Yeasts in Cocoa Bean Fermentation

Subjects: Food Science & Technology

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During the fermentation of cocoa beans, the yeasts produce volatile organic compounds (VOCs). Through reactions associated with amino acid metabolism, yeasts generate important aroma precursors as acetate esters and fatty acid ethyl esters; these are essential in developing fruity flavors and aromas in the final product (usually chocolate). In addition, some yeasts may have pectinolytic and antifungal activity, which is desirable in the post-harvest process of cocoa. The main yeast species in cocoa fermentation are *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and *Hanseniaspora opuntiae.* These produce higher alcohols and acetyl-CoA to make acetate–esters, compounds that produce floral and fruity notes.

yeast | cocoa bean | fermentation | flavor precursors | starter cultures

1. Introduction

Yeasts have been involved in the fermentation of products for thousands of years in the production of wine, bread, sake, chocolate, and other fermented foods. Some of them have been produced for more than 10,000 years [1][2]. It has been shown that increasing yeast diversity in food fermentations increases the sensory complexity and diversity of aroma compounds found in the final products. Aromatic compounds play many key roles for yeasts, as survival strategies, defense mechanisms, and cellular communication. Humans have used their production to enhance the flavor and sensory attributes of fermented foods [2]. One of the most relevant fermentations in which yeasts are involved is the fermentation of the cocoa bean [3].

Cocoa is the fruit of the *Theobroma cacao* L., which is a perennial tree native to the South American tropical region. The dry fermented cocoa bean is the raw material for chocolate production and is composed of two cotyledons and an embryo, enveloped in a sweet and white mucilaginous pulp [4][5]. Based on Statista data [6], the global cocoa production scenario for the period 2021–2022 is 4,955,000 metric tons. Leading cocoa-producing countries for the same period are the Ivory Coast, Ghana, Indonesia, Nigeria, Cameroon, and Brazil [7]. There are three main cocoa varieties: *Forastero* (bulk or ordinary accounts for 95% of world cocoa production), *Criollo*, and *Trinitario* [4][5].

When the cocoa beans are removed from the pod, the pulp is degraded by a spontaneous fermentation conducted by yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Several authors have found that good quality in fermented dry cocoa beans was correlated with good on-farm agricultural and post-harvest practices, bean selection, placenta removal prior to fermentation, and blending of the cocoa bean pulp mass [8][9][10]. In addition, well-performed fermentation is a prerequisite for producing high-quality chocolate [11]. Several studies have shown that yeasts produce various aromatic precursor compounds, such as alcohols and esters, which positively contribute to the aromatic profile of the chocolate [11][12][13][14][15][16]. The ethanol produced by yeast strains during cocoa fermentation is used as a carbon source for acetic acid bacteria and triggers diverse biochemical reactions inside cocoa bean that drives the aroma and flavor precursors in cocoa cotyledons [17]. Cocoa aroma is the result of various reactions that occur during the processing of beans and is related to the cocoa genotype, as well as environmental conditions, microbial diversity during fermentation, and subsequent processing steps, mainly drying and roasting [18]. Furthermore, cocoa flavor comprises of non-volatile compounds (polyphenols, carbohydrates, alkaloids, and proteins) and volatile compounds (esters, phenols, alcohols, aldehydes, ketones, furanones, and pyrazines) [19].

2. Cocoa Bean Fermentation and Biochemical Transformations on Cocoa Bean during Fermentation

Fermentation is essential for developing flavor and reaching the final acidity of cacao beans [5][20][21][22]. Four different methods are used to ferment cocoa beans: platform, box, heap, and basket fermentation. The selection of the fermenting method is related to the region of cocoa production [22]. The cocoa bean fermentation process involves the degradation of the mucilaginous pulp surrounding the beans by complex microbial interactions, mainly by yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (BAA). Other microorganisms such as spore-forming bacteria (*Bacillus* and *Paenibacillus*), enterobacteria, and filamentous fungi are also present; however, their role remains unclear [4][5][20][22][23][24]. Cocoa pulp is a rich medium for microbial growth. It consists of water (80–90%), sugars, mainly glucose, sucrose, and fructose (0–15%), citric acid (1–3%), and pectin (1–1.5%). Proteins (0.5–0.7%), amino acids, vitamins (mainly vitamin C), and minerals (K^+, Na^+, Ca^{+2}, Mg^{+2}, Fe^{+2}, and Zn^{+2}) are also present [4][18][20][22][23]. There are two important phases in the fermentation of cocoa beans, anaerobic, and aerobic. The anaerobic phase lasts about 48–72 h after cocoa pod breaking and involves yeast and LAB strains [25]. The aerobic phase occurs after approximately 48 h of fermentation with the growth of AAB strains [4][20].

