A Review of Flavour Formation in Continuous Beer Fermentations*

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ABSTRACT

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The attractive prospect of a continuous beer fermentation system consists mostly of the accelerated transformation of wort into beer. Although continuous beer fermentation has been studied as a promising technology for several decades, the number of industrial applications is still limited. The major obstacle hindering the extensive industrial exploitation of this technology is the difficulty in achieving the correct balance of sensory compounds in the short time typical for continuous systems. This paper offers an integral view on the particularities of continuous systems, which may impart beer a sensorial character distinct from conventionally fermented counterparts. The main groups of flavour active compounds are discussed from the perspective of possible control strategies by means of process parameters and strain selection.

Key words: Beer, continuous, control, flavour, fermentation, reactor.

INTRODUCTION

Continuous immobilized cell systems are undoubtedly suitable for maturation of young beer and alcohol free beer production. This can be also illustrated by industrial examples[35,67,96]. However, wider application of this technology has not taken place and it can only be speculated whether it is the necessary equipment conversion or the disbelief of the brewers that is responsible for the state of the art.

Regarding continuous main fermentation with immobilized or free yeast cells, the situation is rather different. The main fermentation has encountered problems of different origin such as engineering (carrier choice, reactor design, production inflexibility), microbial (upstream hygiene, long-term process asepticity), physiological (immobilization induced metabolic shifts, yeast mutation, aging) and economic (carrier cost, costs for skilled supervision)[2,45,59,92,112,113,120]. It is clear, that some of the problems were met in one system, but not in others. Concerning troubleshooting, some solutions are easy to suggest (e.g. clogging in packed-bed reactor? → use reactors with forced circulation, high carrier cost? → use wood chips, spent grains, corncobs etc.), while others such as yeast physiology require a more comprehensive understanding of the issue. In general, the complexity of continuous main fermentation and its obstacles have often resulted in an unbalanced flavour of the final product[51,117]. The objective of this review is to summarize the knowledge on the origin of the flavour active compounds in beer and to discuss the strategies of controlling and adjusting their formation during continuous beer fermentation.

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PARTICULARITIES OF CONTINUOUS FERMENTATION SYSTEMS

The increased volumetric productivity in continuous fermentation systems is achieved through a controlled contact of fermentable substrates with a high concentration of free and/or immobilized biomass. Evidence that the continuous mode of reactor operation, immobilization, aging and mutation of cells provokes different physiological responses when compared to batch free cell systems have been frequently observed[43,116]. These differences have to be taken into account when considering continuous beer fermentation (Table I).

Continuous mode of reactor operation

During traditional batch beer fermentation, brewing yeast adapt their relatively versatile metabolism to a changing external environment. Different metabolic fluxes belong to distinct phases recognizable on the growth curve (Fig. 1). The metabolic changes associated with entry into individual growth phases are exerted at the level of gene expression and enzyme activity. The ability of yeast to sense the changing external environment initiates the induction or repression of specific genes corresponding to the actual growth phase, while the modulation of metabolic pathways is mediated through stimulatory or inhibitory effects of intracellular metabolites. Events such as dissolved oxygen and substrate depletion trigger the selective and sequential assimilation of individual compounds, e.g. fermentable sugars and wort amino acids[42], as well as the formation of metabolic by-products in a coordinated fashion. Thus the beer flavour results from a mixture of aerobic and anaerobic metabolic products produced during growth phases of different intensity.

Contrary to batch fermentation, at steady-state conditions in a continuous culture the cells are not exposed to

Table I. Multidisciplinary factors affecting beer flavour in continuous fermentation systems.

Factors affecting beer flavour	Comments on flavour impact
Operational parameters	
Temperature	Increasing the production of growth related by-products (VDKs, higher alcohols, acetaldehyde) and enzyme activity (esters)
Dissolved oxygen	Increasing the production of growth related by-products (VDKs, higher alcohols, acetaldehyde) and inhibiting ester formation
Residence time	Enhancing diacetyl removal and increasing attenuation
Wort composition	Complex (e.g. higher extract increases formation of volatiles; optimum FAN reduces diacetyl formation etc.)
Microbial and physiological factors	
Genetic background of specific strain	Complex (strain selection and genetic manipulation represent a powerful tool for flavour adjustment)
Viability/aging	Unclear, yet expected to be rather negative
Mutation/selection	Unclear, yet expected to be rather negative
Immobilization induced changes	Complex and varying in a wide range (case dependent)
Hygienic considerations	Negative impact of contamination
Engineering and technological factors	
Mass transfer rate	Volumetric oxygen mass transfer coefficient (k_La) data would allow more sophisticated control of flavour active compounds
Reactor design	Proper reactor type for given growth requirements can facilitate flavour control (heat transfer, clogging, channelling)

