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Alternative, Biological Methods in the Flow Profile: Characterization of Bioreactors

In order to determine the flow profile in continuously and batch operated bioreactors, alternative methods were evaluated, using microorganisms (yeasts) as tracer material whose fermentation kinetics were similar to those of the starter cultures. The Modified Raffinose Test thereby proved to be an efficient method when dealing with larger quantitative differences of bottom- and top fermenting yeasts, whereas the IGS2 314 PCR-Capillary Gel Electrophoresis Test delivered trustful results when utilizing mixtures of two strains of top-top, or alternatively two strains of bottom-bottom fermenting yeasts. Both methods are therefore suitable in order to evaluate the flow profile via a step function test, in which the fermentation strain is substituted by a different (tracer) strain. The presence of tracer yeast in the starter yeast culture could also be clearly detected via RealTime PCR analyses. Yet this test was found to be too sensitive in order to detect larger increments (20 % [w/w]). Hence, this test is rather suitable for the judgment of flowing patterns via the impulse function test, where a small amount of tracer material is being injected into the fermenter, before its presence is being detected subsequently.

Descriptors: flow profile, residence time, bioreactor, biotracer, yeast differentiation, continuous fermentation, *Saccharomyces pastorianus ssp. carlsbergensis*, bottom-fermenting yeast, TUM 34/70, TUM 128, TUM 224, *Saccharomyces cerevisiae*, top-fermenting yeast, TUM 68, TUM 184, TUM 210, Real-Time PCR, IGS2-314 PCR, capillary-electrophoresis

1 Introduction

The flow profile evaluation in bioreactors is a current research topic of interest [58]. The quality and homogeneity of the product highly depends on the flow present in the system. A quantitative method to char-acterize the reactor's current dynamics is therefore desired [37].

At present knowledge about the flow pattern in beer fermentation tanks is mainly based on experience. The complex physical- and biochemical interactions of processes, as well as the lack of appropriate measurement methods, have led to little experimental analysis so far [48].

Schuch was able to qualitatively describe the flowing pattern in beer fermentation tanks with the light-section-process [68], whereas *Meironke* obtained quantitative results with the Ultra Sonic Doppler Method [48]. However, the complexity of those methods and the necessity of sophisticated equipment made their usage in routine control applications economically unfeasible.

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Therefore special tracer substances are commonly used in the industry, in order to determine the fluid dynamics roughly, via the analysis of local residence times. So far especially salts, coloring-, and radioactive substances are being employed – in water – for residence time determinations, since their presence would influence the regular fermentation process – in substrate – and hence its flowing pattern.

Due to this fact, this widely used method can never reflect realistic situations during the fermentation processes, where flows are influenced significantly by thermo dynamical-, physical-, and biochemical reactions and interactions.

Furthermore the usage of potential harmful tracer materials, which may not be completely removable out of the plant, hinder their application in the food and beverage industry.

This paper therefore proposes and evaluates the use of biological tracers, more specifically, yeasts for regular yeast (beer) fermentations as alternative. The substitution or addition of the main fermentation organism, by one with similar or identical wort decomposing properties as tracer, shall thereby enable the monitoring without influencing the flow patterns significantly.

The first step in this research was therefore the comparison of fermentation characteristics of different yeast strains under standard conditions. Afterwards, different alternative methods were developed and tested, in order to differentiate the organisms quantitatively. Subsequently one suitable system was chosen in order to monitor the residence time in a continuously operated reactor at the Research Center Weihenstephan.

2 Background of flow influences and calculations

Depending on the particle size and agglomeration rate, yeast sediments at different speeds. Single yeast cells are round or oval shaped and measure approximately d ≈ 5–7.5 µm in diameter, resulting in low Reynolds numbers during sedimentation (Re << 0.1) [42]. In this case the sedimentation velocity can be calculated according to Stokes. Especially top fermenting yeast cells tend to form cell clusters, normally consisting of 8–10 cells which may lead to different flow characteristics. Therefore, methods in which mixtures of both: top-bottom and top-top/bottom-bottom fermenting yeasts could be differentiated were desired in this research.

During the beer fermentation process yeast metabolizes wort into ethanol, carbon dioxide and biomass.

(1) $$OE = \frac{(A*2{,}0665 + E_r)*100}{A*1{,}0665 + 100}$$ [8, 36]

OE is (Weight of extract in cold wort)/(Total weight of cold wort) [WW %]

A is the Alcohol per weight [WW %]

Er is real extract [°Plato]

The reaction is exothermic and the released energy is partly used by the yeast to reproduce itself [3]. Natural, turbulent convection occurs during the beer fermentation process, due to temperature gradients caused by the cooling jacket and the exothermic fermentation reaction, as well as the production of CO_2 [48]**.** The low temperature around the fermentation tank's wall and the heat transfers from the inside out causes torus horns. The variation in temperature around the jacket creates torus rings constantly changing in size and position. A vertical stream steadily flows from the surface to the bottom of the cone near the walls surface. Similarly, a vertical stream flows in the opposite direction through the middle of the tank [24]. Different temperature- and concentration gradients, as well as agglomeration effects during the fermentation process, constantly affect fluid density and fluid viscosity. The sedimentation velocity changes and the flow profile is varied throughout the course of the process. Energy variation in the bioreactor occurs through heat conduction/convection, radiation, mass transfer and work done on the system.

(2) $$\frac{dE}{dt} = Q_{in} - Q_{out} + H_{in} - H_{out} + \sum W_{others}$$

Q is the heat transfer

H is energy transfer through mass processes

W is other energy terms (e.g.: friction)

Liquid heat transfer coefficients are significantly greater than liquid heat transfer conductivities, which is the reason why they can be omitted. Due to relatively low temperatures radiation also plays a minimal role in heat transfer and can therefore be neglected as well. The heat transfer can therefore approximately be described by Fourier's law:

(3) $$Q = -\lambda A \left(\frac{\delta T}{\delta s}\right)$$ where

λ is the heat conductivity [W/(m x K)]

A is the cross sectional area [m^2]

(δT/δs) is the temperature gradient along path s [K/m]

Systems in which the path is not known reduce the quotient of heat conductivity and length to the heat transfer coefficient α.

(4) $$\alpha = \frac{\lambda}{s} = f(Nu)$$

The heat transfer coefficient is a function of the Nusselt number and can be described by empirical correlations depending on the employed system and on the convection type (natural, or forced). The Archimedes number portrays thereby the dominating mechanism. Systems with Archimedes numbers Ar >> 1 are dominated by free convection, whereas systems with Ar << 1 are dominated by forced convection. Archimedes numbers Ar ≈ 1 need to consider free and forced convection in their Nusselt correlations, so as to whether buoyancy forces act in the same or opposing directions.

