Detection and control of strictly anaerobic, spore-forming bacteria in sugarbeet tower extractors

Nachweis und Kontrolle von strikt anaeroben, Sporen bildenden Bakterien in Extraktionstürmen der Rübenzuckerindustrie

Günter Pollach, Walter Hein, Alexander Leitner and Peter Zöllner

Clostridial fermentation during extraction contributes to an elevated content of volatile fatty acids in the process stream of a sugar factory and thus to impairment of molasses quality. Furthermore, unpleasant odor and elevated TOC emissions are bad side-effects. Hop products, introduced to the sugar industry by Zuckerforschung Tulln (ZFT), seem to be useful in controlling such misfermentation. A dosage can be triggered by a signal from an online hydrogen detector, when Clostridia appear. With periodic application of hop products, a parallelism between the appearance of butyric acid and an unknown, UV absorbing substance was observed, which was identified as uracil. Uracil has not yet been described in the context of microbiology of sugar production, but in contrast to volatile fatty acids, no inhibition of yeast growth has been observed. However, due to its characteristic UV absorption at 258 nm, it can serve as an HPLC-indicator to detect Clostridial fermentation in tower extractors.

Keywords: uracil, thermoanaerobacter, clostridium, hop acids, hydrogen

Clostridien-Gärungen bei der Zuckerrübenextraktion können zu einem erhöhten Gehalt an flüchtigen Fettsäuren im Prozessstrom von Zuckerfabriken und damit zur Beeinträchtigung der Melassequalität beitragen. Des Weiteren können sie sich durch Geruchsbelästigungen oder erhöhte TOC-Emissionen ungünstig auswirken. Die von der Zuckerforschung Tulln (ZFT) in die Zuckerindustrie eingeführten Hopfenprodukte erscheinen zur Bekämpfung solcher Fehlgärungen geeignet. Eine Dosierung kann beim Auftreten von Clostridien auf Basis einer Online-Wasserstoffdetektion ausgelöst werden. Durch periodische Anwendung von Hopfenprodukten wurde eine Parallelität zwischen Buttersäure und einer unbekannten UV-absorbierenden Substanz beobachtet, welche als Uracil identifiziert wurde. Uracil wurde bisher nicht in Zusammenhang mit der Mikrobiologie der Zuckererzeugung beschrieben, zeigte aber im Gegensatz zu flüchtigen Säuren keine Hefehemmung. Es kann aber auf Grund der charakteristischen UV-Absorption bei 258 nm als HPLC-Indikator für Clostridien-Gärungen in Extraktionstürmen herangezogen werden.

Stichwörter: Uracil, Thermoanaerobacter, Clostridium, Hopfensäuren, Wasserstoff

1 Introduction

Compared with the numerous papers dealing with aerobic micro-organisms growing on agar-plates, rather little is found in the technical literature on the growth and control of strictly anaerobic bacteria in sugarbeet extractors. At the end of the 19th century there were heated debates, whether hydrogen, appearing in extractors, stems from a pure chemical reaction only or from microbial activity [1, 2]. A massive growth of H_2S-forming organisms in the tower extractors of Austrian sugar factories about 40 years ago triggered a lot of publications about a newly-found organism, which was named *Clostridium thermohydrosulfuricum* [3, 4]. A comprehensive overview of the literature is found in the quoted papers. In these articles as well as in a work on ensilage of pressed pulp [5], *Clostridium thermosaccharolyticum* was described as a micro-organism appearing in sugar factories. When in 1993 a detonation occurred in a German sugar factory, this could be associated with Clostridial activity [6]. Finally, in 1997, the content of butyric acid rose massively in molasses at an Austrian sugar factory, and this could be attributed, based on a literature survey, to the growth of *C. thermosaccharolyticum* in press water [7].

In 1993 and 1994 initiatives were taken to change the taxonomic classification as well as the names of both Clostridia. *C. thermohydrosulfuricum* was then renamed *"Thermoanaerobacter thermohydrosulfuricus"* [8] and *C. thermosaccharolyticum* changed to *"Thermoanaerobacterium thermosaccharolyticum"* [9]. For the sake of compatibility with earlier papers in sugar technology and to have the possibility of employing the collective name "Clostridia", the original names will be used.

The growth of *C. thermosaccharolyticum* is easily recognized by the smell of butyric acid in press water. On the other hand, the growth of *C. thermohydrosulfuricum* is easily overlooked, when fresh water is acidified with H_2SO_4 instead of SO_2. In this case the organism does not form much H_2S, but only lactic acid, acetic acid, hydrogen, CO_2 and ethanol. A detection of this micro-organism is difficult when SO_2 is absent, because lactic acid is formed predominantly by thermophilic bacilli. Furthermore, acetic acid may be a sequence product of nitrate utilization by bacilli [7], and the determination of gas and ethanol is no routine procedure in the course of microbiological control of extractors. On the other hand, white sugar is tested routinely for anaerobic micro-organ-

isms and since 1958 methods for the detection of thermophilic anaerobic bacteria, even H_2S-forming ones, are included within the framework of ICUMSA activities [10]. Therefore, it seems appropriate to seize the opportunity of improving Clostridia detection in extractors. As well as the risk of a carry-over into the end product, drawbacks for the extraction as well as for the environment arise.

