Chapter 3

Natural Flavours Obtained by Microbiological Pathway

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Additional information is available at the end of the chapter

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Abstract

In the last years, the demands for natural flavours have dramatically increased. To fulfil the consumer requests, researchers are looking for new and alternative methods to obtain qualitative aroma compounds by utilising microbiological pathways. Some microorganisms like lactic acid bacteria or yeasts are capable of synthesising specific flavours corresponding to diacetyl and acetaldehyde as secondary metabolites. By supplying the culture media with flavour precursors and optimising the primary culture media, high amount of specific flavours could be obtained. Also, the biosynthesis of each specific flavour is influenced by the type of amino acids and sugars involved in the bioprocess. Thus, by changing the ratio of amino acids and sugars in the culture media, different amounts of flavour can be obtained. In this context, monitoring the compositions of the culture media and fermentation conditions is crucial in obtaining high amounts of a qualitative-specific aroma.

Keywords: natural flavours, microorganism, metabolic pathway, fermentation

1. Introduction

Generally, the first major source of flavour is the extraction from plant biomass due to consumer preference for "clean" and "organically" produced aromas and fragrances. Taken into account the significant differences between the price of synthetic and non-synthetic manufacturing, the microbial flavour production is considered [1]. Consequently, in the last years, the main focus of researchers in the field was the identification of a suitable biosynthetic pathway and the optimal culture medium design for an efficient production.

Lactic acid bacteria (LABs) are an important class of microorganism for flavour manufacturer. LABs are very important for the dairy industry, being extensively used in fermented food production. During the fermentative processes, LABs influences the sensory properties of the

final products, also including the flavour development. The flavour production is very much substrate and strain dependent, and the presence of the flavour precursors and regulatory responses may influence the balance of the flavour biosynthesis from a secondary metabolite product to the main compound [2]. Strains like *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* are industrially used for flavour biosynthesis as a sole microorganism or in coculture with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [2].

At the same time, the flavour biosynthesis and the changes in metabolic pathway are linked to environmental conditions. From a technological point of view, the metabolic changes are very important for the volatile compound biosynthesis, as well as for the microorganism, in order to obtain energy and to maintain the $NAD^+/NADH^+H^+$ balance [3]. It is obvious that there are major differences in flavour profiles between utilised complex and standard media. *S. thermophilus* LMG18311 biosynthesize 2,3-pentanedione and acetic acid only in standard media, but *Bacillus subtilis* CICC 10025 biosynthesize higher amounts of acetoin in media consisting of acidified molasses and soybean hydrolysate, because soybean hydrolysate is a more optimal nitrogen source for acetoin production for this strain [2]. Diacetyl is almost exclusively synthesised by LAB and is the key flavour compound naturally produced by the *Leuconostoc* sp. [4].

2. The influence of the culture medium composition on flavour biosynthesis

2.1. The influence of nitrogen

The LAB strains are able to survive starvation due to their capacity to utilise another energy source rather than carbon. The starvation conditions decrease the organism ability to synthesise ATP with generation of proton motive force (PMF) and also slow down the accumulation of necessary nutrients to maintain viability over time. As an additionally carbon source, the LABs are capable to catabolise amino acids which provide building blocks, cofactor recycling and limited energy source [5]. The LAB inability to synthesise many of the amino acids required for protein synthesis needs the supplementation of the culture media with high amount of essential amino acids [6], since the amino acid catabolism is a major process for flavour formation. Proteolytic enzymes from LAB play an important role in degradation of proteins by producing free amino acids. These amino acids contribute directly to flavour formation being precursors for catabolic reactions [7, 8]. The conversion of amino acids to aroma compounds by LAB is essentially initiated by a transamination reaction, which requires α-ketoacid as the amino group acceptor, pathway demonstrated for lactococci, mesophilic lactobacilli and thermophilic LAB [9].

By amino acid catabolism, LABs are able to synthesise flavours. In the first step of the pathway, the amino acids are involved in dehydrogenation and transamination reactions with the formation of α-ketoacids, compounds which have a fundamental effect on flavour type and amount. Further, by decarboxylation reaction, α-ketoacids are transformed in aldehydes (**Figure 1**).

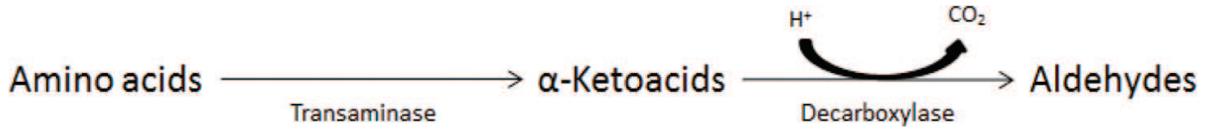

Figure 1. The pathway for the conversion of amino acids to aldehyde.

From decarboxylation reaction, a proton is consumed in the process, and the product is exported from the cell, resulting in an increase of the intracellular pH [10]. Additionally, the aldehydes are transformed into alcohols or carboxylic acids by dehydrogenation, a majority of these compounds being flavour compounds. Several enzymes, for example, α-ketoacids, can thus be considered as intermediates involved in both biosynthesis and degradation of amino acids. Since branched-chain amino acids (Val, Ile, Leu), aromatic amino acids (Trp, Tyr, Phe) and sulphur-containing amino acids (Cys, Met) are important precursors of flavour compounds, the genome of *Lactococcus lactis* IL1403 was screened for gene-encoding enzymes of the biosynthetic pathways for these amino acids. At least 12 aminotransferases of the *Escherichia coli* are found to be encoded in the *L. lactis* IL1403 genome sequence. By knowing the enzyme and metabolic pathway, new potential flavours are expected to be biosynthesise for industrial applications [11].