Forced convection, as opposed to free convection, occurs in beer fermentation tanks due to pressure gradients. Carbon dioxide bubbles, built in the fermentation tanks, have a greater pressure than the fluid surrounding them, as shown by the Young Laplace equation. As such carbon dioxide bubbles float to the surface. *Delente* showed that CO_2 bubbles are mainly built in the tank cone, forcing them upwards through the middle of the tank [22]. Already dissolved CO_2 diffuses into the upward moving bubbles. The latter mechanism describes the heat transfer through convective mass transfer as dictated by the Stefan Maxwell equation. Gas and liquid diffusion coefficients can be described for systems with known path length through the Fuller equation or empirical correlations, respectively. If the path becomes unknown, the diffusion coefficient is reduced to the mass transfer coefficient. The mass transfer coefficient is a function of the Sherwood number and can be found depending on the present flow in the system

(5) $$\beta ij = \frac{1}{\delta ij} = f(Sh)$$

β ij stands for the mass transfer coefficient

δ ij stands for the diffusion coefficient

Sh stands for the Sherwood number

Unknown paths occur through free convection. The Rayleigh number can be used to determine whether mass transfer is governed entirely by diffusion or a mixture of it and convection [56].

The high complex mechanisms occurring in fermentation tanks can be investigated by measuring the concentration response of inert tracer material as a function of time. Characteristic concentration profiles have been developed depending on the governing flow profile and the inert material feed stream [39, 40, 58].

As illustrated in figure 1, the residence time can be determined by either performing a step experiment, in which the infeed flow is substituted by one containing a tracer, or by an impulse test, in which the tracer material is being injected into the fluid. Depending on the applied test and the prevalent flow, different response functions can be expected as shown [11, 49].

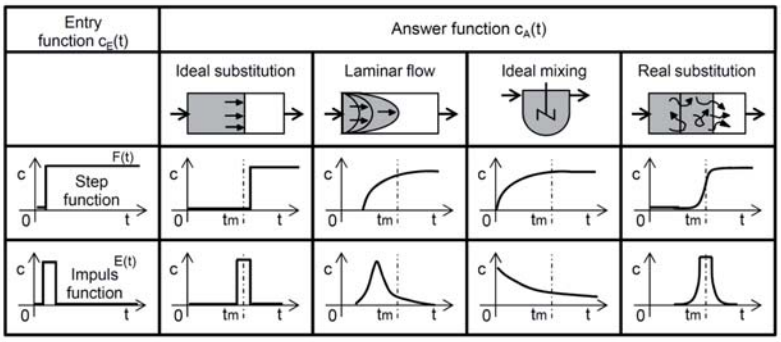

Fig. 1 Control and answer functions for different flows [58]

In a step experiment the tracer material concentration is therefore described by (6)

$$F(t) = \frac{cT\,(t)}{cT_0} \quad \text{(6)}$$

where

F(t) is the inert material sum distribution at time t [–]

cT (t) is the outlet inert material concentration at time t [mol/m³]

cT_0 is the set inert material concentration [mol/m³]

Similarly, a step change in inert material concentrations is described by equation (7).

$$E(t) = \frac{cT\,(t)}{cT_0} \quad \text{(7)}$$

where

E(t) is the inert material distribution density [–]

cT (t) is the outlet inert material concentration at time t [mol/m³]

cT_0 is the set inert material concentration [mol/m³]

Sum distributions and initial concentration measurements allow a calculation of the inert material concentration at any time t.

3 Brewing yeast differentiation

In this research different yeast strains were used as tracer substances, in order to measure their residence time in a continuously operated bioreactor. Therefore appropriate differentiation methods were mandatory.

The most commonly used yeasts in the beverage industry are Saccharomyces strains, more specifically of the species *S. cervisiae*, *bayanus* and *pastorianus*. Those yeasts can be categorized into the sensu stricto complex.

Bottom fermenting yeasts, used in the brewing industry are hybrids, categorized in genus *S. pastorianus* ssp. *carlsbergensis*, whereas top fermenting yeasts belong to the *S. cervisiae* type. For the production of specialty beers, sometimes *Dekkera* spp. types are used additionally for maturation [7].

Brewers often distinguish their yeasts in bottom- and top fermenting yeasts, which vary physiological, morphological and in means of fermentation characteristics.

Bottom fermenting yeasts (*Saccharomyces pastorianus* ssp. *carlsbergensis*) may be identified via the microscope as single cells or as pairs. The maximum growing temperature of bottom fermenting yeast is 34 °C [3]. Contrary to top fermenting yeasts, bottom fermenting yeasts are known to ferment well, if cold temperatures of approximately 5 °C are being applied. Bottom fermenting yeasts contain the enzyme β-Fructosidase and α-Galactosidase, which enables them to completely metabolize raffinose. Furthermore bottom fermenting yeasts have a comparatively poor sporulation ability, which can usually be detected after 72 hours [22].

Top fermenting yeasts (*Saccharomyces cervisiae*) usually form cell joins during the fermentation of up to 8–10 single cells or more. They can grow in temperatures up to 39,8 °C, but tend to sediment at temperatures below 10 °C [3]. Furthermore top fermenting yeasts can metabolize raffinose comparatively only to one third, since they do not contain the enzyme α-Galactosidase. Therefore melibiose cannot be decomposed further on. Additionally, top fermenting cells tend to sporulate already after 48 hours [6, 7].

Due to those differences, numerous methods were developed in the past, in order to differentiate these yeasts on genus and strain level. All standard methods, which are described precisely in the literature, are summarized in the table 1 below.