2.1. Anaerobic Phase of Cocoa Bean Fermentation

2.1.1. Yeast

The first stage of cocoa bean fermentation involves the growth of yeasts mostly belonging to the genera *Hanseniaspora*, *Saccharomyces*, *Candida*, *Kluyveromyces*, *Kazachstania*, *Meyerozyma*, *Rhodotorula*, *Wickerhamomyces*, and *Pichia* [16][26][27]. Yeasts are the microorganisms that predominate this process during the first 24 h of fermentation, and subsequently, their population decreases [4][21]. Yeasts are favored by the initial acidity of the cocoa pulp (pH 3.6), the concentration of citric acid, the low oxygen levels, and environmental temperature ranging from 25–35 °C [4][20]. Yeast metabolizes glucose, fructose, and sucrose present in the cocoa pulp, yielding ethanol and carbon dioxide [28]. Yeast central metabolism begins with the basic conversion of sugars to pyruvate, producing ATP and reduced NADH cofactors. Under aerobic conditions, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase and directed to the citric acid cycle.

The anaerobic conversion of pyruvate to ethanol is a two-step process. First, pyruvate is converted to acetaldehyde by pyruvate decarboxylase (PDC), releasing carbon dioxide. Next, acetaldehyde is converted to ethanol by alcohol dehydrogenase (ADH). This oxidoreductase type can catalyze the reversible interconversion of alcohols and the corresponding aldehydes or ketones (**Figure 1**A) [17]. Some yeast species can produce organic acids, including acetic, phosphoric, oxalic, malic, and succinic [27][29]. Yeasts also contribute to the development of the characteristic flavor of chocolate due to the generation of volatile compounds [30]. Furthermore, it has been reported that some yeast strains such as *Pichia kudriavzevii* can hydrolyze the pectin present in the mucilaginous pulp surrounding the cocoa bean since they can produce pectinolytic enzymes [25].