Theoretically, there are three pathways for the formation of α-ketoglutarate by bacteria using glutamate, citrate and pyruvate [9].

2.1.1. Amino acids' first specific degradation pathway

In the first step, the glutamate dehydrogenase pathway produces α-ketoglutarate directly from oxidative deamination of glutamate, utilising NAD^+, NADP or both as cofactor. NADP-dependent activity was detected in most *Lactobacillus plantarum* strains and in several *Lactobacillus lactis*, *Lactobacillus paracasei* and *S. thermophiles* strains, whereas NAD^+-dependent activity was observed in only a few *L. lactis* and *S. thermophilus* strains. Moreover, it has been demonstrated that the ability of LAB to produce aroma compounds from amino acids is closely related to their glutamate dehydrogenase activity [9, 12]. Literature reports showed that conversion of amino acids to aroma compounds by LAB was limited by the lack of α-ketoacid acceptor for transamination reactions. Indeed, the addition of α-ketoglutarate to culture medium enhanced their aroma by increasing the amino acid catabolism (**Figure 2**).

α-Ketoglutarate is the best α-ketoacid acceptor for amino acid transamination by *L. lactis*. Another α-ketoacid that can also be used is pyruvate, but the aminotransferase activities were 40 times lower than with α-ketoglutarate. However, for some lactobacilli strains, pyruvate appeared to be an acceptor as efficient as α-ketoglutarate. A *L. lactis* strain genetically modified overexpresses a gene encoding a catabolic glutamate dehydrogenase, which catalyses the deamination of glutamate to α-ketoglutarate and, therefore, greatly increased the conversion of amino acids to potent aroma compounds [9]. *Pediococcus pentosaceus*, *Lactobacillus brevis*, *Lactobacillus curvatus* and *Lactobacillus fermentum* inoculation leads to the conversion of glutamine to glutamic acid and NH_3 [13].

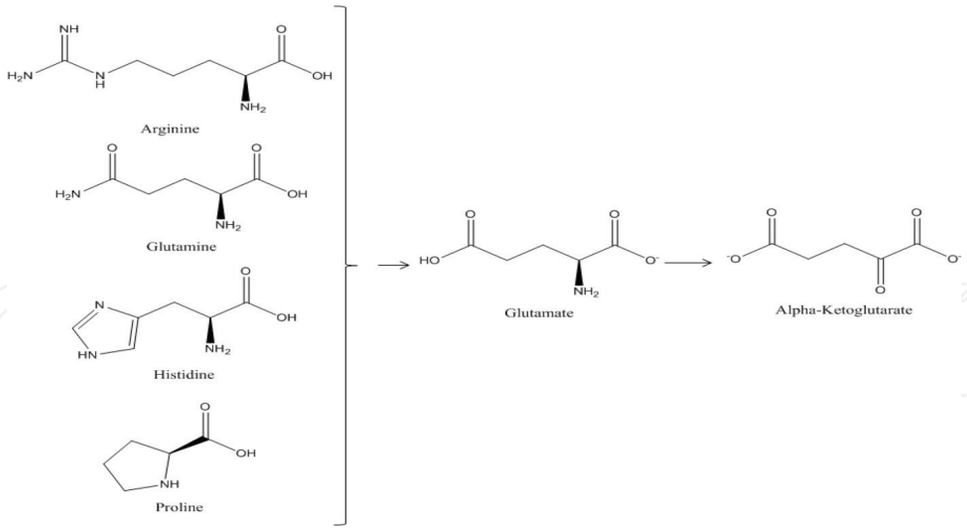

Figure 2. The glutamate dehydrogenase pathway of the amino acids.

Different amino acids have diverse amino peptidase, with characteristic activity on amino acids [6]. *Lb. fermentum* IMDO 130101 possesses an arginine deiminase pathway which is modulated by environmental pH. This converts arginine into ornithine *via* citrulline while producing ammonia and ATP [14] but at the same time has the ability to catabolise arginine to α-ketoglutarate by glutamate formation (**Figure 2**).

The proline catabolism by *Saccharomyces cerevisiae* also leads to flavour biosynthesis, the intermediary compound being glutamate, which is further degraded to aroma compounds [15].

2.1.2. Amino acids' second specific degradation pathway

The second possible pathway is the citrate-oxaloacetate pathway, which leads to α-ketoglutarate production from citrate and glutamate, by successive action of citrate permease, citrate lyase and aspartate aminotransferase (AspAT). Citrate permease allows citrate uptake inside the cells with the citrate catabolism initiation by transforming citrate to oxaloacetate. Oxaloacetate can then be transformed into aspartate and α-ketoglutarate, in the presence of glutamate, by an aspartate aminotransferase. For *L. lactis* species, only the diacetylactis subspecies possesses citrate permease and citrate lyase, but in this subspecies, oxaloacetate is mainly decarboxylated to pyruvate, which is then transformed to lactate, acetate, and diacetyl [9] (**Figure 3**).

L. lactis IFPL326 strain showed the highest aminotransferase activity towards isoleucine, which is a specific substrate for the *Lactococcus* branched-chain aminotransferase. This LAB in combination with other strains which has α-ketoacid decarboxylase with high specificity for branched-chain degradation can be used for the obtaining of high yield of isoleucine-derived volatile compounds (2-methyl-1-butanol, 2-methylbutanal and phenylacetaldehyde) in the incubated milk [12, 16]. For example, 2-methyl-1-butanol is one of the components of the black truffle (*Tuber melanosporum*) aroma. Some *Lactobacillus helveticus* strains have been capable of diacetyl biosynthesis from α-aceto-α-hydroxybutyrate, an intermediate of isoleucine metabolism [17].