Table 1 Common methods for yeast differentiations according to [34, 35]

Method	Degree of Differentiation
Physiological- and Morphological Methods	
Standard Methods	Genus, Species [9, 43, 62]
Miniaturized Commercial Systems	Genus, Species [5]
Chemotaxonomical Methods	
Total Fatty Acid Analyze	Species [65, 71]
Protein-Fingerprinting	Species, Strain [1] [38]
Mass Spectroscopy based Methods	Species, Strain [59, 69, 72]
Fourier transformed Infrared Spectroscopy	Species, Strain [72, 76]
Immunological Methods	
Technique based on mono-clone Antibodies	Species, Strain [1, 41]
Molecular-genetic Methods	
Sequencing	Species [5, 44, 71, 75]
Karyotype determination	Strain [4, 23, 27, 32, 47, 53, 54, 55, 64]
RFLP with DNA	Strain [10, 15, 20, 26, 27, 28, 31, 46, 47, 61, 64, 73, 74]
Fluorescent/Chemical luminescent in-situ Hybrids	Genus, Species [63, 70, 77]
PCR-RFLP of the 5.8S ITS rDNA Region	Species [5, 10, 27, 29, 45, 50, 61, 64, 74, 75]
PCR-DGGE, PCR TGGE	Species, Strain [19, 60]
Real-Time PCR	Species, Sub-species [12, 13, 14, 17, 21, 25, 57]
RAPD-PCR	Strain [30, 32, 66]
Microsatellite PCR	Strain [16, 33, 66]
AFLP-PCR	Strain [67]
δ-Sequence PCR	Strain [15, 18, 23, 46, 61, 66]
PCR-DHPLC	Strain [34]

As illustrated in table 1, there are numerous methods in order to differentiate yeasts on various taxonomic levels. Many of those are used regularly in order to identify pure single cultures. The goal of this research in contrast was, to quantitatively identify defined mixtures of fermenting yeasts (starter culture) and tracer yeasts, in order to determine residence times and therefore present flow dynamics. For this purpose a selection of common methods were modified as shown below.

4 Apparatus for flow profile evaluations

Besides the laboratory development, and evaluation of different methods in order to detect tracer organisms, one promising concentration profile test was chosen to be evaluated in the continuously operated pilot plant, developed by *K. Müller-Auffermann* [51].

As simplified illustrated in figure 2, the plant consists of a series of cylindroconical reactors, which contain inverse shaped and bottom opened pipes, though, in this case, the fermentate is being discharged. The feed of the inoculated wort is done via the tank top and each vessel contains a purge valve, located at the bottom of the cone, in order to discharge sediments and particles.

The flow rate was adjusted, to achieve theoretical residence times of 24° hours in each tank. In this research only the first reactor, and therefore one fermentation day, was monitored.

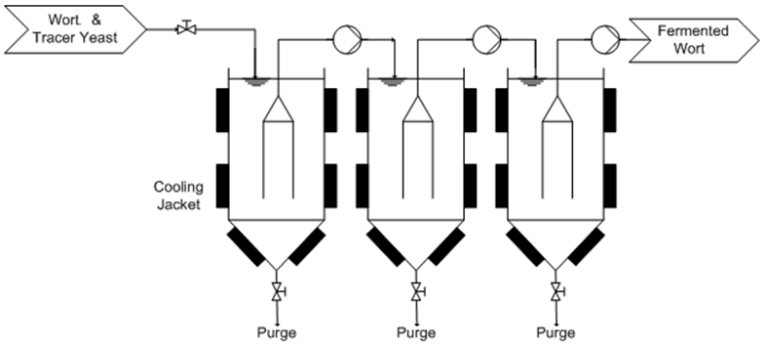

Fig. 2 Continuous fermentation concept developed by K. Müller-Auffermann [51]

5 Experimental

5.1 Materials

The following yeast strains were used throughout the here presented evaluation: The yeast strains of the species *Saccharomyces pastorianus* ssp. *carlsbergensis*: TUM 34/70, TUM 128, TUM 224 (all three strains can be used for lager or pils beer types) and the species *Saccharomyces cerevisiae*: TUM 184 (used for Alt beer types), TUM 210 (used for ale beer types) and (used for wheat beer types).

The organisms were provided by the Yeast Center of the Research Center Weihenstephan, and the necessary wort was taken from the Weihenstephan State Brewery under standard conditions.

5.2 Wort fermentation analysis

The previously mentioned yeast strains were used in finding desired similar extract/wort decomposition properties in order to maintain a constant flow profile. Specially designed small scale tanks with a 2:1 height to diameter ratio were used [52]. Wort was added up to the 2 l mark (60 % total volume) and the tanks were closed using a steel clamp. The gas valve was opened to allow a precise pressure adjustment. The fermentation conditions were 15 °C, 0.5 bar and 30 million Cells/ml. The fermentation behavior was tracked during five days. Extract levels were measured with the MEBAK II 2.10.6.2 method. pH-values, so as, alcohol quantity, free amino acids (FAN), fermentation by products and vicinale diketone were tracked using the MEBAK II 2.14, MEBAK II 2.10.6.3, MEBAK II raw material 3.1.4.5.5.2, MEBAK III 1.1.1 and MEBAK III 1.2.1 method, respectively. A pair of yeast strains with similar wort decomposing characteristics was chosen and utilized for evaluating alternative methods in the determination of tracer yeast's profile concentration.

5.3 Method I: tracer identification by the evaluation of metabolic differences

The following tests are based on the different reaction kinetics of top- and bottom fermenting yeasts, with similar wort reaction kinetics.

5.3.1 The modified raffinose test

Top fermenting yeasts decompose raffinose to one third of the value done by bottom fermenting yeasts [2]. The MEBAK 10.4.5.1 raffinose test describes quantitatively the method in order to analyze pure top- and bottom fermenting yeast strains. By keeping the initial wort and yeast mass constant and measuring the CO_2 production, it can easily be determined whether top or bottom fermenting yeasts are present in the system.

In this research the raffinose test was initially done for all yeasts in order to determine the strain`s CO_2 production. The test was then modified by using different yeast fractions in increments of the top- and bottom fermenting yeasts, using the strains with the greatest difference in CO_2 production.

The raffinose solution was prepared by a 90 % [w/w] water, 10 % [w/w] raffinose-pentahydrat mixture. TUM 34/70 and TUM 210 were propagated in sterile larger beer wort in the laboratory before usage. The propagated yeasts were cooled down to 5 °C and centrifuged at 4000 rpm during 5 min (= 1932 g) separately. The sediment was then collected with an applicator, blended with a 0.1 % [w/w] peptone (from Casein; Supplier: Fluha Analytical) bidistilled sterilized water solution in a ratio of 1:10, before homogenization with a magnetic stirrer.

After appropriate washing, the solution was repeatedly centrifuged at 5 °C at 4000 rpm during 5 min (= 1932 g). The sediment was recollected with a spattle, added once more to a 0.1 % [w/w] peptone solution in a ratio of 1:10, and homogenized with a magnetic stirrer (5 min; 100 rpm, stirrer length: 60 mm).

The cell amount was subsequently adjusted to be 200 million yeast cells/ml in the Raffinose (Penthydrate; Supplier: Merch) solution.

The mixture was incubated one hour at 20 °C under atmospheric conditions and added to Einhorn's fermentation saccharometers. The flasks where filled and CO_2 formation was tracked by measuring the displaced volume each half hour at 20 °C.