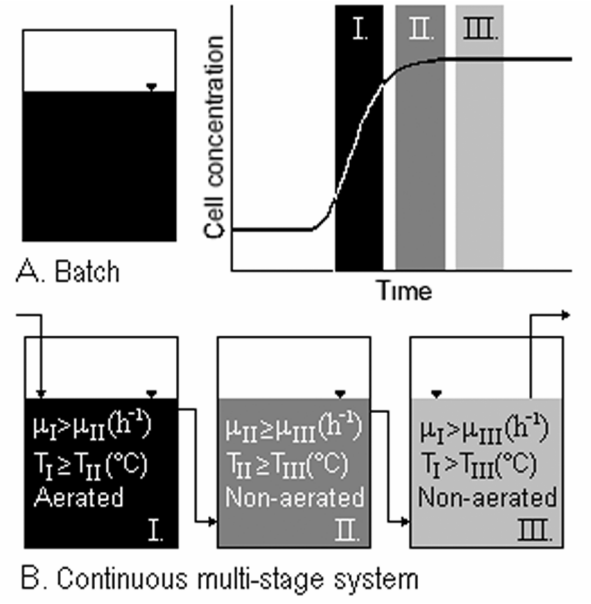

Fig. 1. Possible reactor configurations for beer fermentation. A. Batch reactor – distinct phases on growth curve characterised by different specific cell growth and product formation rate. The final beer has a balanced flavour; B. Continuous multi-stage reactor system – different specific cell growth rates (μ), corresponding to areas of specific growth rates on batch growth curve, are mimicked in a series of reactors, where the cellular activities are controlled by process parameters (T, aeration). Product quality is comparable with batch fermented beer.

significant alterations in the reaction environment[55]. Hence, the microbial population of the continuous systems lacks the distinct growth phases of a batch culture. Therefore, the batch fermentation is mimicked either in continuous tubular reactors with plug-flow[65] or in a series of agitated reactors[6,37] (Fig. 1). Often, the complete continuous beer fermentation systems consist of an agitated reactor for primary fermentation followed by a packed-bed reactor, with more or less ideal plug-flow[10,121]. A flavour profile similar to conventional beers can be achieved by inducing different growth conditions in a series of usually two or three reactor vessels, where the high cellular activity in the first reactor decreases in subsequent stages. This can be achieved by means of controlling the process parameters such as temperature, aeration and residence time in each reactor separately. The number of reactors in multi-stage systems results from a compromise between flavour requirements, investment and operational costs[95,110,119].

Mass transfer limitations

In a typical three-phase system (gas-liquid-solid) the external and/or internal mass transfer rate may affect both yeast physiology and beer flavour[71] through limited substrate supply and/or altered concentrations of metabolites in the vicinity of the immobilized biomass.

Considering oxygen supply, it has to be stated, that continuous primary beer fermentation does not require an intensive aeration. On the contrary, its excess causes product deterioration; hence precise aeration is a crucial parameter for the formation of flavour active compounds and long-term cell viability. Consequently, the knowledge of the volumetric oxygen mass transfer coefficient (k_La) is essential in order to accomplish a thorough understanding of the aeration impact on flavour formation.

Internal mass transfer limitations of nutrients can occur when cells are entrapped in a polymer matrix. The cells entrapped in gels are exposed to different micro-environmental conditions and exhibit modified metabolic activities. The degree of limitation is given by the position of the cell, bead size and polymer structure. These mass transfer limitations are the probable explanation of the often-observed decrease in immobilized cell growth rate, specific productivities and changed by-product formation as compared to free-cell cultures[28,88,89].

The pre-formed porous (e.g. sintered glass) and non-porous carriers (e.g., DEAE-cellulose, wood chips, spent grains) do not have the additional gel diffusion barrier. However, depending on the porosity of the carrier and on the degree of colonization, internal mass transfer limitations may occur[62]. Cells adhered in a single layer to DEAE-cellulose showed similar metabolic activities[88] while yeast cells attached to spent grains in a multilayer biofilm had a lower specific substrate consumption rate than free cells in the same system[8].

Physiological changes caused by immobilization

Changes in metabolic functions (substrate uptake, product formation, enzyme expression and activity) of the immobilized cells have been reported because of complex micro-environmental conditions resulting from cell immobilization[34,84,100,116].

It has been shown that immobilized cells exhibit increased levels of DNA, structural carbohydrates[19], glycogen[28], fatty acids[34], as well as modifications of gene expression levels, cell proteome, cell wall and cell membrane composition[68,81,108]. Not surprisingly, the alterations of plasma membrane composition have a marked impact on several enzymes, sensor proteins, transporters and membrane fluidity. This can result in increased ethanol tolerance[32,40] and altered sugar and amino acid uptake[82,108]. Hence, it is expected that immobilization induced changes of cell physiology in turn may cause flavour variations.