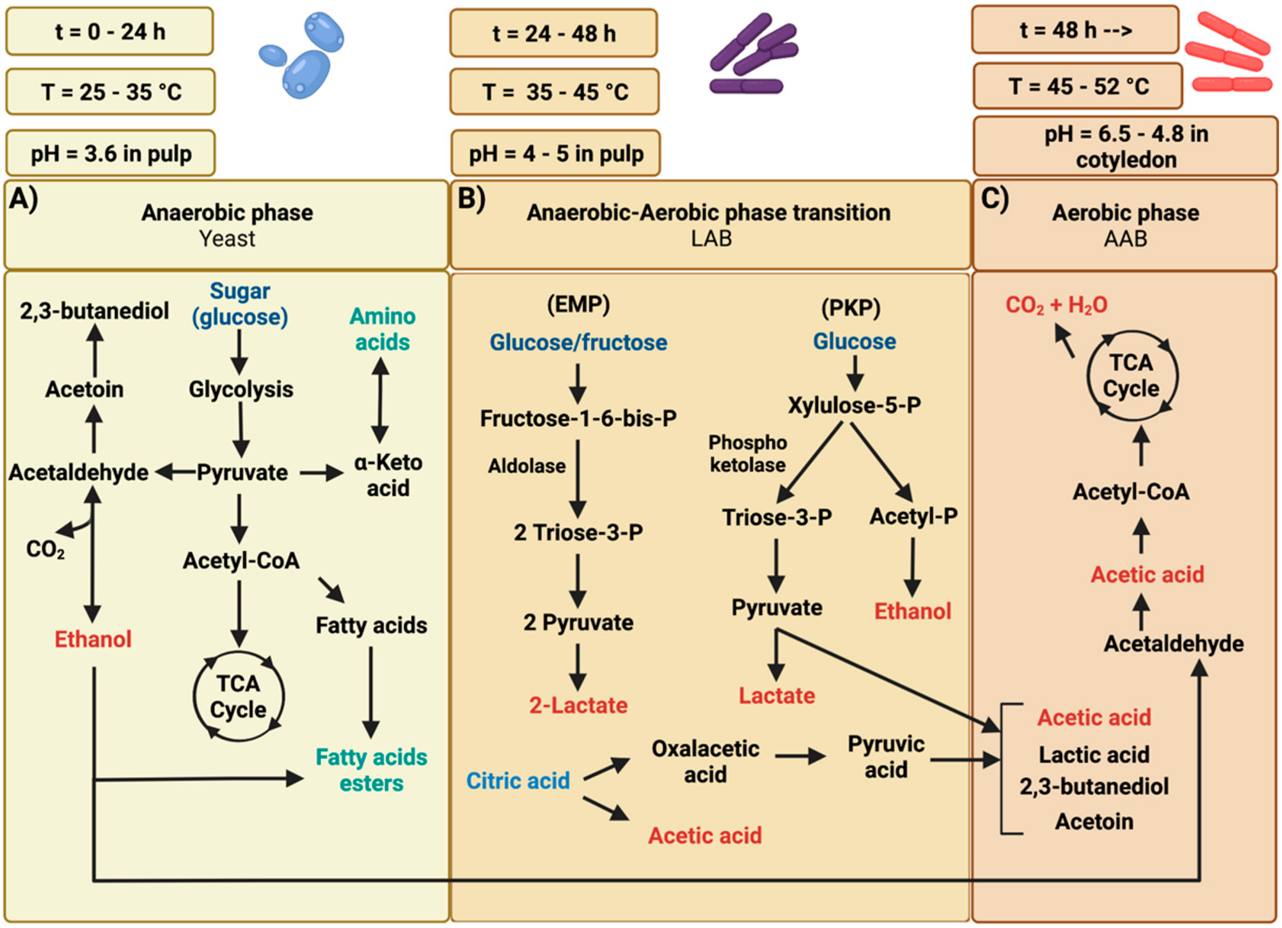

Figure 1. Main phases of cocoa fermentation. (**A**) Yeasts produce ethanol from sugar (glucose), fermenting it to pyruvate through glycolysis to obtain ATP, reduce equivalents production, and produce ethanol and carbon dioxide. (**B**) LAB strains utilize glucose through the Embden–Meyerhoff–Parnas EMP pathway (Homofermentative LAB) or phosphoketolase PKP pathway (Heterofermentative LAB). (**C**) Lastly, AAB strains oxidize ethanol produced by yeasts to acetic acid.

2.1.2. Lactic Acid Bacteria (LAB)

LAB is a group of Gram-positive bacteria whose main product of fermentable carbohydrate metabolism is lactic acid [31]. The LAB population increase when some of the pulp and lixiviate have drained mainly due to pectin degradation, and the yeast population decreases. Carbon dioxide production favors this increase in LAB populations by the yeasts and by the release of vitamins and other nutrients from the autolysis of yeast cells during cocoa fermentation [28]. The most abundant species after 24 h of fermentation are *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, and *Lactococcus lactis* [5][18][25][32][33]. During cocoa fermentation, LAB utilizes glucose via the Embden–Meyerhof pathway. The homofermentative LAB strains use glycolysis or Embden–Meyerhof–Parnas pathway (EMP) and yield more than 85% lactic acid. However, other species utilize glucose via the known pentose phosphoketolase pathway (PKP), hexose monophosphate shunt, or 6-phosphogluconate pathway producing only 50% lactic acid, and other metabolites such as ethanol, acetic acid, glycerol, mannitol, and CO_2, as shown in **Figure 1**B [20][34]. LAB strains can consume fructose and metabolize citric acid. In the case of fructose, it is metabolized homofermentative (glycolysis) or heterofermentative (phosphoketolase pathway) to pyruvate, while citric acid is metabolized to acetic acid and oxaloacetic acid [5]. Oxoloacetic acid is converted into pyruvate, which will yield either lactic acid, acetic acid, or pyruvate metabolites as 2,3-butanedione (diacetyl; buttery notes), 2,3-butanediol, and 2-butanone (acetone; buttery notes) [5]. Some LAB strains can metabolize citric acid yielding diacetyl, acetoin, and butanediol [35].

2.2. Aerobic Phase of Cocoa Bean Fermentation

On the third day of fermentation, when the pulp of the cocoa beans has been decreased, and both the temperature and the amount of air inside the fermentation mass have been increased, the environmental conditions are favorable for the proliferation of AAB. These bacteria metabolize the ethanol produced during yeast growth as their primary carbon source [18][36].

