time, their activity has changed conditions of the medium, and other microorganisms join in [16, 30, 31].

Yeasts play an important role in the pulp degradation process. Cocoa pulp can be fermented to produce an alcoholic beverage. Yeasts demonstrate pectinolytic activity. The secondary products of yeast metabolism involve organic acids, aldehydes, ketones, higher alcohols, and esters. The production of glycosidases enzymes is important and affects the quality of beans and, subsequently, that of chocolate [32].

Lactic acid bacteria. Lactic acid bacteria comprise a group of microorganisms linked by the formation of lactic acid as the main metabolite. They are a product of carbohydrate fermentation. Depending on the amount of this product, they can be homo- or heterofermentative. They share similar morphological, physiological, and metabolic characteristics. They are Gram-positive, catalase and oxidase negative, not mobile, and they do not form spores. They can be anaerobic, microaerophilic, and airborne [33].

Lactic acid bacteria can appear at the onset of fermentation. However, they increase their number and become active only when the pulp with its sugars begins to hydrolyze and leave the fermentative system, which is boosts yeast metabolism. The main species that have been isolated so far include *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus cellobiosus*, *Leuconostoc mesenteroides*, *Lactococcus (Streptococcus) lactis*, *Pediococcus spp*, and various species of *Bacillus*.

As for heat-resistant flora, *Lactobacillus curieae*, *Enterococcus faecium*, *Fructobacillus pseudoficulneus*, *Lactobacillus casei*, *Weissella paramesenteroides* and *Weissella cibaria* have also been registered, but to a lesser extent. They exist during the first 72 h of fermentation and reach their peak in 36 h.

Their maximum growth period is 16–48 h. The prevailing species include *Lactobacillus plantarum* and *Lactobacillus fermentum*. Lactic acid bacteria mostly produce lactic acid, but they also generate small amounts of alcohol and acetic acid from fructose and glucose. In addition, they can use citric acid to produce acetaldehyde, diacetyl, mannitol, acetic acid, and lactic acid [34–36].

Acetic acid bacteria. Acetic bacteria are Gram-negative and belong to the *Acetobacteraceae* family. They are strict aerobics, non-spore-forming, ellipsoidal or bacillus-shaped. They may occur in pairs or in chains. Members of the *Acetobacter* genus are so common due to their ability to grow in ethanol environment. Acetic bacteria are known to partially oxidize a variety of carbohydrates and to release various metabolites, e.g. aldehydes, ketones, and organic acids, in different media. For a long time, they have been used to perform specific oxidation reactions via processes called “oxidative fermentations” [37]. The first step in the production of acetic acid is the conversion of ethanol from a carbohydrate by yeasts. The second step is the oxidation of ethanol to acetic acid by acetic acid bacteria [38].

During cocoa fermentation, the population of yeasts and lactic acid bacteria decays, thus creating an aerobic environment favorable for the growth of acetic bacteria. The temperature reaches approximately 37°C. The increase in temperature triggers protein hydrolysis and acidification of the beans. As a result, ethanol dissolves into acetic acid, carbon dioxide, and water. Some strains appear at 24 h and reach their growth peak at 88 h. After 120 h, they can no longer be detected. They begin to disappear when the mass reaches 50°C. Part of the generated acid volatilizes, while the rest enters the bean and is responsible for killing the germ [39]. These bacteria play a fundamental role in the generation of volatile compounds that affect the quality of chocolate. The *Acetobacter* and *Gluconobacter* geni are usually observed during fermentation, the most common being *Acetobacter aceti* and *Acetobacter pasteurianus*, as well as the recently discovered *Acetobacter ascendens*, *A. rancens*, *A. xylinum*, *A. lovaniensis*, *A. xylinum*, *A. peroxydans*, and *Gluconobacter oxydans* [16, 40, 41].

Fermentation stages.

Stage I. During the first stage of fermentation, the volume of the pulp that surrounds the beans reduces the diffusion of oxygen within the medium. This is where the beans will be fermented in anaerobic conditions. During this stage, first yeasts and then lactic bacteria consume sugars and organic acids from the pulp, thus producing ethanol, lactic acid, etc. [25].

The yeast population starts with 10^7 CFU/g pulp and reaches a maximum of 10^8 CFU/g pulp. After that, it starts to decline until it reaches the bottom level of 10 cells per gram of pulp. Yeasts are prevailing microorganisms, and their depectinization activity causes liquefaction of the pulp with its subsequent

drainage, or “sweating”. The pulp loses its viscosity and lets in air [42]. As a result, the simple sugars of the mucilage, namely sucrose, fructose, and glucose, turn into ethanol. The pectin degrades, causing the texture of the bean to change, and eliminates citric acid. The yeasts which are generally responsible for metabolizing this acid are *Candida* spp. and *Pichia* spp., which generate an alkaline pH. This parameter, together with alcohol and oxygen, coincidentally inhibits the yeasts and their activity, but contributes to the development of lactic bacteria. Yeasts also form such organic acids as acetic, oxalic, phosphoric, and malic acids. They help reduce pH fluctuations [43, 44].