However, the direct effects of immobilization are very difficult to conclude from the literature, since the information concerning the physiological conditions of immobilized yeast is rather complex due to different matrices, variable system configurations and strain specificity of the observations. Therefore, when evaluating the most suitable support for continuous beer fermentation with immobilized yeast, besides engineering and economic aspects, the mechanism of immobilization should be taken into account. Most of the supports combine various mechanisms[12]; however, adsorption can be considered the most gentle immobilization method, because it resembles the natural biofilm formation.

Aging of yeast in continuous cultures

The yeast *Saccharomyces cerevisiae* has a limited replicative lifespan determined by its genes and influenced by environmental factors. Brewing yeast cells are capable of a finite number of divisions (10 - 30 divisions) before entering a non-replicative state termed senescence, leading to death and autolysis[73]. Yeast display an array of changes during aging including decrease of viability[5], increase in size, cell surface wrinkling, increase of generation time, increasing bud scar number and decreased metabolic activity[38,60,85]. The study of the aging process of brewing yeast strains also has a practical significance. Serial fermentations can select for an undesirable subpopulation enriched with elderly cells. The aged brewing yeasts show changed flocculation characteristics[5,74,86,107] and fermentation performance[15,74]. It is believed that the performance of lager strain begins to degenerate after 10 serial repitchings[39].

Taking into consideration the long periods of time (several months) that immobilized cells are spending in a continuous reactor[91,110], the question of the immobilized cell aging process is even more relevant. The viability and fermentation capacity (vitality) of immobilized brewing yeast in continuous fermentations have already been reported to decrease[10,37,45,46,72]. However, there is little known about the impact of senescence and aging of immobilized yeast in continuous fermentation on product quality. Hence, elucidating the influence of the aging process on cell vitality and fermentation performance would be of a great practical importance. As a consequence, proper measures to ensure stable fermentation performance of the bioreactor could be taken. Nevertheless, it can be anticipated that yeast strains with low maximum lifespan potential would not be appropriate for continuous fermentations or immobilized systems.

Mutation of yeast in continuous cultures

The spontaneous genetic mutation of yeast is a common phenomenon manifesting as alterations at both the morphological and biochemical levels. Most of the spontaneous mutations which occur in brewing yeast cells are "petite" mutations. During the brewing process, the frequency of genetic drift and mutation is considerable. Petite mutations leading to respiratory deficiency occur spontaneously with a frequency of 0.5 to 5% among yeast harvested from fermenters, but figures as high as 50% have been reported for stored yeast[59]. Prolonged storage, starvation[33] and ethanol stress[36,41] are known to increase the occurrence of petite mutations. Physiologically, petites exhibit an altered cell membrane and cell wall morphology. Respiratory-deficient yeast can lead to reduced fermentation rate and ethanol production, decreased cell viability, inappropriate flocculation behaviour and flavour defects[23,59].

It is expected, that spontaneous mutations may become even more evident under the conditions of continuous culture. The genetic drift of producing strains in continuous non-immobilized fermentations was particularly feared. The observations during laboratory scale experiments reported approximately 50% mutated cells after nine months of continuous cultivation with a deleterious effect on beer flavour[92]. Conversely, no mutation of the yeast was observed in other industrial scale continuous beer fermenters operating over several months[2,6]. Given that there was no clear evidence of mutation in continuous fermentation systems, it can be speculated that mutations are either strain specific and/or the majority of the mutations do not provide any advantage.

Comparing to continuous free cell systems the situation in immobilized cell reactors is complicated by the effect of the immobilization method. In reactors with carrier to cell surface interaction (adsorption, adhesion and attachment) as a prevailing immobilization mechanism, the genetic drift of the biocatalyst can be caused by selection pressure based on surface interactions[9]. However, there is little known on the risk of selecting a subpopulation, with surface properties favouring cell retention, not to speak about other possible accompanying physiological changes. In the case of predominantly mechanical retention of immobilized cells (entrapment, encapsulation), the fear of genetic drift seems to be less relevant owing to a large amount of biomass already present in the system at start-up.

FLAVOUR COMPOUNDS AND THEIR CONTROL DURING CONTINUOUS BEER FERMENTATION

The flavour profile of a beer is an important attribute in evaluating the feasibility of a continuous beer fermen-

Table II. Concentration of selected flavour active compounds in continuously fermented beer vs. conventionally fermented beer (continuous/batch).