Acetic Acid Bacteria (AAB)

AAB dominates this phase of cocoa bean fermentation; in recent years, these bacteria have been extensively studied due to their significant contribution to cocoa bean fermentation [37][38][39]. AAB conducts ethanol and lactic acid oxidation to acetic acid. Acetic acid is considered one of the main metabolites produced by an exothermic reaction oxidizing ethanol to acetic acid (**Figure 1**C). The rise in temperature to 40–52 °C, decrease in pH from 6.5 to 4.8 in the cotyledon, and penetration of acetic acid and ethanol to the cocoa bean is the cause of the death of the embryo, promoting their inactivation and increasing the permeability of the cell wall of the grain and the release of precursor molecules of cocoa color and flavor precursors [4][18][25][38][39][40]. The diversity of AAB is practically limited to two genera: *Acetobacter* and *Gluconobacter* [25]. *Acetobacter pasteurianus* is the most identified AAB during cocoa bean fermentation in Ivory Coast [41][42], Cameroon [16][43], Honduras [44], and Brazil [45][46].

2.3. Biochemical Transformations on Cocoa Bean during Fermentation

The biochemical transformations that occur inside the cocoa bean are driven mainly by the production of ethanol, lactic acid, and acetic acid, and an increase in temperature during fermentation provoked by the oxidation of ethanol by AAB [5][20][21][47][48][49]. Acetic acid penetrates the bean and induces a drop in the pH of the cotyledons (approximately 6.5 to 4.8). This low pH of the cotyledons, combined with the presence of non-dissociated acetic acid and ethanol and the heat effect during fermentation, causes the embryo's death (**Figure 2**) [20][21][47][50] damages the cotyledon's internal structure to prevent the germination of cocoa beans. The physicochemical modifications result in desirable enzymatic and non-enzymatic conversions and the release of compounds from the cocoa bean.