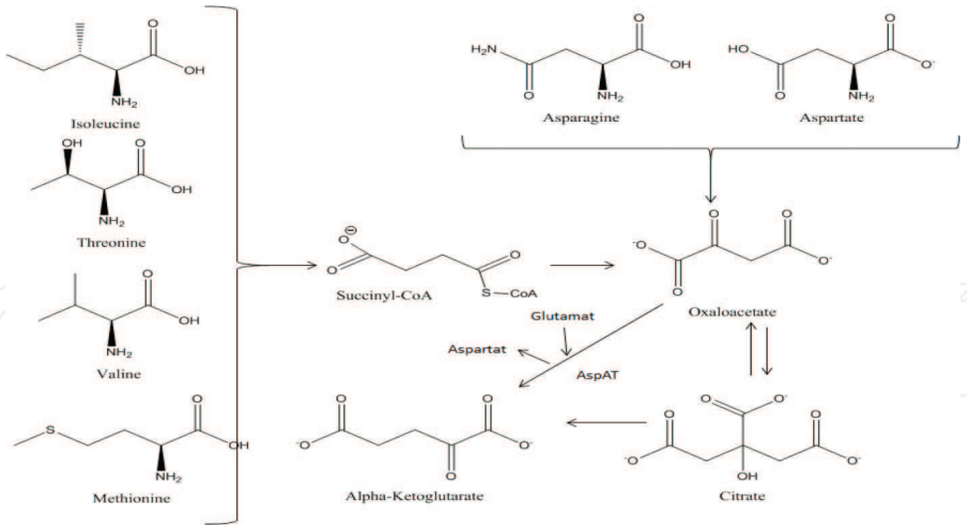

Figure 3. The citrate-oxaloacetate pathway of the isoleucine, threonine, valine and methionine (AspAT—aspartate aminotransferase).

Other research show that isoleucine catabolism leads to the formation of α-keto-β-methyl valerate [10].

On the other hand, the valine catabolism by non-oxidative enzymatic decarboxylation leads to the formation of α-ketoisovalerate in *L. lactis* fermentation that uses α-ketoisovalerate decarboxylase [10, 16]. The high specificity of the *L. lactis* α-ketoisovalerate decarboxylase permits to be a key controlling step in the formation of branched-chain aldehydes. *Lactococcus* strains combined with *L. lactis* IFPL730 for incubation in milk lead to production of aldehydes, without the necessity of exogenous α-ketoglutarate addition, and the production of different flavour compounds, like 2-methyl-1-propanol, 2-methylpropanal (straw fragrances) and 2-methyl-1-propionic acid (rum-like odour), was observed [12, 16].

In another study, the *L. lactis* aromatic aminotransferase converts aromatic amino acids but also leucine and methionine. The methionine conversion was in lower concentration than isoleucine, leucine and valine [16]. Aminotransferase activity requires α-ketoglutarate with the formation of 4-methylthio-2-ketobutyric acid which can be converted to methane-thiol, *via* a thiamine pyrophosphate-dependent decarboxylase that produces 3-methylthiopropanal [11], dimethyl sulphide and dimethyl disulphide. It is important to mention that methional is a notable flavour used in potato-based snacks, while dimethyl disulphide has a garlic-like aroma. During cheese ripening, cystathionine β-lyase can convert methionine to various volatile flavour compounds, but in bacteria its physiological function is the conversion of cystathionine to homocysteine, which is the penultimate step of methionine biosynthesis. In other researches, beside aroma abovementioned, obtained from methionine catabolism, phenylacetaldehyde (with honey-like aroma) was identified by *Lb. plantarum* UC1001, *S. thermophilus* and *Lb. helveticus* biosynthesis [12].

The biosynthesis of the diacetyl from aspartate by some *Lactobacillus* strains has been reported by Garde and co-workers [17]. Aspartic acid under the aminotransferase action may generate acetoin and diacetyl by *Lactobacillus casei* GCRL163 [5] and *Lactobacillus* strains. Thage and

co-workers [18] demonstrated that three *Lb. paracasei* subsp. *paracasei* strains (CHCC 2115, 4256 and 5583) had different expression of aspartate aminotransferase activities against aspartate. Another study made by Skeie and co-workers [19] shows that all the LAB strains with citrate metabolism can biosynthesise diacetyl and acetoin by aspartate metabolism with the formation of the unstable α-acetolactate.

LAB protein degradation determines the formation of free amino acids that vary in their concentration over time. Leucine has been reported to be dominant amino acid in Cheddar cheese after 6 months of maturation [5]. Leucine catabolism leads to the formation of α-keto-isocaproate [10] and generates aroma like 3-methylbutanal (cheesy, chocolate, malt), 3-methylbutanoic acid (cheesy, sweaty), phenylacetaldehyde and 2-hydroxy-4-methyl pentanoic acid methyl ester [18]. On the other hand, under *Lb. plantarum* UC1001, *S. thermophilus* and *Lb. helveticus* catabolism of lysine results in hexanoic acid, with a cheesy aroma [12].

