Flavour formation in continuous fermentations

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Introduction

The attractive prospect of continuous beer fermentation system consists in the continuous and rapid transformation of wort into beer in a multistage reactor system. Although continuous beer fermentation has been considered as a promising technology since the 1960s, by contrast, it has found only a limited number of industrial applications.

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 (Hyttinen et al., 1995; Van Dieren, 1995; Pajunen, 1996). 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. 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, payroll costs for skilled supervision). It is clear, that some of the problems were met in one system, but not in other. The same applies to troubleshooting, some solutions suggests themselves (e.g. clogging in packed-bed reactor $\rightarrow$ reactors with forced circulation, carrier cost $\rightarrow$ wood chips, spent grains, corncobs etc.), while others require comprehensive understanding of the issue (yeast physiology). In general, the complexity of continuous main fermentation and its obstacles often resulted in an unbalanced flavour of the final product (Linko et al., 1998).

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. Evidences 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 (Junter et al., 2002). These differences have to be taken into account when considering the continuous beer fermentation.

Continuous mode of reactor operation

During traditional batch beer fermentation the brewing yeast adapt their relatively versatile metabolism to changing external environment. Different metabolic fluxes belong to distinct phases recognizable on the growth curve. 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 (Jones and Pierce, 1964), 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 significant alterations in reaction environment (Masschelein and Andries, 1995). 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 (Pajunen et al., 1989) or in a series of agitated reactors (Bishop, 1970; Inoue, 1995). Usually, 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 (Yamauchi et al., 1995a; Brányik et al., 2006). A flavour profile similar to conventional beers can be achieved by inducing different growth conditions in a series of two or more 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 (Van De Winkel et al., 1993; Yamauchi et al., 1994a; Virkajärvi and Kronlöf, 1998).

Immobilization induced mass transfer limitations

In a typical three-phase system (gas-liquid-solid) such as continuous immobilized cell beer fermentation, the external mass transfer rate may affect both yeast physiology and beer flavour (Pilkington et al., 1998) through limited substrate supply and/or altered concentration of metabolites in the vicinity of the immobilized biomass.

Considering oxygen supply it has to be stated, that the 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 oxygen mass transfer coefficient (k_La) is essential in order to accomplish a thorough understanding of the aeration impact on flavour formation transferable between different reactor arrangements.

Internal mass transfer limitations of nutrients occur when cells are entrapped e.g. in a polymer matrix. The cells entrapped 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 (Galazzo and Bailey, 1990; Taipa et al., 1993; Šmogrovičová and Dömény, 1999).

The pre-formed porous (sintered glass) and non-porous carriers (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 (Norton and D'Amore, 1994). Cells adhered in a single layer to DEAE-cellulose showed similar metabolic activities (Šmogrovičová and Dömény, 1999) while the multi-layers biofilm yeast attached to spent grains had lower specific substrate consumption rate than free cells in the same system (Brányik et al., 2004a).

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 (Hilge-Rotmann and Rehm, 1991; Shindo et al., 1994; Van Iersel et al., 2000). It has been shown that immobilized cells exhibit increased levels of DNA, structural carbohydrates (Doran and Bailey, 1986), glycogen (Galazzo and Bailey, 1990), fatty acids (Hilge-Rotmann and Rehm, 1991), as well

as modifications of gene expression levels, cell proteome, cell wall and cell membrane composition (Parascandola et al., 1997; Vidgren et al., 2003; Shen et al., 2003a). 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 (Jiang et al., 2002; Hallsworth, 1998) and altered sugar and amino acid uptake (Shen et al., 2003b; Vidgren et al., 2003). 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 (Cashin, 1996); however, the adsorption can be considered the most gentle immobilization method, because it resembles the natural biofilm formation.