As of today, in some countries, *e.g.* Austria or Germany, intentional fermentation takes place in the tower extractor, when *e.g.* pulp drying is the limiting factor for the beet slicing capacity [11]. In this case, beet processing can be enhanced and disadvantages are taken into the bargain, but should be kept at a minimum. A precondition for fermentation-aided extraction is the acidification of fresh water with H_2SO_4 instead of SO_2, because on employment of SO_2 massive Clostridial fermentation would occur and cause problems by H_2S odor and gas formation. The significance of sugar losses is reduced, compared with technical and environmental problems, in factories with intentional fermentation. Microbial factory control has to be modified in this case, because a lot of microbiological tests applied under "sterile" operation are not suitable. To cite an example, the proposed nitrite assay [12, 13] for the detection of bacterial activity often gives negative results in the case of a heavy fermentation.

Ideally, to improve pulp pressing, one would wish to have a so-called "homofermentative" lactic acid fermentation, with only lactic acid formed from sugar. Under these aspects, Clostridial fermentation is a misfermentation, because various metabolic products such as CO_2, H_2, acetic acid, butyric acid and ethanol are formed as well as lactic acid, even under the omission of SO_2.

The various products have the following disadvantages:

- Acetic acid is – in contrast to lactic acid – a volatile acid and is emitted during pulp drying. Recently, the major contribution of acetic acid to TOC in the exhaust fumes of pulp drying, stemming from molasses, has been shown in a technical paper [14]. This environmental aspect has to be considered for bacterially-formed acetic acid in pulp in the same way.
- Hydrogen poses the risk of detonation on welding, *e.g.* after repairing a defect. This danger is known from the literature [6].
- Juice, saturated with CO_2, will liberate gas in the heating circuit and thus cause fluctuations in the mixers, which must be compensated for by increasing the dosage of defoaming agents, as reported in the literature [15].
- If Clostridial spores pass over into white sugar at a higher rate and microbiological limits are exceeded, awkward complaints may result.
- *C. thermosaccharolyticum*, which grows, due to its temperature sensitivity, in the uppermost part of the tower extractor and in the press water circuit [5], forms butyric acid [16], which is enriched in the molasses. The technical literature refers to a yeast growth inhibition by butyric acid [17, 18], as well as elevated contents of volatile acids in molasses, due to an intentional fermentation during extraction [19]. If beet molasses is used in fermentation, the original pH value of 7.5–8 is adjusted to a slightly acidic pH value of 5. When a fermentation plant performs a so-called "hot-acidic purification" [18], butyric acid is partly removed, but an unpleasant smell occurs. If a "cold purification" is employed [18], the volatile fatty acids remain dissolved to a greater extent and thus cause an increased hindering effect on fermentation.
- But beside molasses, butyric acid is relevant for white sugar. An investigation about the sensory properties of white sugar judged this volatile fatty acid to be particularly significant, amongst other volatile fatty acids [20].
- *C. thermohydrosulfuricum,* the second anaerobic spore-forming type of micro-organism, was found due to H_2S-formation on the employment of SO_2 in extractors [3]. It is extremely thermally resistant and thus cannot be suppressed effectively by elevating the extraction temperature. This organism forms ethanol instead of butyric acid and was intended for ethanol production [21]. During sugar manufacture, ethanol passes into vapor condensates and ends up as TOC-emission. In the absence of SO_2, the presence of this type of micro-organism cannot be noted easily, because tests for ethanol and acetic acid are carried out very infrequently and normally lactic acid is used as an indicator. As yet, no on-line sensors for hydrogen have been established in sugar factories.

Due to the numerous drawbacks caused by Clostridia, the type of fermentation during beet extraction should not be a question of pure chance. According to a report in the literature, the use of formalin in beet extraction obviously does not select a gas-free type of residual fermentation, because a higher consumption of defoaming agent was necessary, compared to hop products [15]. On the other hand, when hop products were applied to a DDS extraction trough, it was noted that acetic acid was eliminated better than lactic acid [22]. Subsequently, the authors tried to repress Clostridial acetic acid and consequently all unwanted metabolites of Clostridia in favor of lactic acid, and the first trials have shown a success [7].

The goal of the present paper has been to confirm these results and, additionally, identify a method for on-line detection of Clostridia. In the course of the investigations, unexpected findings were made, which will be described in this paper. With liquid chromatography a substance was found that forms in parallel with Clostridial growth. Its structure and identity should be elucidated, in order to judge its influence on sugar production.

2 Materials and methods

2.1 Samples from the process stream of sugar factories

In general, collective samples were used for investigations and balance trials. For samples of press water, raw juice and thin juice a well-tried deep freezing system was employed [23].

2.2. Synthetic nutrient solution for pure cultures

According to "Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ" [24], a synthetic nutrient solution for cultivation of Clostridia was prepared from 10 g Bacto-Tryptone (Difco Nr. 0123-17-3), 10 g sucrose, 2 g yeast extract (Difco Nr. 0127-17-9), 0.2 g $FeSO_4 \cdot 7\ H_2O$, 0.2 g Na_2SO_3, 0.08 g $Na_2S_2O_3 \cdot 5\ H_2O$ per L of distilled water, adjusted to pH = 7 and autoclaved for 20 min at 121 °C. This medium was cooled down after sterilization to inoculation temperature with as little movement as possible and bred without stirring to avoid oxygen entering. For maintenance and a first propagation of cultures, this nutrient solution was used in a semi-liquid state, with the addition of 3.5 g Agar-Agar (Bacto-Agar, Difco Nr. 214010) per L.