5.3.2 4 °C Saccharose decomposition test

This test was developed, based on the information that top fermenting yeasts metabolizes sugars significantly slower than bottom fermenting yeasts, if fermentation temperatures below 10 °C are being applied [3]. The used method and materials follows the method described in the *"Modified Raffinose Test"*. A 10 vol. % saccharose-peptone solution was used as substrate, a fermentation temperature of 4 °C was chosen, and that the CO_2 production was monitored in 8 hour periods over the course of 48 hours.

5.3.3 The modified 37 °C test

The 37 °C test is based on the different metabolic properties of bottom and top fermenting yeasts, resulting in different temperature ranges for the reproduction optima. Bottom fermenting yeast strains do not reproduce at 37 °C [6], while top fermenting yeasts do. In the 37 °C test pure yeast quantities were dispensed on agar plates and the existence of top fermenting yeast strains could be qualitatively determined based on the colonies formed after three to five days. The 37 °C test was modified by using different yeast fractions in 20 wt% increments (cell amount determined via Thoma-Cell-Chamber) of the bottom fermenting yeast TUM 34/70 and the top fermenting yeasts TUM 68 and TUM 184. Furthermore different dilution rates of the tracer organisms were tested (1 cell/ml; 10 cells/ml; 100 cells/ml; 1.000 cells/ml 10.000 cells/ml; 100.000 cells/ml) in order to determine the ideal amount of cell colonies for a statistical interpretation. After a five-day incubation period at 37 °C the colonies on the plates were once again counted. The cell number difference was used to determine the contained bottom fermenting yeast percentage. For assurance, samples were also incubated at lower temperatures (27 °C) in which both, bottom and top fermenting yeasts may form colonies.

5.4 Method II: tracer identification by the evaluation of genetic differences

The following tests are based on the different genetic properties of the tested yeast strains, which possess similar wort reaction kinetics. This method allows bottom- and top fermenting yeasts, so as bottom-bottom-, and top-top fermenting yeast strains, to be differentiated. Therefore potentially a more precise analysis can be obtained, due to the fact that bottom-bottom and top-top fermenting yeasts form similar cell cluster sizes and hence theoretically influence the system identically.

5.4.1 IGS2-314 standard PCR – capillary gel electrophoresis

The DNA isolation was performed according to the modified instageneTM matrix protocol as described in detail below. Hence 200µl of a dense yeast cell suspension were added to a 1.5 ml Eppendorf reaction tube and centrifuged 1 min at 13 000 rpm (= 15.115 g) (Hettich MIKRO 200). The dense yeast suspension was prepared by collecting 150 ml of the desired cropped yeast, and establishing the cell number with the help of a Hemocytometer, according to the MEBAK III 10.11.4.4 procedure. 30 ml from the yeast solution were then diluted to 30 million cells/ml with sterilized water, and yeast mixtures were prepared in 20 % [w/w] increments subsequently, by diluting the adjusted cell suspension with sterile water.

200 µl of the InstaGeneTM matrix were added to the cell pellet and incubated for 30 min at 56 °C in a thermomixer (Eppendorf Thermomixer comfort). The pellet was then vortexed for 10 seconds. Subsequently it was incubated in a thermomixer for 8 min at 95–97 °C and centrifuged for 1 min at 13 000 rpm (= 15.115 g) (Hettich MIKRO 200). The DNA concentration was measured using the Nanodrop ND 2000 (Thermo Scientific) and was set to be 25 ng/µl. The excess sample material was stored for the Polymerase Chain Reaction test at –20 °C.

DNA copies were obtained through the standard Polymerase Chain Reaction (PCR), amplifying partial sequences of the IGS2 rDNA regions [34, 35]. Thereby 2.5 µl of sample DNA was added to an 25 µl assay. The assay was composed of 12.50 µl Genaxxon RedTaq Mastermix 2X, 7.00 µl AmpuWa, and 1.50 µl of each primer IGS2_314 fp and IGS2_314 rp with a 10 pmol/µl to reach a primer concentration of 600 nM. The temperature of the thermocycler (Eppendorf Mastercycler ep Gradient) was adjusted, so that a first heating to 94 °C for 30 s, 40 cycles of a denaturation (94 °C, 30 s), annealation (54 °C, 30 s) and elongation (72 °C, 40 s) resulted. A final elongation step was performed at 72 °C for 5 min and finally cooled down until reaching 4 °C [34, 35].

Capillary gel electrophoresis can separate PCR-products according to their length of base pairs through capillary pores, filled with a highly purified gel when exposed to an electric field. The capillary gel electrophoresis was performed using the Agilent 2100 Expert BioAnalyzer, so as a capillary Electrophoresis-Kit Agilent DNA 1000 Kit. IGS2-314 PCR-products were added to the DNA-chips of the Agilent DNA 1000 Kit according to the kit manual. The capillary electrophoresis run was subsequently performed also according to the DNA 1000 kit settings. Electrophoresis graphs were collected from the Agilent 2100 Expert BioAnalizer, and after a visual pre-check of PCR-products and primer-dimer bands, they were evaluated using bionumerics 6.5 (Applied maths). Defined mixtures of the top fermenting yeasts TUM 210 and TUM 184 were selected for this experiment. In addition to their similar wort fermentation profiles, these two yeast strains show specific IGS2-314 PCR-product bands that can be quantified according to their PCR-product concentration using the Agilent Technologies BioAnalyzer 2100 Expert software version B.02.03.SI307. One band (band with base pair length of 165bp) is specific for TUM 210 and one band (band with base pair length of 193 bp) is specific for TUM 184 (electropherogram not shown). Additionally the bottom fermenting yeast strains TUM 224 and TUM 128 were selected for further experiments. Here the yeast strain TUM 224 shows one specific IGS2-314 PCR-product band with a base pair length of 224, so that mixtures of both bottom-bottom fermenting yeasts could be detected quantitatively.