Table 1. 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 reduce diacetyl formation etc.)
Microbial & 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 & Technological factors	
• Mass transfer rate	– Oxygen mass transfer coefficient (k_La) data would allow a more sophisticated control of several flavour active compounds
• Reactor design	– Proper reactor type for given growth requirements can facilitate flavour control (heat transfer, clogging, channelling)

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 are capable of a finite number of divisions (10 - 30 divisions) before entering a non-replicative state termed senescence, leading to death and autolysis (Powell et al., 2000). Yeast display an array of changes during their aging including decrease of viability (Barker and Smart, 1996), increase in size, cell surface wrinkling, increase of generation time, increasing bud scar number and decreased metabolic activity (Motizuki and Tsurugi, 1992; Jazwinski, 1990). The study of the aging

process of brewing yeast strains has also a practical significance. Serial fermentation can select for an undesirable sub-population enriched with elderly cells. The aged brewing yeasts show changed flocculation characteristics (Soares and Mota, 1996) and fermentation performance (Powell et al., 2003; Deans et al., 1997). It is believed that the performance of lager strain begins to degenerate after 10 serial repitchings (Jenkins et al., 2001).

Taking into consideration the long periods of time (several months) that immobilized cells are spending in a continuous reactor (Virkajärvi and Kronlöf, 1998; Tata et al., 1999), the question of the immobilized cell aging turns to be even more relevant. The viability and fermentation capacity (vitality) of immobilized brewing yeast in continuous fermentations have already been reported to decrease (Kronlöf et al., 1989; Kronlöf and Linko, 1992; Inoue 1995; Pilkington et al., 1999; Brányik et al., 2006). However, there is little known on the impact of senescence and aging of immobilized yeast in continuous fermentation on product quality. Hence, elucidating the influence of 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 Hayflick limit (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 both at morphological and biochemical level. Most of the spontaneous mutations which occur in brewing yeast are “petite” mutation. During the brewing process, the frequency of genetic drift and mutations is considerable. Petite mutations leading to respiratory deficiency occur spontaneously with the frequency of 0.5 to 5% among yeast harvested from fermenters, but figures as high as 50% have been reported for stored yeast (Morrison and Suggett, 1983). Prolonged storage, starvation (Heidenreich and Wintersberger, 1998) and ethanol stress (Ibeas and Jimenez, 1997; Jimenez et al., 1988) are known to increase the occurrence of petite mutations. Physiologically, petites exhibit an altered cell membrane and cell wall morphology, while respiratory-deficient yeast can lead to reduced fermentation rate and ethanol production, decreased cell viability, inappropriate flocculation behaviour and flavour defects (Morrison and Suggett, 1983; Ernandes et al., 1993).

It is expected, that the 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 cell after 9 months of continuous cultivation with deleterious effect on beer flavour (Thorne, 1968). Conversely, no mutation of the yeast was observed in other industrial scale continuous beer fermenters operating over several months (Ault et al., 1969; Bishop 1970). 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 vast majority of mutations does not provide any advantage.

Comparing to continuous free cell systems the situation in immobilized cell reactors is complicated by the effect of immobilization method. In reactors with carrier to cell surface interaction (adsorption, adhesion, attachment) as a prevailing immobilization mechanism, the genetic drift of the biocatalyst can be caused by selection pressure based on surface interactions (Brányik et al., 2004c). However, there is little known on the risk of selecting a sub-population, 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 present in the system already at start-up.

Flavour control in continuous beer fermentation

The flavour profile of a beer is an important measure in evaluating the feasibility of a continuous beer fermentation 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 2. 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. The comparison is depicted on selected compounds; however, it is needless to say that beer flavour is not merely a question of analytical determination (Table 2). The mutual ratios of flavour active compounds in continuously fermented beers and in their batch counterparts are depicted on Fig. 1. As it can be seen, in most of the cases the multiples of compound concentrations do not match exactly, the most scattered being the values of the total diacetyl and esters (Fig. 1).

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 and taste in concentrations above the flavour threshold (0.15 ppm). 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 identically from α-acetohydroxybutyrate. This chemical conversion is considered the rate limiting step of VDK formation. In late 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 (Bamforth and Kanauchi, 2004; Bergen et al., 2005). Immediate regulation of valine and thus diacetyl precursor biosynthesis occurs at the level of enzyme activity. When 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 (Petersen et al., 2004).