2.3 Pure cultures

Pure cultures of *Thermoanaerobacterium thermosaccharolyticum* (Basonym *C. thermosaccharolyticum*) with the strain numbers DSM 571 und DSM 572, as well as a pure culture of *Thermoanaerobacter thermohydrosulfuricus* (Basonym *C. thermohydrosulfuricum*) with the strain number DSM 567 were ordered from DSMZ [24] and cultivated in the synthetic culture media. The more temperature-sensitive strains 571 and 572 were cultured at

60 °C and strain 567 at 65 °C. After growing on, the cultures proved robust and rather insensitive against oxygen.

2.4 Butyric acid, acetic acid and ethanol in press water

In the course of this project, numerous samples were assayed for butyric acid. To reduce analytical expenses, the fact was used that samples low in dry substance content, such as press water, can be analyzed directly by HPLC after microfiltration, without distillation. Separation was carried out on a cation exchange column in H^+-form (Aminex HPX-87H, Biorad). The eluent was H_2SO_4 at a concentration of 5 mmol/L, detection was carried out with a UV/VIS-detector at 210 nm. Samples were filtered through a membrane, pore size 0.45 µm, before analysis. On chromatography, butyric acid showed a retention time of 19.8 min.
At 16.8 min, an unknown peak showed up which was initially interpreted as propionic acid due to the almost identical retention time. Later on, an additional HPLC-line was available for these studies (*Dionex*, model Summit), equipped with a diode array detector (200 to 598 nm). The peak in question did not correspond to propionic acid, but for the time being to an unknown substance. It proved to be nonvolatile and was detected, so to speak, through saving of a distillation for press water pretreatment.

2.5 Investigations with high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS)

2.5.1 Materials

Sulfuric acid (p.a.), barium acetate (p.a.), methanol (HPLC grade) were supplied by Merck (Darmstadt, Germany). 4-Acetylbutyric acid and uracil were purchased from Sigma (Deisenhofen, Germany). For HPLC investigations, water (conductivity of 18 $M\Omega^{-1} \cdot cm^{-1}$) was purified with an Elgastat water purification system (Bucks, UK).

2.5.2 Sample preparation

Due to the high sulfuric acid concentration in the mobile phase, the mass spectrometer (MS) could not be coupled on-line to the cation exchange HPLC column. Therefore, analyte fractions of four subsequent HPLC runs were collected off-line, using UV detection at 258 nm. By reinjection of 10 µL of these combined sample fractions, purity of the combined analyte fractions was checked. To remove the sulfuric acid, an equimolar amount of barium acetate was added. After 3 h at 6 °C, the precipitated barium sulfate was removed by filtration. The solvent of the filtrate was removed in a stream of nitrogen and the residue redissolved in 500 µL of water. 50 µL of this solution was injected into the reversed-phase HPLC-MS/MS system.

2.5.3 High-performance liquid chromatography

All HPLC analyses were performed on an Agilent HPLC system, model 1100 series (Agilent, Waldbronn, Germany), either coupled to an UV-detector (210 nm, 258 nm, 200–400 nm) or to a tandem mass spectrometer. Chromatographic separation was achieved on an Aminex HPX87H 300 × 7.8 mm cation exchange column (Bio-Rad, Vienna, Austria) protected with a 40 mm × 4 mm guard column, or on a HP/HPV RP-8 150 × 3 mm reversed-phase column (Waters, Milford, MA, USA). 0.005 mol sulfuric acid at a flow rate of 0.6 mL/min and 10 mmol ammonium acetate respectively in methanol-water (50:50, v/v) at a flow rate of 0.5 mL/min was used. UV detection (210 nm, 258 nm and 200–400 nm) was used with both columns, while the MS was only coupled to the reversed-phase column.

2.5.4 Tandem mass spectrometry (MS/MS)

MS and MS/MS analyses were performed on a PE Sciex API 365 MS/MS instrument (Applied Biosystems, Toronto, Canada), equipped with an electrospray interface (ESI). In the positive and negative ion mode voltages of 4300 V and –3500 V were applied, respectively. For product ion MS/MS experiments, the protonated molecular ion (positive ion mode) and the deprotonated molecular ion (negative ion mode) were selected as precursor ions, which were collisionally activated with an energy of 11 eV in the positive and 13 eV in the negative ion mode. Nitrogen was used as collisional gas. In the LC-MS mode the HPLC flow was split in a ratio of 1:50 before entering the ESI interface. For direct-inlet MS experiments, a syringe pump (Harvard Apparatus, South Natick, USA) was used at a flow rate of 5 µL/min.