Yeasts have become focus of numerous cocoa bean fermentation studies since they release pulp degradation enzymes. Moreover, they are also the main producers of esters and higher alcohols, which can contribute to the complex mix of aromatic volatile compounds that make up the cocoa aroma. The main yeasts that generate these volatile compounds are *Candida* sp., *Kluyveromyces marxianus*, *Kloeckera apiculata*, *S. cerevisiae*, and *S. cerevisiae* var. *chevalieri* [45].

The second phase of this stage involves several hydrolytic reactions that occur within the cotyledons. As the fermentation continues and the pulp drains, more oxygen enters the system, thus creating the optimal conditions for the growth of lactic bacteria [42]. They colonize the cocoa mass, degrade the glucose of the pulp into lactic acid, and assimilate the remaining citric acid.

Several studies on microbial fermentation indicate that two most prevalent species in this process are *Lactobacillus plantarum* and *Lactobacillus fermentum*. They also produce acetate esters from acetic acid, which give different tones to cocoa-based products [31]. Lactic bacteria can reach a population of 6.4×10^7 CFU/g pulp. At first, they increase the acidity by producing citric acid, but then they lower the pH by releasing products that are not acidic. Lactic acid bacteria are able to metabolize malic acid. These bacteria have no major proteolytic activity and can only ferment two types of amino acids: serine and arginine. After all these reactions, the environment is totally aerobic, which allows for the growth of acetic bacteria [7, 34, 46].

Stage II. During the second stage, the environment is oxygenated, and the pH has decreased due to the removal of some components and variability of the remaining compounds. At last, acetic acid bacteria can convert the previously obtained ethanol into acetic acid via the oxidation of alcohol. The optimal temperature of the acetic fermentation process is between 28°C and 30°C, and the optimum pH is 4.5. The oxidation of ethanol is carried out in two stages. First, ethanol is oxidized into acetaldehyde. Second, the acetaldehyde becomes acetic acid. Other products include ethyl acetate, butanol, isopropanol, intermediate acetaldehyde compounds, and organic acids [47].

The formation of acetic acid is very important at this stage of the process. It occurs due to the activity of acetic

bacteria. The exothermic reactions of the bacteria raise the temperature of the mass. The population reaches its peak at 1.2×10^7 CFU/g pulp and falls down after three days of activity precisely because of the high temperature it generates. In some cases, the population can reach 3.5×10^3 CFU/g pulp [16, 48].

As the volume of oxygen increases, the pH reaches 3.5–5.0, and the temperature becomes 45–50°C. Under these conditions, several aerobic spores of *Bacillus* bacteria may appear in the fermentation. After the pile of beans has been stirred, one can detect the presence of *Bacillus licheniformis*, *B. megaterium*, *B. pumilus*, *B. pumilus*, *B. coagulans*, *B. circulans*, *B. subtilis*, *B. cereus*, and *B. megaterium*. Most of them are heat-tolerant and can survive during drying and roasting. They are capable of producing numerous enzymes, both proteolytic and lipolytic, which catalyze reactions. However, they give cocoa unpleasant taste and smell since they degrade proteins and fats by producing chemical substances that can distort the flavor [49, 50].

CONCLUSION

The fermentation stage is considered the most important process in the transformation of cocoa to chocolate. The changes that occur in its volatile and aromatic compounds trigger structural changes in the composition. The changes are due to the activity of various microorganisms. Their main objective is to kill the germ and thus stop the metabolism of the bean. The resulting alcohol is broken down into acetic acid and

other acids, which produce desirable sensory properties that will be accentuated during drying and roasting.

Yeasts, lactic bacteria, and acetic bacteria play a fundamental role in the fermentation process. By knowing the main strains, their action parameters, main reactions, and products, food scientists can improve this process to obtain cocoa beans with a better chemical composition.

Each strain of microorganisms requires a separate research with regard to the variety of cocoa beans. The species vary from zone to zone, and plantations in different parts of the world are unlikely to have similar characteristics.

CONTRIBUTION

Roberto Ordoñez-Araque and Julio Urresto-Villegas compiled the manuscript. Edgar Landines-Vera and Carla Caicedo-Jaramillo collected the data, checked the structure, and performed the final review.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to the publication of this article.

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