L. lactis subsp. *diacetylactis* and *Lactococcus lactis* subsp. *cremoris* strains used in the cheese manufacturing are able to degrade phenylalanine and leucine in the presence of citrate and glutamate. This is possible due to the fact that this strains use α-ketoacids (pyruvate and α-ketoglutarate) as acceptor for transamination reaction, produced from citrate metabolism. To balance the α-ketoglutarate biosynthesis (because this is the best acceptor for *L. lactis* amino-transferase and the pyruvate is an enzyme used in many pathways), a selection of a strain with a high aspartate aminotransferase activity and low oxaloacetate decarboxylase activity may be introduced into co-fermentation [9]. From the phenylalanine catabolism resulted in phenylacetaldehyde, a floral aroma and a key odour compound in hard and semihard cheese varieties [18]. This aroma in combination with p-cresol, phenyl-ethanol, indole and skatole can result in undesirable odour that contributes to putrid, faecal or unclean flavours in cheese. By using a specific strain, undesirable flavours can be avoided [11]. *L. lactis* degrades 49% of initial phenylalanine with the biosynthesis of phenyl-lactate, phenyl-acetate, benzaldehyde (which has an almond-like odour) and phenyl-ethanol (with a floral odour) and 22% of initial leucine in milk fermentation with the formation of the hydroxyl-isocaproate and isovalerate (menthol aroma) [20]. *Lb. plantarum* UC1001, *S. thermophilus* and *Lb. helveticus* can produce 2-phenethyl alcohol (rose-like aroma) and phenylacetaldehyde (floral fragrances) along with flavours named from phenylalanine catabolism. These three strains can also produce benzaldehyde from tryptophan catabolism [12]. Tyrosine is degraded by *Brevibacterium linens* 47 by phenyl-alanine pathway [21] (**Figure 4**).

2.1.3. Amino acids' third specific degradation pathway

The third pathway is the citrate-isocitrate pathway which utilises the oxidative branch of the tricarboxylic acid cycle leading to the production of α-ketoglutarate from either pyruvate or citrate with the action of pyruvate dehydrogenase, pyruvate carboxylase, citrate synthase, aconitase and isocitrate dehydrogenase. Pyruvate dehydrogenase and pyruvate carboxylase are necessary to degrade pyruvate into acetyl-CoA and oxaloacetate, respectively, both used by citrate synthase to synthesise citrate. Citrate is then transformed by aconitase into isocitrate, which is finally oxidised to α-ketoglutarate by isocitrate dehydrogenase [9].

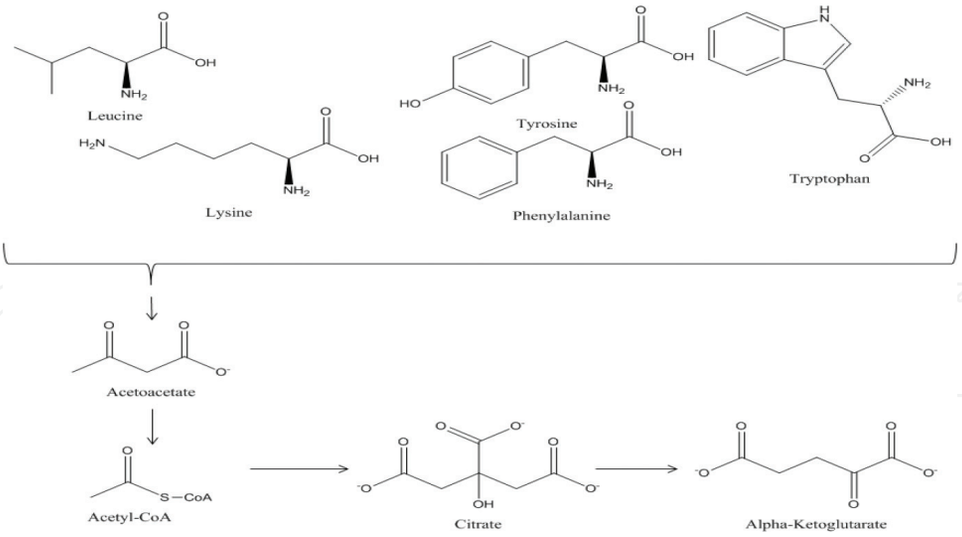

Figure 4. The citrate-oxaloacetate pathway of the leucine, lysine, tyrosine, phenylalanine and tryptophan.

Pediococcus acidilactici and *P. pentosaceus* can convert alanine to pyruvate by α-ketoacid intermediary pyruvate which further is converted to flavour compounds [22]. The diacetyl and acetoin are produced *via* citrate metabolism by citrate-positive LAB (*L. lactis*, *Lb. casei*) through aspartate catabolism described by the L-aspartate-L-alanine-pyruvate steps [23]. The degradation pathway of the alanine by *Lb. plantarum* UC1001, *S. thermophilus* and *Lb. helveticus* leads to the production of acetic acid and ethanol, while from glycine catabolism resulted in acetic acid [5, 12]. Other microorganisms degrade glycine to pyruvate with the formation of serine as intermediary compound, and then the pyruvate is used as a precursor to flavour biosynthesis (**Figure 5**) [24]. By oxidative deamination of the serine under the *Lb. plantarum* UC1001 metabolism, acetic acid is detected [5, 12], while *P. pentosaceus* and *P. acidilactici* are able to produce diacetyl from pyruvate and L-serine [22].

Cysteine is catabolised by α-ketoacid enzyme with synthesis of 3-mercaptopyruvate, which by elimination of hydrogen sulphide, lead to the obtaining of pyruvate, used as a precursor for flavour biosynthesis.

Lb. helveticus and *S. thermophilus* can produce acetaldehyde from threonine by the breakdown of the amino acid with threonine aldolase into glycine and acetaldehyde [17]. Acetaldehyde levels increase together with threonine levels, in cheeses during ripening. Branched aldehydes are produced from the catabolism of branched amino acids, but they do not accumulate in cheese because they are quickly converted into the corresponding alcohols [7]. *Lb. plantarum* UC1001, *S. thermophilus* and *Lb. helveticus* can produce propionic acid from threonine catabolism [12].

2.2. The influence of carbon sources

The carbon source is very important for the microbial growth because it is the principal resource for energy production. In the same time, for aroma biosynthesis sugars with a low-molecular weight are requested to be used, which are at the same time a flavour precursor.