2.6 Yeast inhibition test

Within the framework of this project, orientating fermentation tests were carried out in order to study the inhibiting effect of metabolites on bakers yeast production. A nutrient solution concentrate was prepared from 25 g Bacto-Pepton (Difco Nr. 211677), 15 g malt extract (Difco Nr. 0186-01-5), 15 g yeast extract (Difco Nr. 0127-17-9) and 50 g sucrose per L and autoclaved for 20 min at 121 °C. The substance to be tested was dissolved and added to 20 g nutrient concentrate in a 250 mL beaker, the pH value was adjusted to 5.5 after addition of a sufficient amount of distilled water. Then 0.5 g of dried yeast (Dr. Oetker) was added as a suspension (2%) and the net weight was adjusted to 100 g with distilled water. The beakers were capped with aluminum foil and their contents were incubated at 30 °C with vigorous stirring on a multiple magnetic stirring device. Impairment of yeast growth was judged by measuring the optical density at 600 nm.

2.7 On-line hydrogen detection

Up to now no on-line devices have been employed at sugar factories to monitor Clostridial growth; only *Draeger* tubes or handheld appliances have been used [6]. During the present investiga-

Table 1: Device for on-line measurement of hydrogen

Manufacturer	Draeger
Transmitter	Polytron® 2 for electrochemical sensors
Interface	4–20 mA for connection with central unit
Sensor	Measurement of hydrogen in air
Max. measuring range	0.3% (v/v)
Gas dilution	300 L/h fresh air + 100 L/h tower extractor headspace
Gas dilution pumps	KNF pump N 86 KNE + KNF pump NMP 30 KNE
Measuring range diluted	1.2% (v/v)
Temperature range	–20 to +40 °C
Calibration	With calibration adapter and measuring gas
Measuring gas	H_2 in N_2, 0.3% (v/v)

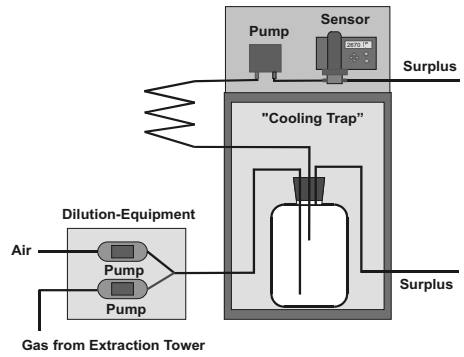

Fig. 1: Schematic representation of the device for on-line measurement of hydrogen

tion, a device already available for online measurement of hydrogen was adapted and equipped with a gas dilution appliance and a dephlegmator. Table 1 gives the technical data, Figure 1 is a schematic representation of the device.

3 Results

3.1 Identification of the unknown substance

3.1.1 HPLC analyses

Near the end of the 1999 campaign with butyric acid measurement in press water by HPLC at the Hohenau sugar factory, a parallelism between butyric acid formation and that of an unknown substance showed up (Fig. 2). The major fluctuations in butyric acid content were due to a periodic application of hop-β-acids, followed by blanks, as already reported in an earlier paper [7]. The parallel fluctuation of the unknown substance was also attributed to microbial activity, and for a short period this substance was judged to be propionic acid. With the aid of a diode array detector (DAD) it could be shown that the substance had – besides the UV absorption at 210 nm (through a limitation to 200 nm not unambiguously a maximum) – a second marked maximum at 258 nm, which propionic acid does not show (Fig. 3). Additionally, the unknown substance was not found in the distillate after a sample pretreatment by distillation, in contrast to the volatile butyric acid. Gas chromatographic control analyses by an external laboratory confirmed the butyric acid values, obtained by HPLC, and the parallel peak was not visible in GC. Thus the non-volatility of the unknown substance was confirmed again and it could not be the volatile propionic acid. Furthermore, it was possible to characterize the unknown substance by HPLC via retention time and UV spectrum.

Initially some special metabolites, described in the literature, were checked for identity with the unknown substance. Production of 1,2-Propanediol by *C. thermosaccharolyticum* [25] or *Thermoanaerobacterium thermosaccharolyticum* [26] did not fit with the peak. 4-Aminobutyric acid, mentioned in the literature and attributed to unspecified microbial activity during extraction [27, 28], disagreed in the same way. Other presumptions were based on the chromatographic data. Acetylbutyric acid showed a nearly perfect correspondence with respect to retention time and UV maximum, but could not be verified by mass spectrometry.

3.1.2 Investigations with pure cultures

In a recent paper [7], the appearance of butyric acid in beet juces was attributed to *C. thermosaccharolyticum*, based on a literature survey. Within the present study with pure cultures, the unknown substance appeared in parallel with butyric acid and thus can be regarded obviously as a Clostridial metabolite, which appears in technical sugar juices. Figure 4 shows a chromatogram of a nutrient solution of the DSM strain 572 (*C. thermosaccharolyticum*) with a butyric acid peak (*RT* = 20.75 min) and the unknown peak (*RT* = 17.55 min), which showed a corresponding maximum at 258 nm by the DAD. The correspondence between the substances, obtained from press water and a pure culture, is a further confirmation of the appearance of a thermophilic species in sugar factories, as described in the technical literature. *C. thermohydrosulfuricum* shows only traces of butyric acid and a lower but distinct peak for the unknown substance (Fig. 5).