5.4.2 RealTime Polymerase Chain Reaction (PCR) test

The RealTime PCR amplifies DNA fragments across several PCR cycles, generating thousands to millions of copies of a particular DNA sequence. The species *S. pastorianus* ssp. *carlsbergensis* (bottom-fermenting yeast) and the species *S. cerevisiae* (top-fermenting yeast) can be detected within interspecies-species mixed populations using the below described PCR-Systems OGCOXII and UG 300 [33, 34]. The applied primer and probe sequences, PCR-protocols, detection limits and PCR-efficiencies and specificities of the systems are described by *Hutzler* [33,34]. For the DNA isolation, the Modified InstageneTM Matrix Protocol (see 5.4.1) was used. The RealTime PCR Test was performed using the method. Hence, the test was performed using PCR Buffer, 200 µM dNTPs, 400 nM forward primer, 400 nM backward primer, 3 mM $MgCl_2$, 80 µg/µl BSA, 200 nM TaqMan-Probe and 0.025 U/µl Taq Polymerase according to Hutzler [34, 35]. For the detection of top fermenting yeast strains of the species *S. cerevisiae*, the PCR System OGCOXII was used [34, 35]. In order to differentiate bottom fermenting yeast strains of the species *S. pastorianus* ssp. *carlsbergensis*, the RealTime PCR system [34, 35] was used.

In addition a new RealTime PCR system was designed by Riedl for this project, in order to allow the differentiation of top fermenting yeast strains, species *S. cerevisiae* by amplifying a partial sequence of the Met10 gene. The primers for the so called RealTime PCR system Met10_W are: Met10_W f (5'-ACTTCAGCTTGGAAGCAG-CAT-3'), Met10_W r (5'GATGGTAAGTTTCTTTTGAATGTTGGT-3') and the MGB Taqman Probe Met10_W_87MGB (5'- ATAATCGTT-CGTGACAGAGC-3') (Applied Biosystems®; Life Technologies GmbH, Darmstadt, DE). The RealTime PCR parameters were set compatible to the RealTime PCR systems according to Hutzler [34, 35].

For this experiment yeast mixtures of TUM 34/70 (bottom fermenting) and TUM 68 (top-fermenting) in 20 % increments were analyzed using the *RealTime PCR* systems OG-COXII and UG 300. The yeast mixture of TUM 184 (top-fermenting Alt beer yeast) and TUM 210 (top-fermenting ale beer yeast) in 20 % increments were analyzed using the MET10 RealTime PCR-system described above. This system enables the detection of a small concentration of TUM 184 (Alt beer yeast) within a TUM 210 (ale beer yeast) population.

5.5 Method III: experiments in a continuously operated pilot plant

Based on the previous proposed methods the best suitable alternative was chosen and used to analyze its performance in the continuously run pilot plant at the "Research Center Weihenstephan" (see figure 2). In order to simplify the test, only one reactor, in which the residence time is adjusted to be theoretically 24 hours, was monitored in this trial.

Due to logistical reasons the fermenter was run with larger amounts of the industrial propagated TUM 34/70 yeast strain, before the feed stream was entirely substituted by the also industrial propagated TUM 68 strain. Since TUM 68 proved to ferment more extract in 24 hours at 15 °C than TUM 34/70 (see figure 3) it was decided to use slightly lower cell amounts (25 million Cells/ml) as tracer respectively, in order to compensate this effect (the amount was calculated on the base of the volumetric productivity per cell). The feed flow rate and the yeast flow rate were set to 2 l/h. Samples were taken hourly directly from the tank as well as from the fermenter outflow. All samples were immediately treated and analyzed as described in the modified raffinose test.

6 Results and Discussion

6.1 Fermentation characteristics

The extract reduction and the alcohol production were of significant interest in this research, due to their influence on the flowing pattern. Extract and alcohol content correlate inversely proportional. Hence, only the median extract reduction is shown in figure 3 (all trials were performed three times. Due to the very slight deviations [median: approx. 1 %] the error bars are not being illustrated in this figure). The alcohol content, free amino acids, fermentation byproducts and vicinale dicetone were also recorded in order to monitor the process, but are not presented, due to their negligible influence on the flow profile.

As illustrated in the figure 3, the top fermenting (TF) yeast strains TUM 210, TUM 184 and the bottom fermenting (BF) yeast strain TUM 34/70 showed similar fermentation behaviors in the first 24 hours, and were hence chosen for the flow profile characterization with a mix of top fermenting yeast strains and bottom fermenting yeast strains via the "Modified Raffinose Test", the "Modified 37 °C Test" and the "4 °C Decomposing Saccharose Test".

The top fermenting yeast strains TUM 210 and TUM 184 with nearly identical fermentation performance, as well as the bottom fermenting yeast strains TUM 224 and TUM 128, which did not perform similar, but which could be differentiated by specific IGS-2 314 PCR product bands, were chosen for the flow profile characterization via "IGS2 314 PCR – Capillary Gel Electrophoresis". For RealTime PCR mixtures of TUM 210 and TUM 184 (top-fermenting/top-fermenting) and mixture of TUM 34/70 and TUM 68 (bottom-fermenting/top-fermenting) were chosen (see 5.4.2).

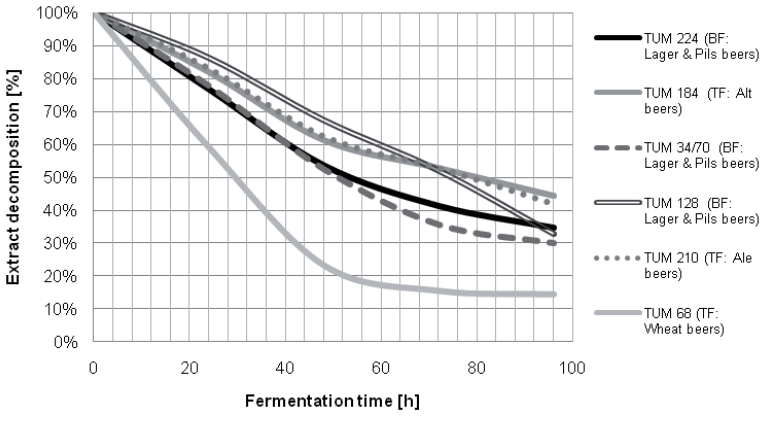

Fig. 3 Median extract reduction as a function of time for different yeast strains.

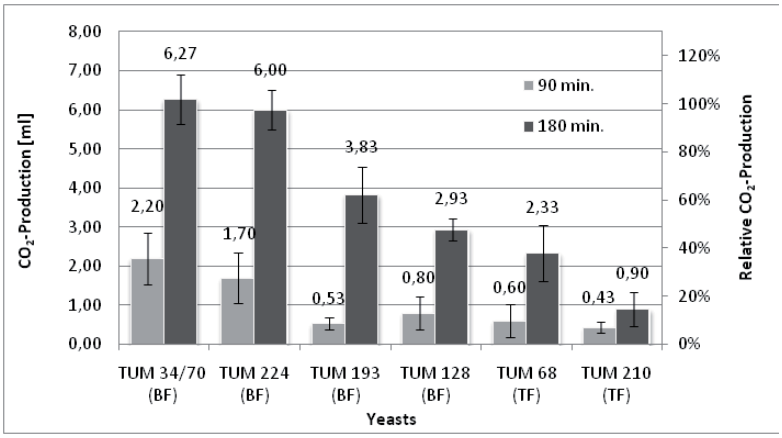

Fig. 4 CO_2 production during Raffinose fermentation by different yeasts

The bottom fermenting yeast TUM 34/70, so as the top fermenting yeast TUM 68, were used in the continuously operated pilot plant at the Research Center Weihenstephan, mainly due to logistical reasons.