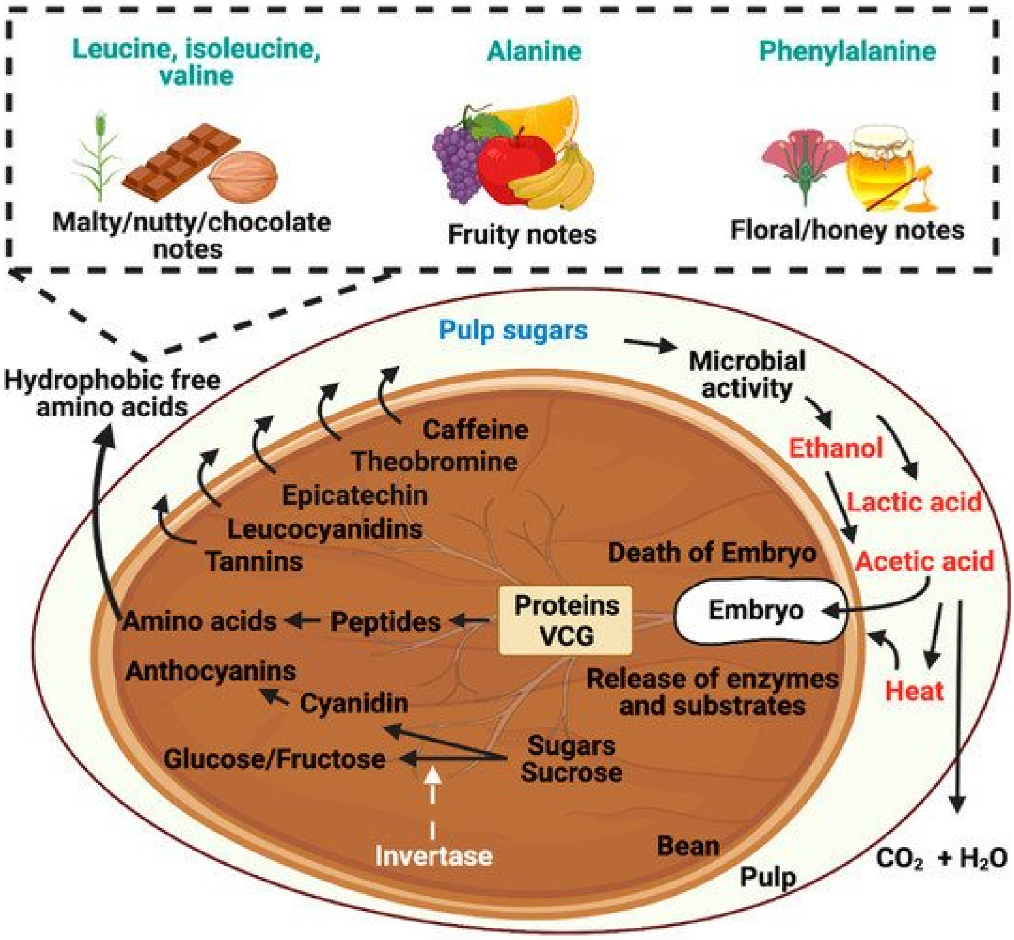

Figure 2. Formation of flavor precursors from hydrophobic-free amino acids. Microorganisms utilize the available substrates present in the cocoa pulp, such as carbohydrates, pectin, and organic acids, to produce the main metabolites of the process, such as ethanol, lactic acid, and acetic acid. Acetic acid penetrates the beans' interior, causing the embryo's death and the release of enzymes and endogenous substrates that, through proteolytic reactions, generate the flavor precursors (amino acids).

Consequently, the different enzymes found inside the cocoa bean can be activated or inactivated gradually during the fermentation and drying processes [5][20][21][47][50][51]. The free amino acids and peptides are formed by proteolytic enzymatic reactions, while reducing sugars, such as fructose and glucose, are products of sucrose hydrolysis by invertase [52]. Peptides and hydrophobic-free amino acids, such as alanine, phenylalanine, leucine, and tyrosine, are precursors that contribute to the cocoa and chocolate flavor formation that develops through acetic acid and lactic acid-induced proteolysis of vicilin-class globulin (VCG). Strecker degradation of each specific amino acid produces a unique aldehyde with a unique aroma, e.g., from alanine; fruity notes (acetaldehyde), phenylalanine; sweet, bitter, and almond notes (benzaldehyde), leucine, malty/chocolate notes (3-methylbutanal), isoleucine; malty/chocolate notes (2-methylbutanal), valine; malty/nutty/chocolate notes (2-methyl propanal) and phenylalanine; floral/honey notes (phenylacetaldehyde) as shown in **Figure 2** [5][53][54]. Cocoa bean's phenolic compounds impart astringency; however, their concentrations decrease significantly during fermentation and drying. Anthocyanins are rapidly hydrolyzed to cyanidins and sugars (catalyzed by glycosidases). Polyphenol oxidases convert polyphenols (mainly catechins) to quinones. The complex of proteins and peptides with polyphenols gives rise to the brown coloration typical of fermented cocoa beans. Methylxanthines impart bitterness. However, their levels decrease by 30% during cocoa bean fermentation [55]. The invertase, optimally active at an acidic pH of 4.5, is active mainly at the beginning of the cocoa bean fermentation and hydrolyses sucrose into the reducing sugars glucose and fructose that serve as flavor precursors [5].

3. Contribution of Yeasts during Cocoa Fermentation

The fresh cocoa pulp is favorable for yeast growth since it consists of an anaerobic environment rich in sugars and a low pH that inhibits the development of other microorganisms [5][56]. Many studies have demonstrated a great diversity of yeast species during cocoa fermentation. The main yeast genera involved in the fermentation process of spontaneous cocoa are *Pichia*, *Saccharomyces*, *Hanseniaspora*, and *Candida*. Other genera found in lower abundance are *Wickerhamomyces*, *Torulaspora*, *Kluyveromyces* and *Rhodotolura* [9][11][13][15][16][26][27][28][29][33][41][45][46][57][58][59][60][61][62][63][64][65][66][67][68][69][70][71][72][73][74][75][76][77][78][79][80]. Concerning the yeast species found in this process, several authors have highlighted that the most frequent are, in decreasing order, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Hanseniaspora opuntiae*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Pichia manshurica*, *Pichia kluyveri*, and *Candida tropicalis* [25][56]. The main activities performed by yeasts during the cocoa fermentation process are the production of volatile organic compounds (VOCs), pectin hydrolysis, and carbohydrate fermentation [56]. Some species may have some antifungal effect [44][81][82] and can metabolize citric acid [29][75].

3.1. Flavor Precursor Formation by Yeast during Fermentation

Yeasts are involved in the production of VOCs, which are essential in developing fruity flavors and aromas. These compounds are also determinants in developing fruity, caramel, or chocolate flavors and aromas [18]. Ho et al. [15] demonstrated that the absence of yeast during cocoa bean fermentation caused the absence of higher alcohols and esters in the fermented cocoa beans. This suggests that yeasts are the leading producers of these compounds. They concluded that yeasts were essential to the cocoa fermentation process. Koné et al. [13] identified 33 VOCs produced by yeasts. The species *P. kudriavzevii*, *S. cerevisiae*, *C. tropicalis*, and *Wickerhamomyces anomalus* were found to produce higher alcohols (isobutanol and isoamyl alcohol), acids (acetic acid and isovaleric acid) and esters (ethyl acetate, isobutyl acetate, and isovaleric acid).