Selected flavour active compounds in beer (ppm)							
Total diacetyl (0.07-0.15)[c]	**Acetaldehyde (10-20)[c]**	**Ethyl acetate (25-33)[c]**	**Isoamyl acetate (1.0-1.6)[c]**	**n-Propanol (600-800)[c]**	**i-Butanol (80-200)[c]**	**Isoamyl alcohols (50-70)[c]**	**Ref.**
0.48/0.3		17.9/17.2	0.9/1.2	15/17.5	10.1/12.5	60.1/70	8[a]
0.7/0.4		33.3/16.8	2.8/1.3	11.6/10.4	11/9.7	51.2/50.9	45[a]
	8.0/4.9	11.3/26.4	0.01/0.08	32.5/9.9	11.1/7.8	47.4/46.7	56[a]
0.08/0.2	16.4/10.5	13.7/15.2	0.65/0.55	19.8/14	18.8/13.8	67.9/62.2	61[a]
1.18/0.3		14.1/14	1.21/1.2	11.3/10		72/59.5	63[a]
		11/19	0.06/2	8/9.8	7.5/16	31/62	78[a]
	17.2/15.9	29.7/21.5	0.3/0.3	36.3/9.9	1.2/0.2	52.1/50.8	90[a]
		31/18	1.3/1.1	12/18	13/14	54/67	1[b]
0.17-0.29/0.03	13-36/8	3.5-11/17.2	0.03-0.2/1.2	16-32/17.5	8-12/12.5	56-71/70	10[b]
0.1/0.12	2.7/2.85	10.8/12.1	3.3/3.1	6.1/6.8	12.1/16.9	53.7/51.7	18[b]
0.02/0.01		11.6/26.7	0.8/2.5	13.3/12.4	10.8/11.5	36.5/39.5	31[b]
	7.5/4.3	23/23.4	0.7/2.0	14.5/9.7	9.4/10.2	49.5/52	110[b]
0.02/0.04	8/9	18/15	1.6/1.8	13.8/11.3	6/11.3	42.3/58	120[b]
		18/18	1.9/2	11.8/12	7.5/11	42/58	121[b]

[a] Continuous main fermentation with immobilized cell system / Conventional main fermentation.
[b] Continuous complete fermentation with immobilized cell system / Conventional complete fermentation.
[c] Range of flavour thresholds in beer[11,22,48,57].

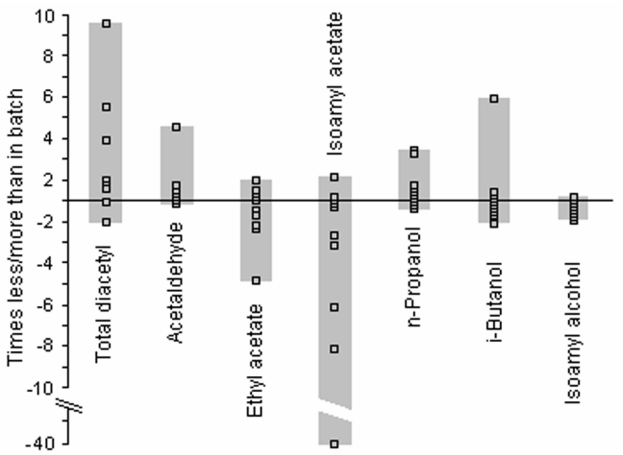

Fig. 2. Multiples of compound concentrations and their distribution in continuously fermented beers comparing to corresponding conventional beers (positive values = times more than in batch, negative values = times less than in batch). Markers represent the individual values obtained from literature used in Table II.

tation technology. Reports allowing the comparison of selected flavour active compounds in conventionally and continuously fermented beers, originated from wort with the same composition and fermented by identical brewing yeast strains, are summarized in Table II. All the selected flavour compounds in continuous beers show a range of variability more or less approaching the values in traditional beers. Therefore the objective of this review is to discuss the multi-disciplinary reasons of these divergences, their consequences and possible means of flavour adjustment (Table I). The comparison is shown on selected compounds; however, it is needless to say that beer flavour is not merely a question of analytical determination (Table II). This can be explained by the fact, that even if the concentrations of certain compounds are below their threshold values, their presence can be important for the overall flavour profile. The mutual ratios of flavour active compounds in continuously fermented beers and in their batch counterparts are depicted on Fig. 2.

Vicinal diketones

The concentrations of two vicinal diketones (VDK), 2,3-butanedione (diacetyl) and 2,3-pentanedione, of which diacetyl is more flavour-active, are of critical importance for beer flavour. Diacetyl has a strong "butterscotch" aroma in concentrations above the flavour threshold around 0.1-0.15 ppm in lager beers[114]. The now accepted pathway is that diacetyl results from the chemical oxidative decarboxylation of excess α-acetolactate leaked from the valine biosynthetic pathway to the extracellular environment. 2,3-pentanedione is formed similarly from α-acetohydroxybutyrate. This chemical conversion is considered the rate limiting step of VDK formation. At the end of the main fermentation and maturation phase, diacetyl is re-assimilated and reduced by yeast to acetoin and 2,3-butanediol, compounds with relatively high flavour thresholds. It seems that various enzymatic systems are involved in the reduction of VDKs by brewing yeast[4,93]. Immediate regulation of valine and thus diacetyl precursor biosynthesis occurs at the level of enzyme activity and synthesis. When the intracellular valine concentration increases, the enzyme responsible for α-acetolactate synthesis is inhibited and total diacetyl formation is reduced. This occurs during the period of valine uptake from wort[70].