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. 1). 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 synthetase responsible for the formation of -acetolactate from pyruvic acid (Shindo et al., 1994).
- An alteration of the amino acid metabolism of the immobilized cells. It is manifested by an unbalanced feed back inhibition of the isoleucine-valine biosynthetic pathway. Nevertheless, the data on amino acid uptake by immobilized cells are often contradictory and influenced by the applied immobilization technique. Lower amino acid uptake by entrapped yeast has been frequently reported (Ryder and Masschelein, 1985; Inoue, 1995) while immobilization by attachment showed uptake rates similar to free cells (Šmogrovičová and Dömény, 1999; Shen et al., 2003b; Kronlöf et al., 1989).
- 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. Despite of this, the results are difficult to transfer between different systems due to the lack of comprehensive engineering data on oxygen transfer rates (k_La) in various types of bioreactors applied for continuous beer fermentation so far.

Table 2. Concentration of selected flavour active compounds in continuously fermented beer vs. conventionally fermented beer (Continuous/Batch).

	Selected flavour active compounds in beer (ppm)						
[Ref.]	Total diacetyl	Acetaldehyde	Ethyl acetate	Isoamyl acetate	n-Propanol	i-Butanol	Isoamyl alcohols
[1][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
[2][a]	0.7/0.4		33.3/16.8	2.8/1.3	11.6/10.4	11/9.7	51.2/50.9
[3][a]	0.48/0.3		17.9/17.2	0.9/1.2	15/17.5	10.1/12.5	60.1/70
[4][a]			11/19	0.06/2	8/9.8	7.5/16	31/62
[5][a]	1.18/0.3		14.1/14	1.21/1.2	11.3/10		72/59.5
[6][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
[7][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
[8][b]	0.02/0.04	8/9	18/15	1.6/1.8	13.8/11.3	6/11.3	42.3/58
[9][b]		7.5/4.3	23/23.4	0.7/2.0	14.5/9.7	9.4/10.2	49.5/52
[10][b]			31/18	1.3/1.1	12/18	13/14	54/67
[11][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
[12][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
[13][b]			18/18	1.9/2	11.8/12	7.5/11	42/58
	Flavour thresholds (ppm)						
[14]	0.07-0.15	10	33	1.6	800	200	70[c]; 65[d]

a- Continuous main fermentation with immobilized cell system / Conventional main fermentation
b- Continuous complete fermentation with immobilized cell system / Conventional complete fermentation
c- 2-Methyl butanol, d- 3-Methyl butanol
[1] – Mensour et al., 1996 ; [2] – Kronlöf et al., 1989; [3] – Brányik et al., 2004a; [4] – Ryder and Masschelein, 1985; [5] – Okabe et al., 1994; [6] – Tapani et al., 2003; [7] – Brányik et al., 2006; [8] – Yamauchi et al., 1994b; [9] – Virkajärvi and Kronlöf, 1998 ; [10] – Andries et al., 1995; [11] – Grönqvist et al., 1989; [12] – Dömény et al., 1998; [13] – Yamauchi et al., 1995a; [14] – Meilgaard, 1975

Several strategies for repressing the excessive VDK formation during continuous main fermentation are possible. Addition of bacterial α-acetolactate decarboxylase, to the fermenting beer converting α-acetolactate directly to acetoin (Godtfredsen et al., 1984) and/or the use of genetically modified brewer's yeast encoding α-acetolactate decarboxylase (Kronlöf and Linko, 1992; Yamano et al., 1995) would lead to lower final diacetyl level in continuously fermented beer. However, the remaining legal obstacles to enzyme supplementation and the lack of consumers' 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 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 cells growth, significant diacetyl formation occurs and its control through growth regulation was in some cases found to be ineffective (Van De Winkel et al., 1993; Yamauchi et al., 1995b). Besides process parameters, the wort free amino nitrogen (FAN) concentration was shown to affect diacetyl production as well. Worts containing both too much and too little FAN gave increased diacetyl contents (Pugh et al., 1997; Petersen et al., 2004). 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 -acetolactate is also strain dependent (Kunze, 1996; Linko et al., 1997), which in turn allows certain diacetyl control by strain selection.