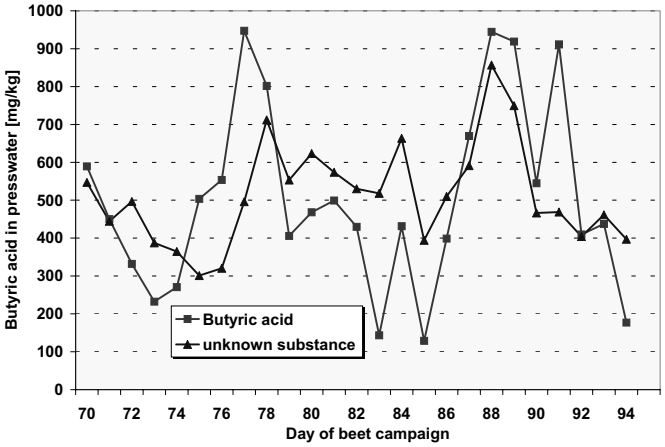

Fig. 2: Butyric acid and a parallel metabolic product

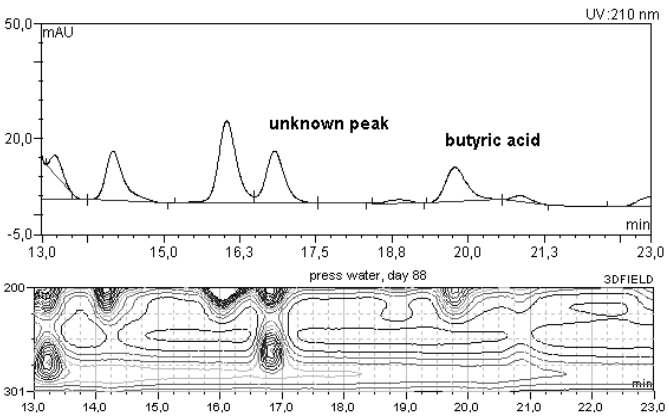

Fig. 3: Chromatogram of press water with the DAD spectrum

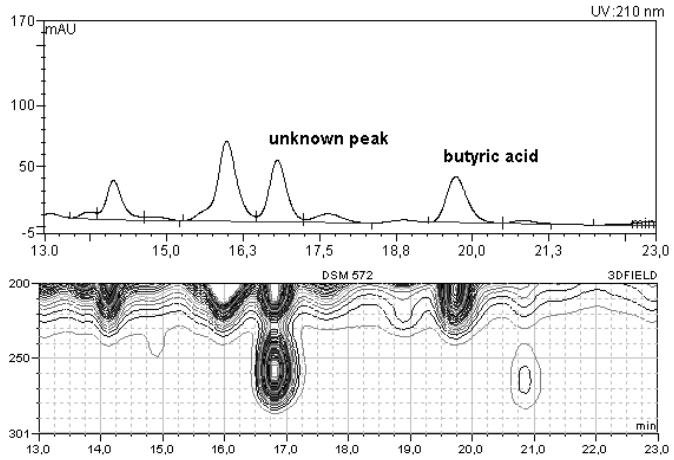

Fig. 4: Chromatogram of a culture of *C. thermosaccharolyticum* (*Thermoanaerobacterium thermosaccharolyticum)*, strain DSM 572, with the DAD spectrum

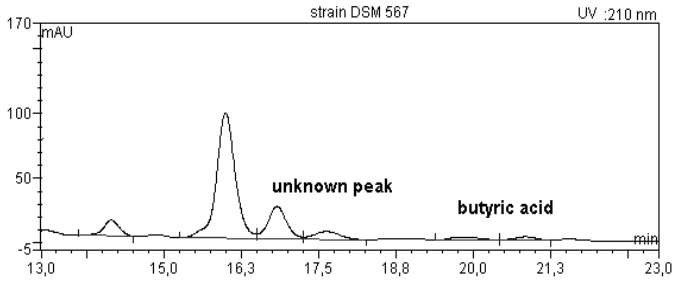

Fig. 5: Chromatogram of a culture of *C. thermohydrosulfuricum (Thermoanaerobacter thermohydrosulfuricus)*, strain DSM 567

After the inclusion of pure cultures it was possible to produce the unknown substance in solution, independent of the beet campaign and the availability of infected juices, in order to identify the non-volatile substance unambiguously by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

3.1.3 Results of LC-MS/MS analysis

3.1.3.1 Structural charcaterization and identification of the target analyte

Following HPLC separation, the molecular mass of the target analyte was calculated from LC-MS measurements in the positive (protonated molecular ion: m/z 113) and negative ion mode (deprotonated molecular ion: m/z 111.0) as 112 g/mol. Product ion spectra of the target analyte contained in the positive ion mode two fragment ions at m/z 70 and m/z 96 (Fig. 6a) and in the negative ion mode (Fig. 6b) one fragment ion at m/z 42.