6.2 Method I: tracer identification by the evaluation of metabolic differences

6.2.1 The modified raffinose test

The modified raffinose test is based on the ability of top fermenting species *S. cerevisiae* to decompose raffinose to one third of the value the bottom fermenting species *S. pastorianus* ssp. *carlsbergensis* [2]. With the established wort fermentation analysis it was obvious, that the top fermenting (TF) yeast strains TUM 210, TUM 184 and the bottom fermenting (BF) yeast strain TUM 34/70 showed similar behaviors during the wort fermentation process. It was hence desired to observe their behavior in Raffinose fermentation to subsequently choose the appropriate pair. Yeasts with the greatest difference in CO_2 production were desired to facilitate their differentiation in mixed quantities. The top fermenting yeast TUM 210 and the bottom fermenting yeast TUM 34/70 showed hereby the greatest difference in CO_2 production and were therefore chosen. The test results can be found in figure 4. All experiments were conducted three times, and the error bars represent the calculated confidence intervals.

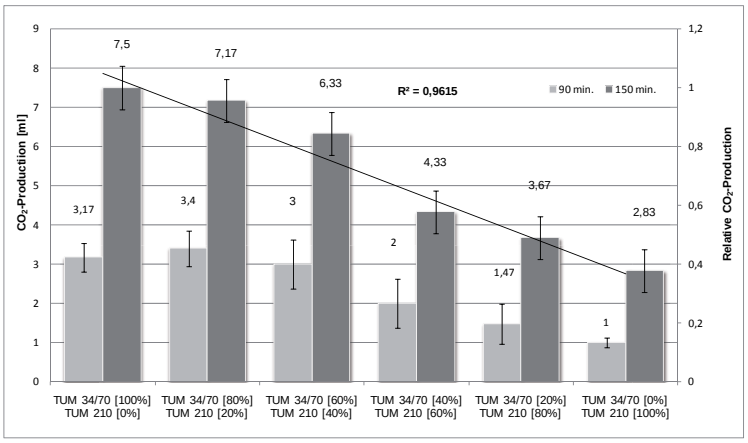

Fig. 5 CO_2 production during Raffinose fermentation for a TUM 34/70-TUM 210 yeast mixture

Upon appropriate yeast pair selection (TUM 34/70 [bottom fermenting] and TUM 210 [top fermenting]), 20 % [w/w] increments were made. As shown in figure 5 the CO_2 production rate decreases, as expected, with increasing TUM 210 mass fractions (All experiments were conducted three times, and that the error bars represent the calculated confidence intervals). The approximate decay function can therefore be used to predict unknown yeast quantities and hence the flow profile.

6.2.2 The modified 37 °C test

The 37 °C test is based on the inability of bottom fermenting yeasts of the species *S. pastorianus* ssp. *carlsbergensis* to reproduce at 37 °C. Top fermenting yeasts of the species *S. cerevisae* are able to grow and build colonies, whereas bottom fermenting yeasts of the species *S. pastorianus* ssp. *carlsbergensis* do not reproduce and remain as single cells. Under the assumption that all (or at least the 95 % = viability rate) top fermenting yeast cells form colonies in the incubated time, it is theoretically possible to quantitatively find their fraction by counting and calculating the difference between the initial and final cell number. However, the achieved results showed to be imprecise. The appropriate dilution rate proved to be the biggest problem. With too many cells on the agar plates counting was impossible because individual colonies were no more visible. On the other hand, low cell numbers could be counted much better, but impeded a correct statistical evaluation. Overgrowing of colonies also exacerbated the comparison.

6.2.3 4 °C saccharose decomposition test

The 4 °C saccharose decomposition test is based on the inability of top fermenting yeasts to reproduce and ferment quickly at 4 °C With the established wort fermentation analysis it was obvious, that the top fermenting yeasts TUM 210, TUM 184 and the bottom fermenting yeast TUM 34/70 showed similar extract reducing behaviors during the wort fermentation process at 15 °C (temperature used in the continuously run pilot plant). Their behavior at 4 °C was therefore analyzed in order to choose the appropriate pair. Yeasts with the greatest CO_2 production difference were desired, due to the simplicity in being differentiated more precisely in mixed quantities. Based on these results, the yeasts TUM 34/70 and TUM 184 were chosen. Mixtures in 20 % [w/w] increments were then prepared and fermented at 4 °C in Saccharose as descripted. However, the CO_2 amounts produced of both yeasts (even after several days) were very low and varied, so that no clear trend could be observed in this case.

6.3 Method II: tracer identification by the evaluation of genetic differences

6.3.1 IGS2 314 PCR – capillary gel electrophoresis

The wort fermentation analysis revealed, that the top fermenting yeasts TUM 210 and TUM 184 had similar fermentation profiles. They were hence chosen, mixed in 20 % [w/w] increments, their DNA was extracted, amplified with the IGS2 314 protocol [34, 35] and analyzed with capillary gel electrophoresis according to 5.4.1. Using the IGS2 314 PCR with subsequent capillary gel electrophoresis both strains show specific band patterns. A highly

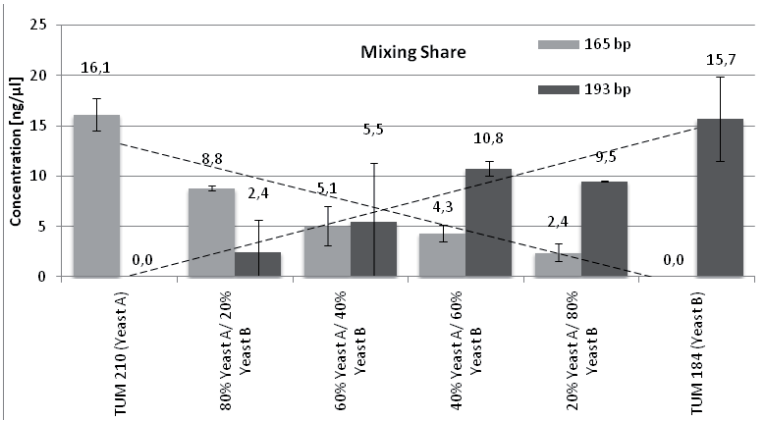

Fig. 6 Base pair concentration of top-top fermenting yeast mixtures TUM 210-TUM184

specific amplicon band was therefore chosen for each strain. One band (band with base pair length of 165 bp) is specific for TUM 210 and one band (band with base pair length of 193 bp) is specific for TUM 184 (electropherogram not shown in this paper). The DNA concentrations were calculated using the Aglient 2100 Expert BioAnalyzer Software according to the band intensity. The results were rechecked using Bionumerics 6.5. The results of three trials including the confidence intervals can be found in figure 6 above.