The amount of total diacetyl formed during continuous main fermentation tends to exceed the concentrations of this immature beer aroma compound in traditionally fermented young beers (Fig. 2). This feature of continuous fermentation systems can be interpreted as:

- An immobilization induced change of cell physiology such as the accelerated expression of the acetohydroxy acid synthase responsible for the formation of α-acetolactate from pyruvic acid[84].
- An alteration of the amino acid metabolism of the immobilized cells. It is manifested by an unbalanced feedback inhibition of the isoleucine-valine biosynthetic pathway. Nevertheless, the data on amino acid uptake by immobilized cells are often contradictory and influenced by the immobilization technique applied. Lower amino acid uptake by entrapped yeast has been reported[37,78,88] while immo-

bilization by attachment showed uptake rates similar to free cells[45,82,88].

- An enhanced anabolic formation of amino acid precursors due to rapid yeast growth. The excess cell growth in continuous fermentation systems is often a result of over-aeration. There have been several studies on the influence of aeration on by-product formation in continuous systems[8,50,111]. Despite this, the results are difficult to transfer between different systems due to a lack of comprehensive engineering data on oxygen transfer rates in various types of bioreactors applied for continuous beer fermentation to date.

Several strategies for repressing excessive VDK formation during continuous main fermentation are possible. Addition of bacterial α-acetolactate decarboxylase, to the fermenting beer, thus converting α-acetolactate directly to acetoin[30] and/or the use of genetically modified brewer's yeast e.g. encoding an α-acetolactate decarboxylase would lead to lower final diacetyl level in continuously fermented beer[46,109,118]. However, the legal regulation regarding enzyme supplementation and the lack of consumer acceptance towards genetically modified yeast favour other methods of diacetyl control. These consist of controlling the operating conditions, process changes, quality control of raw materials and selection of an appropriate yeast strain. Perhaps the most feasible is keeping the yeast growth in immobilized high cell density systems suppressed by optimized process parameters (dissolved oxygen, temperature). However, as long as there is carbohydrate consumption in the reactor system, even at conditions limiting cell growth, significant diacetyl formation occurs and diacetyl control through growth regulation was in some cases found to be ineffective[95,121]. Besides process parameters, the wort free amino nitrogen (FAN) concentration was also shown to affect diacetyl production as well. Worts containing both too much and too little FAN gave increased diacetyl contents[70,75]. Thus maintaining an optimum wort FAN for a given continuous system, characterised by specific cell growth rate, immobilization method and brewery strain, could be of great use. The amount of the produced α-acetolactate is also strain dependent[48,50], which in turn allows some diacetyl control by strain selection.

The diacetyl content above the taste threshold in continuously fermented final beer is often a consequence of an inappropriate effort to shorten the maturation time. Short contact with biomass during the maturation stage disables the sufficient decay and re-assimilation process of diacetyl in beer. Because of this, both increasing the concentration of immobilized cells and prolonging the residence time leads to lower diacetyl concentration in the final beer[10,87]. Another strategy developed in order to speed up the maturation of green beer in continuous beer fermentation systems is the heat accelerated conversion (10 min at 90°C) of all α-acetolactate to diacetyl between primary and secondary fermentation. The diacetyl is then quickly reduced to acetoin during continuous maturation[3,31,66,123].

Higher alcohols

Several higher or fusel alcohols, other than ethanol are formed in beer during fermentation, among which n-propanol, iso-butanol and isoamyl alcohols (2-methyl and 3-methyl butanol) contribute most significantly to beer by alcoholic or solvent-like aroma and a warm mouthfeel. Higher alcohols achieve maximum concentrations during batch fermentation at a time roughly coincident with cell growth arrest and minimum FAN concentration. Their formation takes place by the so-called anabolic and catabolic route. In the anabolic route the 2-oxo acids, arising from carbohydrate metabolism, are decarboxylated to form aldehydes, which are reduced to the corresponding alcohols. Simultaneously, 2-oxo acids also derive from amino acid utilization, which is termed the catabolic (Ehrlich) route to higher alcohol formation[13,64] (Fig. 3). The final concentration of higher alcohols is therefore determined by the uptake efficiency of the corresponding amino acid and the sugar utilization rate. The contribution of each biosynthetic pathway is influenced by wort amino acid composition, fermentation stage and yeast strain[21,47,79]. In addition, some higher alcohols may originate from the reduction of aldehydes and ketones that are present in the wort.