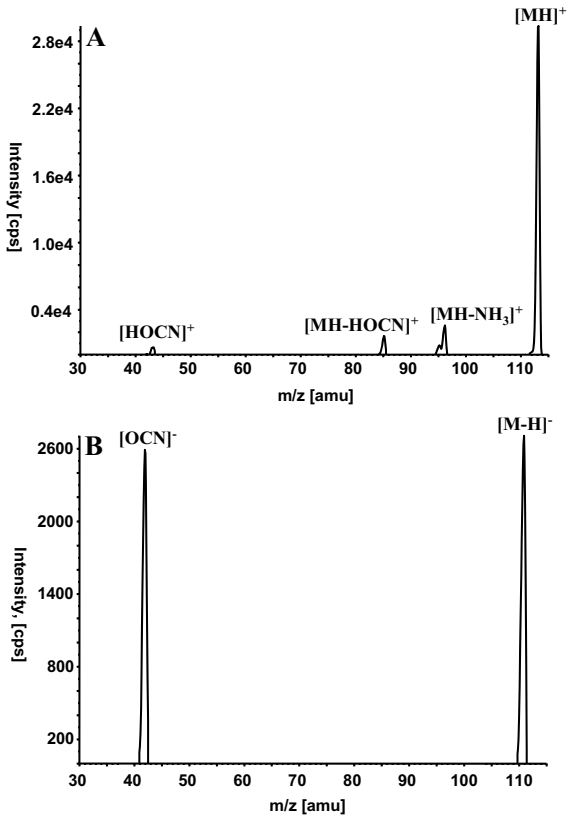

Fig. 6a (above): Product ion mass spectrum of the protonated molecular ion ($[MH]^+$).

Fig. 6b (below): Product ion mass spectrum of the deprotonated molecular ion ($[M-H]^-$).

UV as well as MS data indicate that the target analyte is not 4-acetylbutyric acid, although this compound offers almost identical chromtatographic retention behavior on the cation exchange column. However, its retention time on the reversed-phase column and its fragmentation behavior are completely different from the target analyte. 2-Furanecarboxylic acid, known to be a typical degradation product of carbohydrates, could also be excluded, although its molecular mass is identical with the target analyte, because the UV spectra and the product ion mass spectra are not identical.

The strong absorption at 258 nm in the UV spectrum (Fig. 7) indicates that the target analyte contains an aromatic system. Furthermore, its short retention time on a reversed-phase column reveals its high polarity.

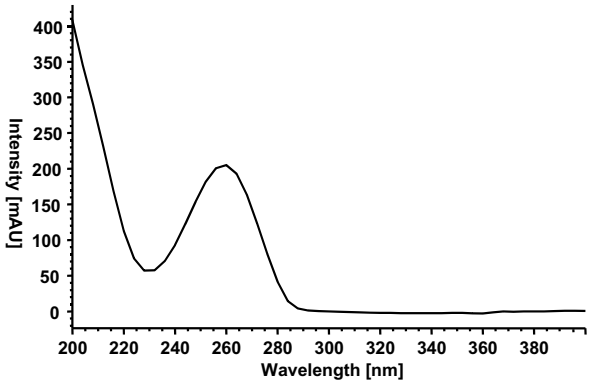

Fig. 7: UV spectrum (200–400 nm) of the target analyte

The fragmentation behavior of the target analyte (Fig. 6a and 6b) is dominated by the cleavage of ammonia and hydrogen cyanate from the protonated molecular ion in the positive ion mode and the formation of cyanate anions in the negative ion mode. Both fragmentation patterns are typical of nitrogen-containing heterocycles, bearing amino-, keto- and hydroxy-groups. On the other hand, fragment ions which are typical of carboxylic acids (cleavage of carbon dioxide and water) are completely absent in the product ion spectra. In view of the even molecular mass, an even number of nitrogen atoms (at least two nitrogen atoms) ought to be present in the target compound.

Based upon this UV and MS information, uracil was proposed as the possible structure of the target analyte.

3.1.3.2 Evidence for the proposed structure

To prove this assumption the UV spectrum, the chromatographic (cation exchange and reversed-phase column) and mass spectrometric behavior (product ion spectra) of a uracil standard were investigated and were found to be completely identical to the target analyte (Fig. 7). Ion formation in the product ion experiments can be rationalized by the following fragmentation pathways and ion structures:

Positive ion mode:	fragment ion at m/z 96 corresponds to the cleavage of NH_3 (ammonia)
	fragment ion at m/z 70 corresponds to the cleavage of HOCN (hydrogen cyanate)
Negative ion mode:	fragment ion at m/z 42 corresponds to cyanate anion.

These convincing results suggest that the target analyte can be unequivocally identified as uracil. By spiking with a defined concentration of uracil standard it was possible to estimate the uracil concentration in a sample of strain DMSZ 571 as approximately 30 ppm.

3.2 Influence of volatile fatty acids and uracil on yeast growth

In these trials the influence of propionic acid, butyric acid and uracil on yeast growth was investigated. In the concentration scaling it was taken into account that uracil in the samples is present only in minute contents (30 mg/kg), compared with the fatty acids. While, according to Table 2, with a fatty acids content of 0.2% already a certain inhibition and with 0.5% a massive inhibition arises, for uracil at the applied concentrations no effect was seen.