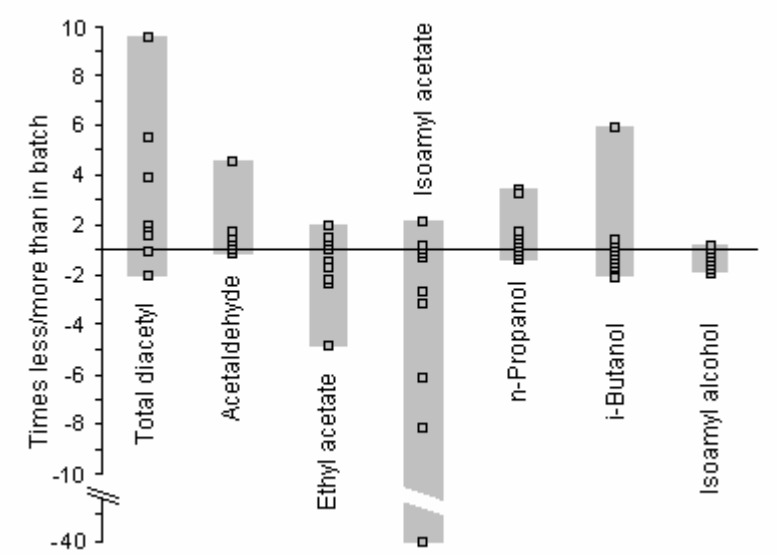

Fig. 1 Multiples of compound concentrations and their distribution in continuously fermented beers comparing to corresponding conventional beers (positive values = times more than in batch, negative values = times less than in batch). Markers represent the individual values.

The diacetyl content above the taste threshold in continuously fermented final beer is most often a consequence of an inappropriate effort to shorten the maturation time. Short contact with biomass during the maturation stage is disabling the sufficient decay and re-assimilation 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 final beer (Šmogrovičová et al., 1998; Brányik et al., 2006). 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 -acetolactate to diacetyl between primary and secondary fermentation. The diacetyl is then quickly reduced to acetoin during e.g. continuous maturation (Grönqvist et al., 1989; Pajunen, 1995; Yamauchi et al., 1995c).

Higher alcohols

Several alcohols, other than ethanol are formed in beer during fermentation, among which n-propanol, iso-butanol and isoamyl alcohol (2-methyl and 3-methyl butanol) contribute most significantly to beer flavour. 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 so-called anabolic and catabolic route. In anabolic route the 2-oxo acids, arisen from carbohydrate metabolism, are decarboxylated to form aldehydes, which are reduced to the corresponding alcohols (Oshita et al., 1995). Simultaneously, 2-oxo acids derive also from amino acids utilization, which is termed the catabolic (Ehrlich) route to higher alcohol formation. The final concentration of

higher alcohols is therefore determined by the uptake efficiency of the corresponding amino acids and sugar utilization rate. The contribution of each biosynthetic pathway is influenced by wort composition, fermentation stage and yeast strain (Kruger et al., 1992). Besides, some higher alcohols may originate from the reduction of aldehydes and ketones present in wort.

As it can be seen (Table 2), 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 of increased propanol yield accompanied by lower i-butanol and isoamyl alcohol formation noticeable in some of the continuous systems (Fig. 1). Fortunately, the high taste threshold of higher alcohols makes these differences less significant.

Approximately at the same degree of attenuation in batch and immobilized systems, the differences in higher alcohol formation can be most probably ascribed to different levels of amino acid utilization and yeast growth. Indeed, in immobilized systems (DEAE-cellulose, stainless steel cloth) with enhanced and similar FAN uptake, the formation of higher alcohols was higher and equal to batch systems, respectively (Kronlöf et al., 1989; Shen et al., 2003b). In other continuous immobilized cell systems (alginate, carrageenan, calcium pectate), by contrast, the diminution of higher alcohol formation seems to be proportionally dependent on the reduction of FAN utilization (Ryder and Masschelein, 1985; Dömény et al., 1998; Šmogrovičová and Dömény, 1999). 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. 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 (Tapani et al., 2003).

Control of higher alcohol formation in continuous systems can be well balanced by the choice of an appropriate yeast strain (Romano et al., 1992; Linko et al., 1997), wort composition, fermentation conditions, immobilization method and reactor design (Norton and D'Amore, 1994; Yamauchi et al., 1995a). The majority of these interventions are based on the stimulation of growth intensity, e.g. dissolved oxygen concentration and temperature, enhancing the higher alcohol formation (Šmogrovičová and Dömény, 1999; Landaud et al., 2001; Brányik et al., 2004a). A positive effect on higher alcohol production has been provoked also by the immobilization-induced acceleration of cell metabolism (Shen et al., 2003b; Shen et al., 2004). 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 (Renger et al., 1992; Landaud et al., 2001; Shen et al., 2004). Proper higher alcohol synthesis in continuous systems is crucial also for sufficient ester formation.