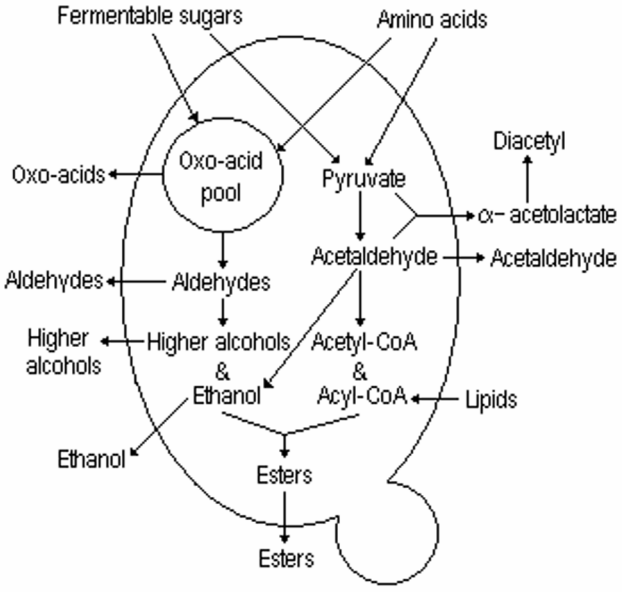

Fig. 3. Simplified metabolic scheme of the formation of the main groups of flavour-active compounds in brewing yeast during beer fermentation.

As it can be seen in Table II, the gap between higher alcohol formation during continuous and traditional beer fermentations does not represent the most serious flavour issue. Yet, there is a trend toward increased propanol yield accompanied by lower i-butanol and isoamyl alcohol formation noticeable in some of the continuous systems (Fig. 2). Fortunately, the high taste threshold of higher alcohols makes these differences less significant.

When comparing beers from free and immobilized cell systems, it seems that the uneven higher alcohol formation can be ascribed to different yeast growth rates, levels of amino acid utilization and mass transfer limitations. Indeed, for cells immobilized by entrapment (e.g. alginate, carrageenan, calcium pectate) the diminution of higher alcohol formation seems to be proportional to the reduction of FAN utilization[18,78,88]. In the case of cells immo-

bilized by attachment (e.g. DEAE-cellulose, stainless steel cloth), by contrast, the similar or slightly increased FAN uptake, led to unchanged or enhanced formation of higher alcohols, respectively[45,82]. These results suggest that fermentations using entrapped lager yeast are associated with FAN uptake limitations and reduced formation of volatiles. Contradicting results were found in an immobilized cell system (aspen chips) with both low cell growth and FAN consumption but with high propanol formation[90]. According to the authors, the high propanol formation is linked with 2,3-pentanedione over-production through α-ketobutyrate, a mutual intermediate of both compounds.

Control of higher alcohol formation in continuous systems can be well balanced by the choice of an appropriate yeast strain[50,77], wort composition (amino acids, lipids, zinc), fermentation conditions (temperature, aeration, residence time), immobilization method and reactor design[14,27,62,121]. The majority of these interventions are based on the stimulation of the growth intensity, e.g. dissolved oxygen concentration and temperature, enhancing the higher alcohol formation[8,49,88]. A positive effect on higher alcohol production has been provoked also by the immobilization-induced acceleration of cell metabolism[82,83]. On the contrary, excess higher alcohol formation can be lowered by growth and/or FAN uptake deceleration e.g. by application of inhibiting dissolved CO_2 concentrations[49,76,83]. Proper higher alcohol synthesis in continuous systems is also crucial for sufficient ester formation.

Esters

The synthesis of aroma-active esters by yeast is of great importance because they represent a large group of flavour active compounds, which confer to beer a fruity-flowery aroma. The esters of beer can be divided into two main groups. The first group comprises acetate esters such as ethyl acetate (fruity, solvent-like), isoamyl acetate (banana) and phenylethyl acetate (roses, honey, apple). The second group of esters are the so-called ethyl or medium chain fatty acid esters e.g. ethyl caproate and ethyl caprylate (both apple-like). Flavour-active esters are the products of yeast acyltransferase activities catalysing the condensation reaction between either acetyl/acyl-CoA and higher alcohols or ethanol (Fig. 3). Several different enzymes are involved in the formation of esters, most of them being alcohol acyltransferases (AATase); however, esterases may also influence the final level of esters in beer. Although different genes encoding ester synthases have been characterised (ATF1, Lg-ATF1, ATF2), the existence of other genes in *Saccharomyces cerevisiae* encoding enzymes catalysing ester synthesis is assumed[25,53,105]. Despite the substantial knowledge on the formation of esters in yeast, the physiological role of these compounds is rather unclear. The ester synthesis in yeast has been hypothesised to serve as a regeneration of free CoA, detoxification of medium chain fatty acids or formation of analogues of unsaturated fatty acids[106].