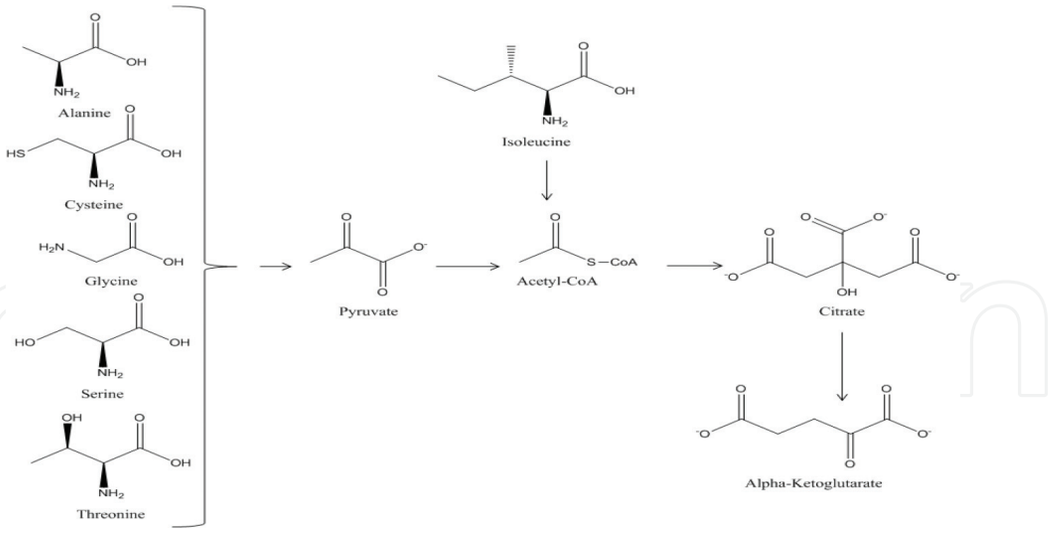

Figure 5. The citrate-isocitrate pathway of the alanine, cysteine, glycine, serine and threonine.

The addition of sucrose in the culture media stimulates the flavour biosynthesis for yeasts and LABs [25]. Di Cagno and co-workers [26] supplemented with sucrose the tomato juice that is subjected to LAB fermentation in order to stimulate the flavour biosynthesis and to reduce the intrinsic flavour acidity of tomatoes. By inoculation of six prebiotic strains in the milk culture media, supplemented with 0.75% fructose (w/v), a desired aroma for the final product was obtained [3].

De Figueroa and co-workers [27] demonstrated that *Lactobacillus rhamnosus* ATCC 7469 can use lactate as the sole energy source and, at the same time, is able to grow with citrate as sole energy source and to produce diacetyl and acetoin. The enzyme activity of this strain is increasing with the increase of temperature from 22 to 37°C. Therefore, the presence of a high pyruvate amount lied to a high production of acetolactate, diacetyl and acetoin. At the same time, when the glucose level is high, diacetyl and acetoin in low concentration are produced by *Lb. rhamnosus* ATCC 7469 [27]. Some LAB species like *Lb. rhamnosus* and *Lb. plantarum* are able to grow on citrate as a single carbon source and consequently to produce diacetyl [19].

In sourdoughs, for example, flavour compounds are produced by LAB and yeasts individually or by their interactions. *S. cerevisiae* produced more volatile compounds than *Candida guilliermondii*, but quantity of volatile flavour compounds can be improved by the addition of glucose, of sucrose and less of maltose. Addition of fructose, glucose or maltose to the dough increases LAB contributions to volatile formation in baking [25]. Escamilla-Hurtado and co-workers [28] prepared a semisolid maize-based culture media and grew a mixed cultures formed by *P. pentosaceus* MITJ-10 and *Lactobacillus acidophilus* Hansen 1748 obtaining 779.56 mg/kg diacetyl after 12 h of exponential growth. *Enterococcus faecium* FAIR-E 198 can grow on xylose, glucose and lactose and converted by biosynthesis the citrate in diacetyl. However, in non-fermentable conditions, the acetoin yield is decreased in the strain fermentation [29]. Also, other species like *Leuconostoc* can use xylose as a sole energy source for diacetyl biosynthesis [30]. *Lb. casei* GCRL163 strain was studied in a medium supplemented with different concentrations of

lactose, but the maximum growth was registered for only 1% lactose in medium with no significant aroma biosynthesis [5].

2.3. The influence of the mineral composition of the medium

The minerals are very important in culture media of the microorganism because they are used as a cofactor in enzymatic activity. All enzymes have a metal as a coordinative element, and the enzyme activity depends on it. At the same time, the salt concentration is very important because it dictates the osmotic pressure and flavour improvements [31]. Similarly, aldehydes can also be generated by chemical oxidation of α-ketoacids catalysed by bivalent cations [16]. Manganese and magnesium sulphate enhanced both biomass and aroma development of 52 different yeasts by obtaining 96.05 mg/L acetaldehyde for *Candida lipolytica* and 3.58 mg/L diacetyl for *Candida globosa* [4].

Recently, two manganese transport systems of *Lb. plantarum* have been characterised. These systems, which are implicated in mineral uptake, convert phenylalanine to benzaldehyde by initiation of a pyridoxal 50-phosphate-dependent aminotransferase. The phenyl-pyruvic acid is obtained after conversion, which is further chemically transformed to benzaldehyde in the presence of oxygen and manganese [11].