Esters

The synthesis of aroma-active esters by yeast is of great importance because they represent the largest group of flavour active compounds in beer (ethyl acetate, isoamyl acetate, ethyl caproate, ethyl caprylate, phenylethyl acetate etc.). Volatile esters are the product of yeast acyltransferase activities catalysing the condensation reaction between acetyl/acyl-CoA and higher alcohols. Several different enzymes are involved in the formation of esters, most of them being alcohol acyltransferases (e.g. AATase I and II); however, esterases may also influence the final level of esters in beer (Mason and Dufour, 2000).

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 (Fuji et al., 1997). Although there is an overlap of the effects of different factors, the central role in beer ester formation seems to have the AATase activity and the regulation of its gene transcription

(Yoshioka and Hasimoto, 1984; Malcorps et al., 1991). In practice, to control the ester formation is rather difficult due to many factors involved in the regulation of activity and gene expression of AATases and regulation of substrate availability (Verstrepen et al., 2003).

The use of immobilized yeast reactors for beer production gives variable amount of esters, depending on the type of the system and operating conditions (Table 2). In few cases, the ester synthesis in continuous system exceeded its traditional counterpart (Kronlöf et al., 1989; Andries et al., 1995). However, the overall tendency in most of the continuous systems indicates a somewhat reduced ester formation, in particular for isoamyl acetate (Fig. 1).

Perhaps the most important factor affecting ester formation in continuous systems is aeration (Van De Winkel et al., 1993; Virkajärvi et al., 1999). Although ethanol production is an anaerobic process, some oxygen is essential for yeast growth, unsaturated fatty acid and sterol synthesis (Masschelein, 1997). In order to supply the optimum oxygen into bioreactors, the knowledge of 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 in continuous beer fermentation systems resulting in excessive or poor ester formation, respectively. Moreover, data on specific ester productivities vs. oxygen supply (Fig. 2) could be transferable between different systems or could at least serve for tentative estimation of aeration demands to avoid flavour problems.

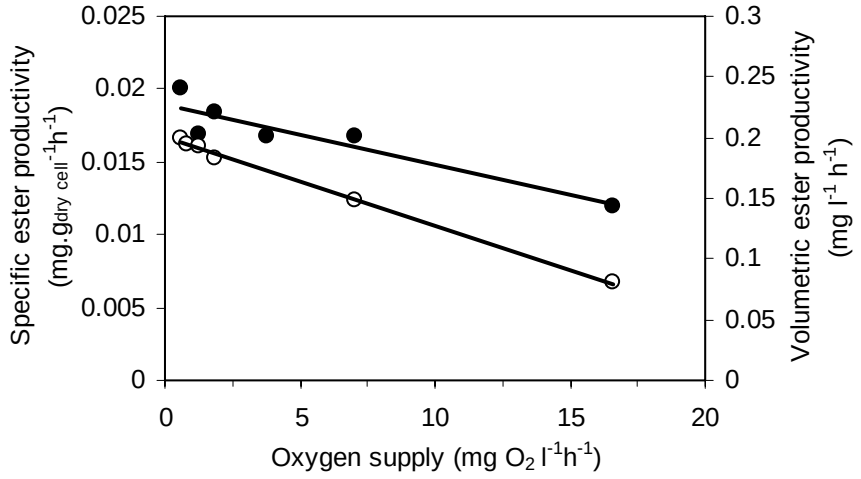

Fig. 2 The influence of oxygen supply on specific (closed symbol) and volumetric (open symbol) total ester productivity in a continuous immobilized cell reactor fed by complex medium (unpublished data).

Apart from aeration, there are other factors affecting ester formation in continuous beer fermentation. Among them, the yeast strain and cell physiology should be stressed out. 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 (Dufour at el., 2003). Thus, selecting a proper production strain suitable for the particularities of a certain continuous fermentation system should be an integral part of a process development.