Fundamentally, two factors are important for the rate of ester formation: the availability of the two substrates (acetyl/acyl-CoA and alcohols), and the activity of enzymes (AATases). For example, higher wort aeration affects esters synthesis through reduced availability of acetyl-CoA (used for growth and lipid synthesis) and inhibition of AATases[26]. Although there is an overlap of the effects of different factors, the central role in beer ester formation seems to be the AATase activity and the regulation of its gene transcription[52,124]. In practice, to control the ester formation is rather difficult due to the many factors involved in the regulation of activity and the gene expression of AATases and regulation of substrate availability[103].

The use of immobilized yeast reactors for beer production gives variable amounts of esters, depending on the type of the system and operating conditions (Table II). In a few cases, ester synthesis in the continuous system exceeded its traditional counterpart[1,45]. However, the overall tendency in most of the continuous systems indicates a somewhat reduced ester formation, in particular for isoamyl acetate (Fig. 2).

Perhaps the most important factor affecting ester formation in continuous systems is aeration[95,111]. Although ethanol production is an anaerobic process, some oxygen is essential for yeast growth and for unsaturated fatty acid and sterol synthesis[54]. In order to supply optimum oxygen into bioreactors, the knowledge of volumetric oxygen mass transfer coefficients (k_La) under real fermentation conditions is indispensable. By determining the k_La it would be possible to avoid the often observed under- or over-aeration of the continuous beer fermentation systems resulting in excessive or poor ester formation, respectively. Moreover, data on specific ester productivities vs. oxygen supply could be transferable between different systems or could serve for the tentative estimation of aeration demands during scale-up and thus avoid future flavour problems.

Apart from aeration, there are other factors affecting ester formation in continuous beer fermentation and important among these is yeast strain and cell physiology. The selection of yeast strain is an important tool in beer ester control, since both the average ester production and the relative proportion of each individual ester is rather strain specific[20]. Thus, selecting a proper production strain, suitable for the particularities of a certain continuous fermentation system, should be an integral part of process development.

Process design strategy should also not neglect the applied immobilization method. Different ester formation rates have been observed in reactors with free, entrapped and adsorbed biocatalysts. Reduced nutrient diffusion to cells entrapped in matrices caused different metabolic behaviour while free and adsorbed cells (DEAE-cellulose) showed significant similarities[88]. By contrast, yeast adsorbed on pre-formed carriers (stainless steel fibre cloth, DEAE-cellulose) produced more esters thanks to either acyl-CoA spared in reduced fatty acid synthesis[81,82] and/or induced AATase synthesis at low concentrations of these fatty acids[99]. Hence, ester formation can be controlled by an appropriately chosen immobilization method, but its possible impact has to be verified for each individual case[113]. Additionally, deterioration of cell physiology during immobilized cell aging and genetic drift[80] during continuous fermentation may also influence ester formation.

Other technological parameters, known to affect ester

production in the traditional batch process also have to be taken into consideration in continuous beer fermentation systems[20,44,103].

They include the following:

- Wort composition, i.e, specific gravity, lipid, zinc and FAN content
- Process conditions i.e., temperature, pH, agitation, reactor design (hydrostatic pressure, dissolved CO_2 concentration, stripping of volatile esters by driving gas in pneumatically agitated reactors) and pitching rate (biomass concentration).

Generally, ester formation is a sensitive process, which is rather difficult to control due to numerous influencing factors involved. The use of mutants and genetically modified yeast with altered ester production profiles might be a promising instrument to control flavour in continuous brewing[94,102].

Organic acids

A large portion of the total organic acids (ca. 50%) in beer is derived from the wort, while the rest is produced or transformed as a result of yeast metabolism[122]. There are organic acids with a short carbon skeleton in beer derived both from the incomplete turnover of the TCA cycle, that occurs during anaerobic growth of yeast, and from the catabolism of amino acids[115]. These organic acids (pyruvate, acetate, lactate, citrate, succinate, malate, oxo-acids) contribute to the reduction in pH during fermentation and confer a "sour" taste to beers[58]. The medium chain fatty acids (C_6 – C_{12}), which are toxic to yeast cells (cell membrane disruption), result either from long-chain fatty acid anabolism under anaerobic conditions and/or are released by the mechanism of cell autolysis. The long chain fatty acids of beer originate mostly from wort and are considered undesirable with respect to taste and foam stability[7,104].

There are only a few studies that deal with organic acid formation during continuous beer production. They report little difference in the total organic acid concentration and pH of traditionally and continuously fermented beers[88,122]. Nevertheless, the contribution of individual organic acids was somewhat different and varied in accordance with the applied fermentation system and process parameters. The control strategy of organic acid composition in continuous multistage beer fermentation is based on the regulation of cell growth and extract consumption rate in individual phases of the system. Since the formation of organic acids is proportionate to fermentation intensity, its control can be achieved by equilibrating the intensity and duration of the respiro-fermentative and fermentative process stages[7,122].