2.4. The influence of temperature

The flavour biosynthesis by microorganism is strongly influenced by the temperature of fermentation. De Figueroa and co-workers [27] demonstrate that *Lb. rhamnosus* ATCC 7469 produce diacetyl and acetoin from citrate within a temperature interval of 22–45°C. The biosynthesis amount of diacetyl increased in the temperature interval between 30 and 37°C with maximum production at 48 h. For the fermentations made at different temperatures, as 22 and 45°C, the maximum aroma biosynthesis was reached at 24 h of incubation, and the level of the acetoin and diacetyl was 4.1 time higher at 37°C than at 22°C. Moreover, the highest efficiency of the conversion of citrate into diacetyl and acetoin was obtained at 37°C. At the same time, citrate transport and incorporation in microbial system reach maximum at the 37°C. Another effect of the temperature is on the enzymatic systems. The activities of citrate lyase and NADH oxidase reach a maximum at 37°C when the temperature is increased from 22 to 45°C [27].

On the other hand, lower incubation temperature tends to selectively promote growth rate of the *Leuconostoc* and *L. lactis* ssp. *cremoris* strains, while the higher temperatures will favour *Lb. rhamnosus* and *L. lactis* ssp. *lactis* strains. The inoculation concentration has also a significant influence on the aroma production. The acetaldehyde biosynthesis by *Leuconostoc* or *Lactobacillus* is not influenced by the temperature changes [32].

For the yeast fermentations (*S. cerevisiae*), the increased temperature from 24 to 30°C leads to increasing of the acetaldehyde biosynthesis. Besides yeast, acetic acid bacteria can biosynthesise acetaldehyde at concentrations up to 250 mg/L. For this fermentations type, acetaldehyde tends to accumulate under low oxygen level and ethanol concentration higher than 10% [33].

2.5. The influence of aeration

The presence of oxygen strongly influences the microbial growth and the flavour biosynthesis. The aerobic microorganism's metabolism is oxygen dependent and is mandatory for flavour biosynthesis pathway. For example, *Lb. casei* grown under aeration conditions leads to higher diacetyl amount biosynthesise in Cheddar cheese than an anaerobic starter culture [23]. Another example is for *E. faecium* FAIR-E 198 strain growth which biosynthesise diacetyl only in aerobic conditions [29].

3. Acetaldehyde biosynthesis pathway

Acetaldehyde besides being a major component of tobacco smoke is the primary metabolite of ethanol [34]. Commercially, it is obtained by Wacker process of ethylene oxidation in strong acid solutions using as catalysts $PdCl_2$-$CuCl_2$ of crude oil, but this method is not very sustainable. The trend demands are for obtained acetaldehyde from renewable raw materials like sugars from biomass or synthesise from lactic acid. Due to its high reactivity derived from containing two conjugated hydroxyls and one carboxylic group, lactic acid (LA) is an attractive feedstock for chemical production, being in torn biosynthesise at low costs by glucose and xylose fermentation. The acetaldehyde may be produced by decarbonylation or decarboxylation of LA in the presence of aluminium phosphates and magnesium aluminate spinels, reaction promoted by acid catalysts [35].

Acetaldehyde represents a secondary metabolite in alcoholic fermentation of yeasts, being a precursor of the ethanol production in beer and wine. It is the most important carbonyl compound produced during alcoholic fermentation in concentrations between 10 and 200 mg/L depending on technological factors, such as culture medium composition, pH, fermentation temperature, aeration and SO_2 concentration and on the yeast strain used [4]. Acetaldehyde is biosynthesized from glucose by the glycolytic pathway enzyme pyruvate decarboxylase. At the beginning, two molecules of pyruvate resulted from glucose glycolysis, and by pyruvate decarboxylation, the secondary acetyl-CoA product is obtained. Furthermore, two acetaldehyde molecules are resulted under alcohol dehydrogenase action on the acetyl-CoA compound (**Figure 6**). The high peak value of acetaldehyde biosynthesis is reached during the early fermentation phases, being then partly re-catabolised by yeast, or is combined with polyphenols or other compounds in the wine being a very reactive compound [36].

Another species that can produce acetaldehyde is the acetic acid bacteria (AAB) characteristic from grape microorganism equipment. This class of microorganism has another biosynthesis pathway, oxidising ethanol to acetaldehyde and acetic acid in concentrations up to 250 mg/L. At ethanol concentrations higher than 10% (v/v) and under low oxygen conditions, acetaldehyde tends to accumulate, in other conditions being oxidised to acetic acid. The yeasts reported to biosynthesize acetaldehyde in high amounts are *S. cerevisiae* with 0.5–286 mg/L and *Kloeckera apiculata* with 9.5–66 mg/L [33].

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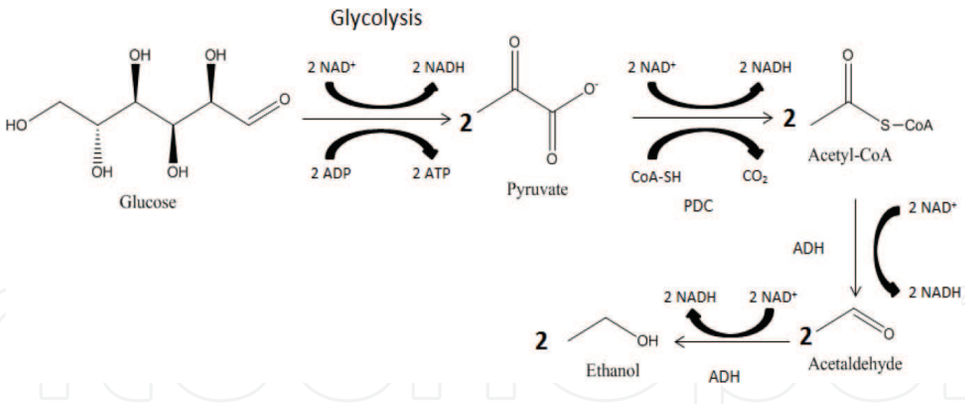

Figure 6. Acetaldehyde biosynthesis pathway. PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase.