Table 2: Influence of volatile fatty acids and uracil on yeast growth

Substance to test	% on culture	Optical density at 600 nm (against water)		Increase in optical density within 5 h
		Starting time	After 5 h	
Blank		0.263	0.365	0.102
Propionic acid	0.1	0.262	0.359	0.097
Propionic acid	0.2	0.270	0.338	0.068
Propionic acid	0.5	0.267	0.283	0.016
Butyric acid	0.1	0.267	0.348	0.081
Butyric acid	0.2	0.271	0.304	0.033
Butyric acid	0.5	0.266	0.277	0.011
Uracil	0.01	0.272	0.370	0.098
Uracil	0.02	0.270	0.362	0.092
Uracil	0.05	0.276	0.373	0.097

3.3 Control of butyric acid formation with hop products

Results from the first trials to control Clostridial fermentation with hop-β-acids during the 1998 campaign have already been published [7]. In this paper, these results will be reproduced and the measures will be fine-tuned. With repeatedly switching on and off the dosage of hop-β-acids, artificial fluctuations of the butyric acid content were generated, which made possible a better indication of coherence (Fig. 8). The fluctuations in the butyric acid content serve as a reference base for other analytical results in Figures 9–11.

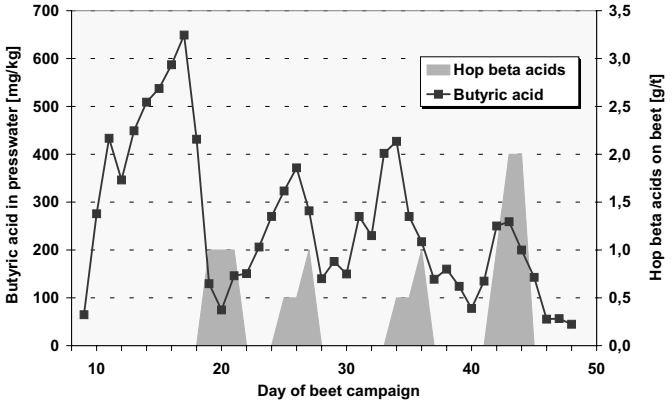

Fig. 8: Control of butyric acid with hop products

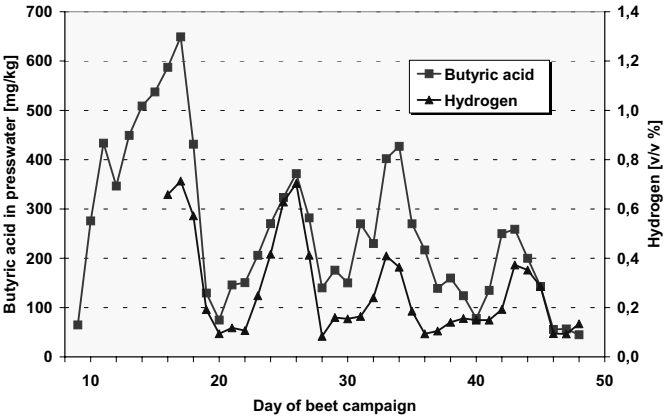

Fig. 9: Butyric acid and hydrogen signal

3.4 On-line detection of hydrogen with a sensor

From the headspace of the tower extractor, which represents a certain gas buffer, and from which hydrogen can only slowly escape via the tower vent, gas was aspirated, diluted in a ratio of 1 part gas + 3 parts fresh air and delivered to the measuring device. As press water forms a circuit with the upper zone of the tower and is fed back to this zone, the hydrogen signal goes in parallel with the butyric acid content of press water (Fig. 9). One has to bear in mind that the ethanol- and hydrogen forming *C. thermohydrosulfuricum* is able to grow in hotter (middle to lower) parts of the tower. Therefore a positive hydrogen signal is not specific for butyric acid, but for Clostridia. Independent of undesired Clostridial metabolites, the hydrogen detector signals a status, which in the case of a defect in the pulp transport elements, and any connected welding work, can be dangerous [6].

3.5 Acetic acid formation

Acetic acid appears within the fermentation equations of both Clostridia. Figure 10 shows the parallelism between butyric acid and acetic acid for *C. thermosaccharolyticum*. Because of the absence of an unpleasant odor, acetic acid itself is not recognized as a disadvantageous product. The drawbacks are a higher TOC emission on pulp drying and corrosion on vapor washers. Fortunately, acetic acid diminishes on control of butyric acid. A stronger production of acetic acid may be caused by *C. thermohydrosulfuricum* in the tower, without parallel production of butyric acid.

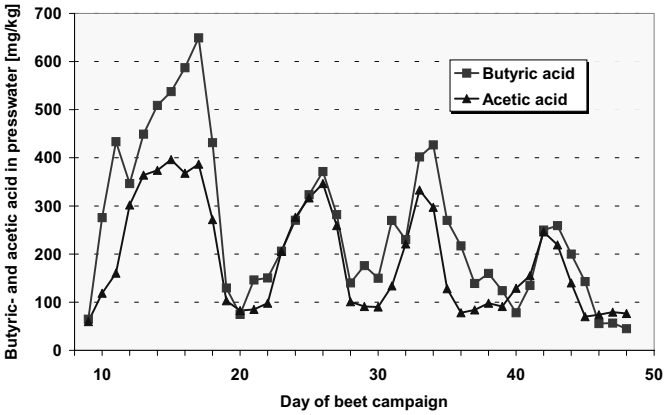

Fig. 10: Butyric acid and acetic acid

3.6 Ethanol formation by C. thermohydrosulfuricum

Figure 11 shows data from the second half of the 1999 beet campaign, which were also evaluated with respect to ethanol. From the graph one can see the parallel appearance of two different Clostridia, where *C. thermosaccharolyticum* forms butyric acid and *C. thermohydrosulfuricum* forms ethanol. Between day 60 and day 85 both organisms appeared in parallel and ethanol was