As illustrated, the pure yeast strain TUM 210 showed the highest absolute concentration of the amplicon band with the size of 165 base pairs. Higher TUM 184 mass fractions showed an increase in its amplicon band concentration, whereas TUM 210 concentrations decreased. The approximate decay functions can therefore be used to predict unknown yeast quantities and hence in order to evaluate the flow profile respectively.

The same procedure was applied in order to differentiate bottom-bottom fermenting yeast mixtures. In contrast to the top fermenting yeasts, were two strains which similar fermentation performances could be chosen, only the bottom fermenting yeasts stains TUM 224 and TUM 128, with different fermentation performances, showed differences in only one specific IGS-2 314 PCR-product band, present exclusively in TUM 224 and absent in TUM 128 (see 5.4.1, electropherogram not shown in this paper). Therefore only those stains could be tested, and only the IGS2 314 PCR-product with fragment length of 212 bp could be monitored. Figure 7 illustrates the results of three independent trials taking the calculated confidential intervals into consideration.

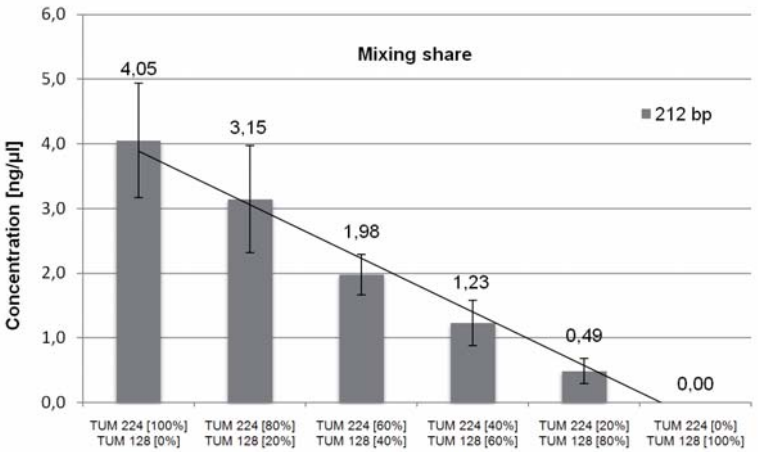

Fig. 7 Base pair concentration of bottom-bottom fermenting yeast mixtures TUM 224-TUM 128

As it can be seen in figure 7 the concentration of the PCR-product with a fragment length of 212 bp, contained exclusively in the TUM 224, clearly decreases with increasing portion of TUM 128. The decay function may therefore be used in order to quantitatively differentiate the increments of the bottom fermenting tracer yeast in a bottom fermenting culture yeast.

6.3.2 Real-Time Polymerase Chain Reaction (PCR) test

The *Real-Time PCR* test amplifies DNA fragments across several orders of magnitude, generating millions of copies of a particular DNA sequence. With the established wort fermentation analysis it was obvious, that the top fermenting yeasts TUM 210 and TUM 184 had similar wort decomposing behaviors, and they were hence chosen for analysis. The Real-Time PCR system Met10_W was used to detect traces of TUM 184 in TUM 210.

The Met10 W *Real-Time PCR* reaction was negative for the pure TUM 210. This means there was no amplification. Upon addition of TUM 184 all compositions (20 %, 40 %, 60 %, 80 % and 100% TUM 184 fractions) reacted positive, using the Met10_W Real-Time PCR. The median C_t-values of triplicates were thereby in between 23,7 and 25,8 (data not shown).

Additionally yeast mixtures of TUM 34/70 (bottom fermenting and TUM 68 (top-fermenting) in 20 % increments were analyzed using the real-time PCR systems OG-COXII and UG 300. The results were similar to the results of the Met10 W Real-Time PCR. Using the PCR system OG-COXII the PCR reaction was negative for the pure TUM 34/70. Upon addition of TUM 68 all compositions (20 %, 40 %, 60 %, 80 % and 100% TUM 68 fractions) reacted positive. The mean C_t-values of triplicates were between 20,8 and 21,6 (data not shown). The application of the PCR systems UG 300 resulted in negative RCR reactions for the pure usage of TUM 68, and turned to be positive, as soon as 20 % of TUM 34/70 or more where added. A quantification of 20 % yeast fraction increments was therefore impossible.

Concluding, it was found that the *Real Time PCR Test* is not suitable for the detection of 20 % [w/w] step increments quantitatively, due to its sensitivity (compare [34, 35]).

Yet, this method is suitable for the judgment of flow patterns, if the impulse function test, where a small amount of tracer material is being injected into the fermenter, is being performed.

Since no suitable PCR-system exists for the differentiation of the here introduced and tested bottom-bottom fermenting yeast stains, this test was only performed for the top-top fermenting and bottom-top fermenting strains as described above.

6.4 Method III: experiments in a continuously operated pilot plant

The modified raffinose test and the capillary gel electrophoresis test proved to be efficient in determining the tracer yeast concen-