Taken into account that lactic acid bacteria are also responsible for acetaldehyde biosynthesis, being a typical flavour component of yoghurt responsible for pungent and fruity flavour [37], this microorganism has attracted the researcher attention. By using recombinant microbial processes for biotransformation approach, Balagurunathan and co-workers [38] engineered an *E. coli* strain for acetaldehyde production from glucose. They introduced the pyruvate decarboxylase from *Zymomonas mobilis* and NADH oxidase from *L. lactis* in the *E. coli* strain genome, and the results confirmed that around 37% of the glucose consumed could be redirected towards acetaldehyde biosynthesis under anaerobic conditions. The mass yield of acetaldehyde obtained is 0.18 g/g glucose, this being the highest mass reported for microbial acetaldehyde production. The main disadvantage of biosynthesise acetaldehyde is the high toxicity on the microbial cells.

4. Diacetyl biosynthesis pathway

Diacetyl (2,3 butanedione) is the typical butter flavour/aroma, commonly found in fermented dairy products, such as butter, sour cream and yoghurt, and important for cheese aroma [39]. Moreover, *L. lactis* is a safe flavour production microorganism [40]. The precursor for diacetyl and acetoin biosynthesis is citric acid, while cow milk is a good substrate which contains 1750 mg citrate/L [41]. Genera as *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Weissella* and others have citrate metabolism property, regulated by different gene expression adapted to the specific microorganism. Different transcriptional factors belonging to DeoR and GntR family mediate transcriptional activation in the presence of the substrate [10].

Citrate metabolism is the principal generator of sensory characteristics of the milk final products. One of the LAB used globally as starter fermentation is *L. lactis* biovar *diacetylactis* where citrate and acetoin/diacetyl pathway increases the intracellular level of pyruvate and is coordinated and expressed at low pH [10].

For LAB, citrate fermentation is involved in cheese flavour and quality. In the first step of citrate metabolism catalysed by citrate lyase, oxaloacetate is obtained. The oxaloacetate is

decarboxylated via oxaloacetate decarboxylase generating pyruvate. In *L. lactis*, genes associated with citrate metabolism are organised in two operons. One operon, citQRP, is involved in citrate transport, and the other operon, citM-citI-citCDEFXG, which is encoded for citrate lyase, is involved in citrate conversion to pyruvate [10, 42]. In this step, the proton motive force is generated (PMF). In LAB enzymatic equipment, two types of oxaloacetate decarboxylase are presented. One type is a soluble oxaloacetate decarboxylase belonging to the malic enzyme family. This enzyme is present in *L. lactis* and *Weissella mesenteroides* and other LAB strains, catalysing the conversion of oxaloacetate from citrate to pyruvate in the presence of divalent metals. The second type is an oxaloacetate decarboxylase membrane complex, which is a biotin-dependent decarboxylase. This consists of four polypeptides and is found in *Enterococcus faecalis* and *Lb. casei* [10].

The pyruvate obtained is condensed by α-acetolactate synthase with the formation of α-acetolactate which is chemically unstable [40]. The α-acetolactate formed by the action of α-acetolactate decarboxylase can be converted to acetoin or diacetyl in a non-enzymatic oxidative decarboxylation reaction, the biosynthesis being more evident at pH 4.5. This pathway could be also completed with additional enzymes such as acetoin/diacetyl reductase and butanediol dehydrogenase (**Figure 7**) [10, 23].

There have been many attempts to redirect the metabolism of various microorganisms for improving diacetyl formation, by classical mutagenesis or directed genetic engineering trying the improvement of by-product formation [43], but the results were not very promising [40].

Liu and co-workers [40] successfully achieved to convert the homo-lactic bacterium *L. lactis* into a homo-diacetyl producer with high titre (8.2 g/L) and high yield (87% of the theoretical maximum) by complete redirection of the metabolism, metal-ion catalysis and respiration activation using glucose as a substrate. In the experiments, they found that almost 90% of the glucose was converted to α-acetolactate without detectable lactate, acetate or ethanol, implying that the glucose flux was successfully redirected to the α-acetolactate formation pathway.

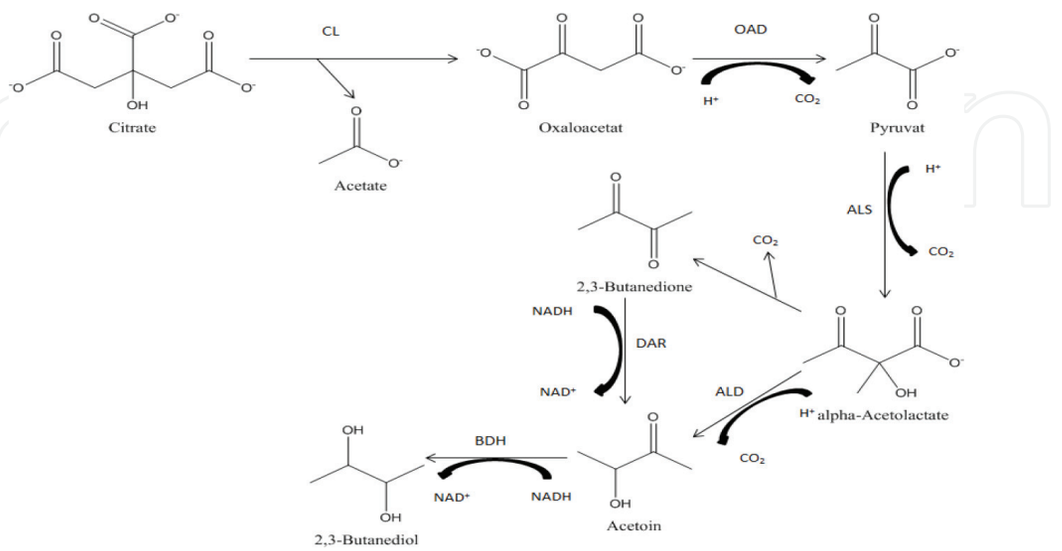

Figure 7. Diacetyl biosynthesis pathway. CL, citrate lyase; OAD, oxaloacetate decarboxylase; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase; DAR, acetoin/diacetyl reductase; BDH, butanediol dehydrogenase.