Carbonyl compounds

Beers made from the same wort and yeast strain in continuous beer fermentations showed slightly increased acetaldehyde content compared to conventional beers (Fig. 2). This supports the assumption, expressed already in connection with higher alcohols and esters, that the majority of continuous systems show excess cell growth and/or over-aeration. Since acetaldehyde is mostly formed during the active growth phase of yeast, its content can be controlled by proper oxygen supply[10]. As well, it can be lowered by prolonged maturation[46].

Several carbonyl compounds present in wort have high flavour potency (3-methyl butanal, 2-methyl butanal, hexanal, heptanal etc.). They contribute largely to the worty off-flavour detected particularly in low-alcohol beer produced by limited fermentation. Beer aldehydes arise mainly during wort production (mashing, boiling) and are partially formed during fermentation from yeast oxo-acid pools both via the anabolic process from carbon source and the catabolic pathway from exogenous amino acids[69] (Fig. 3). Several different yeast enzymatic systems, both NADH and NADPH-dependent, are involved in aldehyde transformation during fermentation: alcohol dehydrogenase isoenzymes[29], branched chain alcohol dehydrogenase[98], aldehyde dehydrogenase[101] and aldo-keto reductases[24]. The activity of these enzymatic systems is strain and condition dependent and their physiological role is still somewhat unclear.

The reduction of wort aldehydes is crucial in alcohol free beer production. Although wort aldehydes are reduced relatively swiftly during batch fermentations, the limited fermentation in continuous systems provoked concerns of process engineers. In fact, a good compromise was found between alcohol formation and carbonyl reduction by optimizing the residence time and temperature of the continuous alcohol-free beer production process. Higher rates of aldehyde reduction were observed at higher temperatures, but residence time also appeared to be an important factor in determining residual aldehyde levels. Indeed, the lower metabolic activity at lower temperatures had to be compensated by a longer residence time in order to achieve the same aldehyde reduction[16,97].

Regarding the influence of immobilization on different enzymatic mechanisms involved in carbonyl reduction, it was found that either they were not affected[17], or the reducing capacity of yeast was improved[100]. The increased alcohol dehydrogenase activity in immobilized yeast was found to be correlated with an immobilization-induced (DEAE-cellulose) higher glucose flux in cells. Since the enzymatic reduction of aldehydes by brewing yeast is coupled to the oxidation of cofactors NADH and NADPH, the higher aldehyde reduction capacity was attributed to efficient cofactor regeneration during faster glycolysis and the pentose phosphate pathway[100].

CONCLUSIONS

The objective of this review was to summarize the knowledge available on formation of selected flavour active compounds in continuous fermentation systems and to review their possible control strategies. The selection of compared flavour compounds was carried out based on the availability of the experimental data. However, it is obvious that it would be an over-simplification to characterize the taste of a beer only by the analytical determination of some components. We are aware that in practice the flavour of one compound may be suppressed or accentuated by another and that the final taste profile results from interplay of the different taste features. When evaluating beer flavour one has to bear in mind that it is also influenced by beer type and circumstances depending on country and fashion.

Technological parameters that affect flavour formation are numerous. Comparisons of different methods of beer flavour control, by means of process parameters and literature resources, rarely provide mass transfer data transferable between different reactor designs. The observed results are therefore unique and valid only for the given configuration. Reliable mass transfer data in different immobilized cell systems, could not only eliminate the often reported excess oxygen supply and/or substrate limitation, but could also contribute to a better understanding of the impact of immobilization and aging on beer flavour.

The selection of brewing strains especially suited to continuous brewing has been underestimated. Yeast strains performing well in traditional batch fermentations were automatically applied in continuous reactors, regardless of a possible mismatch between requirements of the continuous process arrangement, immobilization, aging, flavour production etc. on one side and genetic potential of the yeast cell on the other. The production strain for a continuous beer fermentation system should be carefully selected from a pool of strains in view of the particular circumstances of a certain plant design and taste features of the intended final product. Another promising method of flavour control of beer involves the application of non-recombinant mutants and/or genetically manipulated recombinant brewing yeast strains. Particularly the potential of metabolic engineering using genetic tools is enormous, making possible the future construction of production strains tailor-made for the conditions of continuous beer fermentation.

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Note added in proof

Two additional papers just published that may be of interest are as follows:

Dragone, G., Mussatto, S.I. and Almeida e Silva, J.B., High gravity brewing by continuous process using immobilised yeast: effect of wort original gravity on fermentation performance. *J. Inst. Brew.*, 2007, **113(4),** 391-398.

Silva D.P., Brányik T., Dragone G., Vicente A.A., Teixeira J.A. and Almeida E Silva, J. B., High gravity batch and continuous processes for beer production: evaluation of fermentation performance and beer quality, *Chem. Pap.,* 2008, **62(1),** 34-41.