Process design strategy also should 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 (Šmogrovičová and Dömény, 1999). 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 (Shen et al., 2003) and/or induced AAT synthesis at low concentrations of these fatty acids (Van Iersel et al., 1999). Hence, ester

formation can be controlled by an appropriately chosen immobilization method but its possible impact has to be verified for each individual case (Virkajärvi and Pohjala, 2000). Additionally, deterioration of cell physiology during immobilized cell aging and genetic drift (Sato et al., 2001) during continuous fermentation may also influence ester formation.

Other technological parameters, known to affect ester production in traditional batch process, have to be also taken into consideration in continuous beer fermentation systems (Dufour at el., 2003; Verstrepen et al., 2003):

- wort composition: specific gravity, lipid, zinc and FAN content
- process conditions: temperature, pH, agitation, reactor design (hydrostatic pressure, dissolved CO_2 concentration, stripping by driving gas in pneumatically agitated reactors).

Generally, the 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 (Verstrepen et al., 2001; Vanderhaegen et al., 2003).

Organic acids

A large fraction of total organic acids (ca. 50 %) in beer is derived from wort, while the rest is produced as a result of yeast metabolism. Organic acid formation and excretion contributes to the reduction in pH during fermentation and confers a "sour" taste to beers. The major organic acids secreted by yeast (pyruvate, acetate, lactate, citrate, succinate, malate, α-keto acids) are mainly derived from the incomplete turnover of TCA cycle that occurs during anaerobic growth of yeast and some may originate from the catabolism of amino acids.

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 very similar pH of the traditional and bioreactor beers (Yamauchi et al., 1995b; Šmogrovičová and Dömény, 1999). 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. It can be achieved by equilibrating the intensity and duration of respiro-fermentative and fermentative process stages (Yamauchi et al., 1995b).

Aldehydes

Beers made from the same wort and yeast strain in continuous beer fermentations show a slightly increased acetaldehyde content comparing to conventional beers (Fig. 1). This is supporting 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. Thus, acetaldehyde content has to be controlled by proper oxygen supply (Brányik et al., 2006) as well as it can be lowered by prolonged maturation (Kronlöf and Linko, 1992).

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 partially are formed during fermentation from yeast oxoacid pools both via anabolic process from carbon source and catabolic pathway from exogenous amino acids. Several different yeast enzymatic systems, both NADH and NADPH-dependent, are involved in aldehyde transformation during fermentation: alcohol dehydrogenase isoenzymes (Ganzhorn et al, 1987), branched chain alcohol dehydrogenase (Van Iersel et al., 1997), aldehyde dehydrogenase (Van Nedervelde et al., 1997) and aldo-keto reductases (Ford and Ellis, 2002). The activity of these enzymatic systems is strain and condition dependent and their physiological role is somewhat unclear.

The reduction of wort aldehydes is crucial namely in alcohol free beer production. Although wort aldehydes are during batch fermentations reduced relatively swiftly, the limited fermentation in continuous systems provoked concerns of process engineers. In fact, the fermentation at very short residence time and low temperatures, required for continuous alcohol-free beer production, turned out to be a good compromise between alcohol formation and carbonyl reduction. Higher rates of reduction were observed at higher temperatures, but residence time appeared to be a major factor in determining residual aldehyde levels. Indeed, the lower metabolic activity at lower temperatures was compensated by the longer residence time under the same fermentation conditions (Debourg et al., 1994; Van Iersel et al., 1995).

Regarding the influence of immobilization on different enzymatic mechanisms involved in carbonyl reduction, it was found that either they were not affected (Debourg et al., 1995) or the reducing capacity of yeast was improved (Van Iersel et al., 2000). The increased alcohol dehydrogenase activity in immobilized yeast was found to be correlated with 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 can be attributed to the efficient cofactor regeneration during faster glycolysis and pentose phosphate pathway (Van Iersel et al., 2000).

Conclusions

The objective of this review was to summarize the knowledge 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 upon availability of 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 the final taste profile results from interplay of 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. Comparing different methods of beer flavour control by means of process parameters, the 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 also could contribute to better understanding of the impact of immobilization and aging on beer flavour.

The selection of brewing strain especially convenient for continuous brewing has been underestimated. Yeast strains performing well in traditional batch fermentations were automatically applied in continuous reactors regardless of possible mismatch between requirements of the continuous process arrangement, immobilization, aging, flavour production etc. on one side and genetic potential of the microbe 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, which are tailor-made for the conditions of continuous beer fermentation.

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