5. Flavour determination and quantification

The flavour determination was made by different instrumental analytical methods. The common one is the solid-phase micro-extraction analysis, in which different substrates are used for solid phase, **Table 1**.

Other analysis techniques of flavours are (i) by purge and trap method and (ii) GC-MS separation and identification [7, 52, 53] or (iii) by proton transfer reaction mass spectrometry using PTR-MS [54, 55]. In some cases, the flavour determination was made enzymatically [36, 56] or by derivatisation with dinitrophenylhydrazine (DNPH)-acetonitrile reagent, and then the compounds are analysed by HPLC analysis with detection at 360 nm [34, 57].

Solid-phase material	GC column	Reference
Carboxen/polydimethylsiloxane, 85 μm film thickness, 220°C work temperature	Zebron ZB-624, D-0.25 mm; 1.4 μm film thickness; composition, 94% dimethyl polysiloxane; 6% cyanopropyl-phenyl; 60 m long	[44]
Polydimethylsiloxane with 10% embedded activated carbon phase (PDMS/AC), 50 μm film thickness, 250°C work temperature	HP-5MS capillary column, 5% phenyl methyl silicone, 320 μm × 1.0 μm, 60 m long	[45]
Polyacrylate bonded to silica core, 85 μm film thickness, 220°C work temperature	HP-INNO-WAX polyethylene glycol capillary column, 250 μm × 0.5 μm, 60 m long	[16]
Polydimethylsiloxane fibre, 250°C work temperature	DB5 capillary column, 0.32 μm internal diameter, 1 μm film thickness, 60 m long	[26, 46]
StableFlex divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) coated fibre, 250°C work temperature	ZB-WAXplus polyethylene glycol capillary column, 0.25 mm internal diameter; 0.50 μm film thickness, 60 m long	[47, 48]
Silica fibre covered by Carboxen Polydimethylsiloxane (CAR-PDMS), 75 μm film thickness, 250°C work temperature	CP-Wax 52 CB polyethylene glycol coated, 0.32 mm, 1.2 mm film thickness, 50 m long	[49, 50]
Polydimethylsiloxane divinylbenzene (PDMS_DVB) SPME fibre, 250°C work temperature	SUPELCOWAX™ 10 capillary column, 0.1 mm, 0.1 μm film thickness, 10 m long	[51]

Table 1. The solid-phase micro-extraction conditions for flavour analysis.

6. Applications of natural flavours

Flavour release from food during consumption in the mouth is important in flavour perception and influenced by food matrix [58]. Since food matrix changes biochemically and physically during eating, the food flavour microencapsulation results in controlled release for specific situations. Different natural and synthetic polymers were used for microcapsule fabrication, of which alginate-whey protein compounds have been found to be suitable as vehicle for diacetyl flavour delivery [59].

Nowadays, the delivering of antimicrobial volatiles from polymeric systems, in a controlled manner, gained an increasing interest. In food industry, diacetyl is used not only as an approved food additive but also for food preservation, due to its antimicrobial activities. Diacetyl has been shown to be bactericidal against *E. coli* and *Staphylococcus aureus* at a concentration as low as 100 ppm [60]. The effects of diacetyl on the quality of ground beef were evaluated when diacetyl was used in modified-atmosphere packaging in conjunction with 20% CO_2. A delayed spoilage of ground beef and the maintenance of the fresh colour and odour were observed for this product [61]. The inhibitory effects of diacetyl combining with reuterin, against *E. coli*, *Salmonella enteritidis* and *Listeria monocytogenes* in milk, suggested that these LAB metabolites are potential for pathogen control in dairy products [62].

Strains of *Lactobacillus* and bifidobacteria could produce diacetyl in concentrations up to 30 mg/mL suggesting its potential to exhibit dermal antimicrobial activities [63], with greater sensitivity against Gram-negative bacteria (such as *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Borrelia burgdorferi*, *Salmonella typhi*, *Bartonella* sp., *Klebsiella rhinoscleromatis*, *Vibrio vulnificus* and *Helicobacter pylori*) and fungi as compared to Gram-positive bacteria [60].

Another direction of diacetyl utilisation is related to active packaging systems. Thus, the controlled release of different volatile antimicrobial compounds was tested for packaging obtained from two or more poly(ethylene glycol) polymers of different molecular weights and/or a mixture of poly(lactic acid) and poly(ethylene oxide) [64].

7. Conclusions

In this chapter, data regarding the conditions for flavour obtained by microbial fermentations were presented. The flavour biosynthesis is strongly influenced by growth medium and fermentation conditions and, in addition, is strain dependent. One of the most important factors is the carbon source, which in some cases is flavour precursor. The nitrogen source influences the flavour biosynthesis by the metabolites generated from catabolic degradation pathway. The impact of aeration on the flavour production is significant due to the fact that almost all microorganism strains are aerobic ones and flavour is obtained in the presence of oxygen. The temperatures modulate the amount of flavour biosynthesis, while the mineral composition influences the microbial yield. The knowing of the metabolic pathway leads to the possibility to interfere on the type and the amount of flavour biosynthesised.

The natural aromas obtained by biotechnological routes offer an alternative to the synthetic ones, which appear to be one of the most promising manufacturing techniques for the future.

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