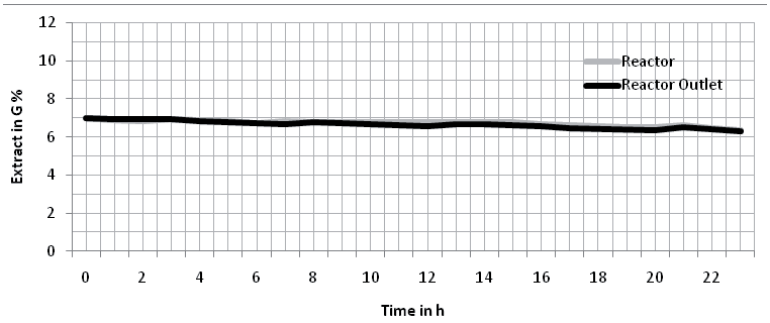

Fig. 8 Wort decay using the continuously run fermentation pilot plant

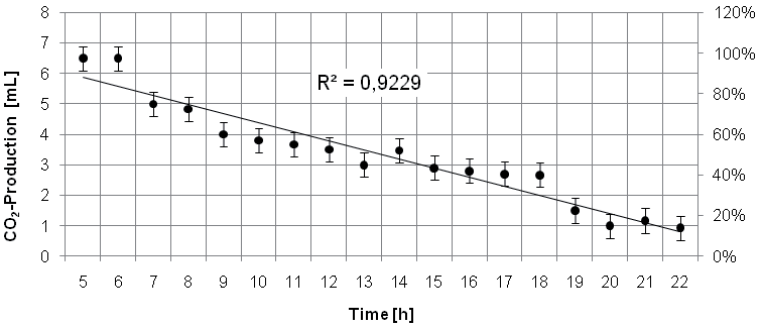

Fig. 9 Modified Raffinose Test of the continuously run pilot plant

tration in the fermenter. However, the simplicity and the low cost of the modified raffinose test increased its attractiveness over the capillary gel electrophoresis. The modified raffinose test was hence chosen in order to evaluate the flow profile in the first reactor of the continuously run pilot plant of the Research Center Weihenstephan.

Due to logistical reasons the fermenter was run with larger amounts of the industrial propagated TUM 34/70 yeast strain, before the feed was entirely substituted by the also industrial propagated TUM 68 strain. As shown in figure 7, the extract degree stayed fairly constant during the 24 hour testing time decreasing only about 0,5 °Plato in average (single samples, also for the extract measurement) were taken each hour during the course of the conducted experiment).

In order to obtain information about the tracer yeast quantity, sample material was taken from the reactor outlet each hour. The *Modified Raffinose Test* was performed and the results were collected as shown in Figure 8 (All experiments were conducted three times, and the error bars represent the calculated confidence intervals).

As shown above, a steady decay in median CO_2 production was observed, indicating the effectiveness of the modified raffinose test for the detection of tracer yeasts with different raffinose fermentation characteristics. This is underlined by the fact, that less CO_2 is being produced in the raffinose solution proceeding, even so the tracer yeast was generally able to reduce the extract content further (see figure 8), and should therefore principally produce more CO_2.

By interpreting the trend line in figure 9, accordingly to the answer functions in the step function experiments presented in figure 1, it can be seen that the present flow in the reactor lead to good, nearly ideal mixing conditions over the course of 24 hours.

7 Conclusions and future work

Alternative methods in the flow profile characterization were evaluated by means of top-bottom, top-top and bottom-bottom fermenting tracer yeast blends, ideally possessing similar wort reaction kinetics. Yeast mixtures were prepared using 20 % [w/w] increments.

Tracking of top-bottom fermenting yeast mixtures was based on their different metabolic kinetics.

The modified raffinose test, in which the produced CO_2 amount of the mixtures was measured, delivered curves that could be used to predict the tracer yeast composition and hence the flow profile. Besides laboratory testing, this method was successfully evaluated in a continuously operated reactor at the Research Center Weihenstephan.

The Modified 37 °C Test, which was based on the different growing range temperatures, revealed to be imprecise. The necessary dilution rate required an excessive number of agar plates, in order to enable correct statistical evaluations. In addition the long incubation times impede its practicability.

The 4 °C saccharose decomposition test was based on the inability of top fermenting yeasts to reproduce at 4 °C. Unfortunately, no clear trend could be observed. A possible explanation might be the significant increase of yeast's sedimentation. This effect may have influenced the fermentation in the Einhorn fermentation tubes, and thereby the CO_2 production rate during the experiments.

In order to differentiate yeasts of similar cell agglomerate sizes and therefore related flow characteristics, top-top and bottom-bottom fermenting yeast mixtures were tracked, based on their different genetic properties.

IGS2_314 rDNA standard PCR and capillary gel electrophoresis proved to be efficient for distinguishing top-top fermenting yeasts with similar wort decomposing properties. A decay curve for the fainting yeast fraction-, as well as an increase function for the substituted yeast, was obtained. Since bottom-fermenting yeasts show in general more similar IGS2 PCR-product band patterns, only one detectable difference (band with fragment length of 212 bp) could be found in two strains (strains TUM 224, TUM 128), which performed differently in wort. Therefore this method could potentially be used to predict the tracer concentration, but seems to be better suitable for top-top fermenting strains. Nevertheless the IGS2 314 PCR – capillary electrophoresis, enables for the first time flow profile control via bottom-bottom fermenting brewing yeast as well.

The applied Real-Time PCR systems were not suitable for the detection of 20 % [w/w] step increments quantitatively. Still, they are suitable for the detection of very small tracer organism concentrations in the starter culture. [34, 35]. Therefore the Real-Time PCR tests may be suitable for impulse function tests, rather than step function tests, to determine residence times.

Concluding, this research reveals that it is possible to detect biological tracer substances in order to determine the present flows in bioreactors.

Bottom- and top fermenting yeasts can be differentiated by selective metabolic tests, such as the Modified Raffinose Test. As shown, this method is not very precise, so that only larger increments of approximately 30 % can be distinguished statistically correct. This method is therefore better suitable for rough flow examinations in continuously operated bioreactors. Here a step function test is the recommended mechanism.

DNA based tests, also allow the appropriate differentiation of bottom-bottom and top-top fermenting yeasts. The IGS2 314 PCR - Capillary Gel Electrophoresis enables the monitoring of both organisms simultaneously and is more precise than tests based on metabolic differences. However, DNA based tests are comparatively expensive; require higher efforts in sample preparation and sophisticated measurement devices are mandatory.

The Real-Time PCR Test revealed positive results for all yeast mixtures that contained 20% or more tracer material. Quantification within the applied mixture range was not possible. Due to the sensitivity of this method, this test seems therefore to be more suitable for the application of impulse function tests in order to evaluate the residence times and therefore the flow in the system. Certainly the growing rates of the organisms as well as the dilution rates have to be taken into consideration, if such a test is being employed.

Generally it can be said, that further research should focus on the application of Real-Time PCR tests and impulse function tests, in order to determine currents more accurately. Here significant genetic differences need to be detected for testing a larger variety of organisms, in order to identify species with identical fermentation profiles and to generate adequate PCR-Kits. Currently the whole genomes of numerous brewing yeasts are being sequenced. Based on the generated data of theses programs more strain specific Real-Time PCR systems should be available in the near future. Upon development of these methods, more complex and industrial size experiments should be conducted.

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