In the metabolism of yeasts, a fraction of the carbon is shuttled to the Krebs cycle, which forms important aroma precursors through reactions associated with amino acid metabolism [17]. Some yeast species such as *Saccharomyces kudriavzevii* produce higher alcohols, either catabolically or anabolically. The catabolic formation by the Ehrlich pathway involves consecutive transamination, decarboxylation, and dehydrogenation of amino acids. The anabolic production is by side products of amino acid biosynthesis starting from pyruvate. Some yeasts produce acetoin from acetaldehyde (green apple notes), which can be further reduced to 2,3-butanediol; similarly, diacetyl can be reduced to acetoin and 2,3-butanediol forming higher alcohol. Additionally, yeasts produce higher alcohols such as 3-methylbutanol and 2-phenylethanol and esters such as ethyl acetate, ethylphenyl acetate, and 2-phenylethyl acetate, contributing to the floral and fruity notes of the cocoa beans (**Figure 3**A) [5][17]. Esters are formed by a condensation reaction between acetyl/acyl-CoA and alcohol. The use of acetyl-CoA or acyl-CoA divides esters into acetate esters and fatty acid ethyl esters (**Figure 3**B). Acetate esters have significantly more influence over flavor and fragrance than the fatty acid counterparts due to their contribution of fruity and floral notes [17].

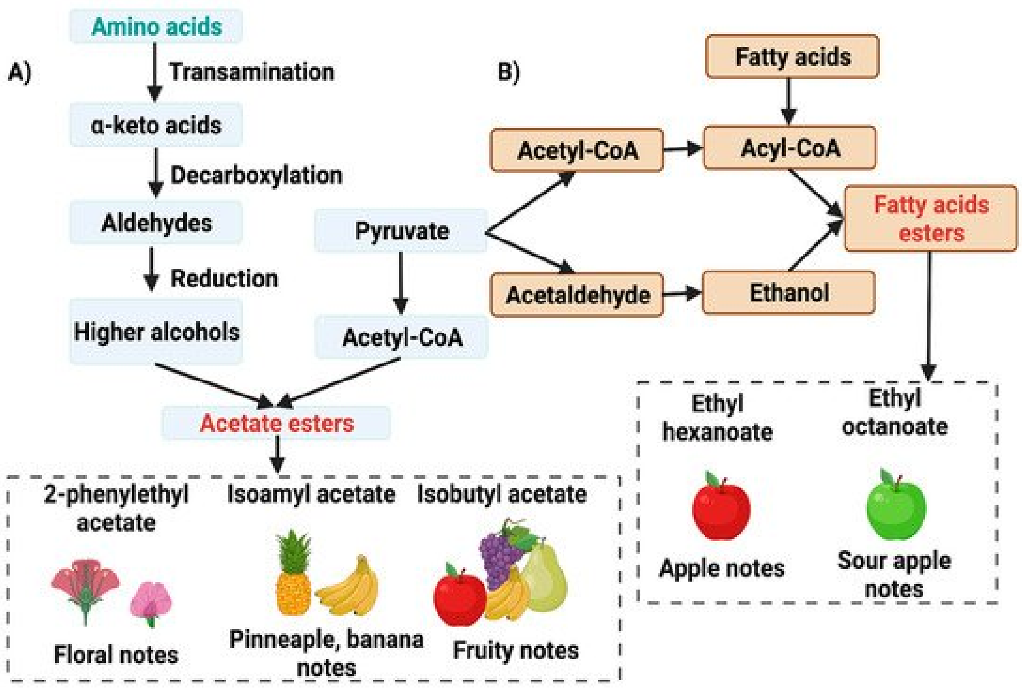

Figure 3. Formation of flavor precursors by yeast during fermentation via (**A**) Amino acids and (**B**) Fatty acids substrates. Yeast autochthonous to the cocoa fermentation process can produce higher alcohols either catabolically, through the Ehrlich pathway involving transamination, decarboxylation, and dehydrogenation of amino acids; or anabolically, as by-products of amino acid biosynthesis from pyruvate during the cocoa fermentation process.

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