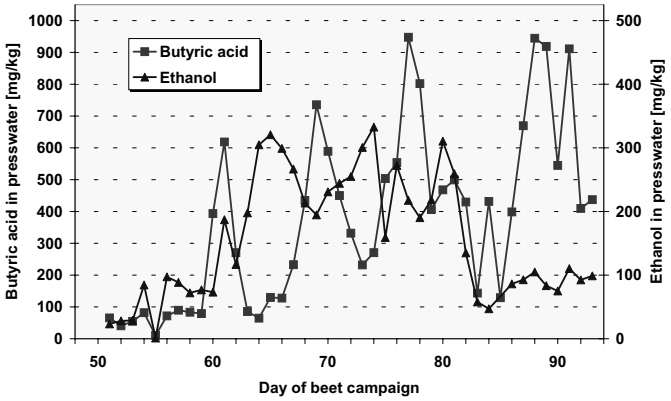

Fig. 11: Ethanol and butyric acid formation by *C. thermohydrosulfuricum*

carried over to press water by pulp. With a more intense hop dosage to the mixer on day 84, the ethanol forming Clostridium was eliminated, but the butyric acid forming one was affected only for a short time.

4 Discussion

At the beginning of the investigation it was assumed that propionic acid – a possible metabolite of Clostridia [29] – appears in parallel to butyric acid in beet extractors, but this assumption was wrong in the light of further analytical investigations. Data obtained by HPLC with a diode array detector showed a substance with the same retention time as propionic acid, but a different UV spectrum with a characteristic maximum at 258 nm. Concerning molasses quality for yeast production, only few scientific answers are available. Because fermentation during extraction could be a possible cause for yeast inhibition, the unknown substance should be identified and its importance for sugar production should be elucidated.

Comparison of literature data, concerning by-products of Clostridial fermentation, with the chromatographic behavior of the unknown substance, which also appears in pure cultures, gave no agreement. The appearance in pure cultures ascertained that the substance stems from Clostridial activity in sugar juices and not from a different source. Investigations with LC-MS/MS unambiguously gave evidence that the unknown UV absorbing substance is uracil.

Subsequent to this evidence, a literature survey concerning uracil was possible. Only selected examples from this survey are discussed here. The usage of uracil by Clostridia is treated by *Hilton* et al. [30], the usage of uracil in general in "Biology of the Prokaryotes" [31]. Secretion of nucleotides into the substrate by microorganisms was also dealt with [32]. Another study emphasizes the UV absorption of uracil and paper chromatographic data are presented [33]. Furthermore, the microbial conversion of pyrimidine to uracil and its secretion into the substrate is addressed [34]. Ultimately, a Japanese patent from 1988 [35], dealing with the production of uracil by Clostridia, proved to be the best literature source to confirm the present finding.

After identification of uracil it was possible to compare its influence on yeasts with butyric acid and propionic acid. It was taken into account that uracil has a high UV absorption and appears in much lower concentrations in the substrate, so it was applied in smaller concentrations. Under these conditions simple trials with Baker's yeast confirmed the well-known influence of butyric acid and propionic acid, but uracil did not show any effect. However, the findings about the appearance of uracil in sugar juices enrich the basic knowledge about microbiological phenomena during sugar production. In a paper dealing with nucleotid cleavage products in technical sugar solutions uracil had been already mentioned as one of several nucleo-bases of pressed beet juice and molasses [36–38], but not in connection with microbiological phenomens.

Hop-β-acids proved well as a measure for selective control of Clostridial infections. With low doses in the range of 1–1.5 g hop-β-acids per tonne of beet, lactic acid fermentation by Bacillus species is selected and Clostridial fermentation is suppressed. These amounts are only rough guidelines and have to be adapted per factory and campaign, based on guiding values.

The on-line hydrogen determination may be used as a guide value, but determination of butyric acid, acetic acid, lactic acid and ethanol at the laboratory is an alternative. A high content of butyric acid indicates for an infection by the more temperature-sensitive *C. thermosaccharolyticum* (max. 60 °C), which can be eliminated by a somewhat higher temperature kept on the water side of the extraction system. A precondition is a sufficient reserve of heating surface.

An elevated ethanol content in raw juice or in press water indicates an infection with *C. thermohydrosulfuricum*. This organism has the highest temperature resistance of all thermophiles, growing in extraction towers (75 °C). Due to the temperature sensitivity of the beet material it is impossible to eliminate this organism by higher temperatures. This Clostridium species is able to grow in hotter parts of tower extractors (mid-tower position) and has to be controlled with biocides.

Both Clostridia species give rise to elevated hydrogen contents in the headspace of the tower, as well as to elevated acetic acid contents in juices and in the pulp. These two findings signal Clostridial activity and have, in case of elevated values, to be interpreted correctly before taking counter measures. With low values of acetic acid and hydrogen, the occurrence of both Clostridia can be ruled out.

Interestingly, the pure culture, which was isolated by *Hollaus* [4] from factory juices and deposited at the DSMZ, forms the same characteristic compound uracil, which was, some thirty years later, isolated from sugar factory press water. This finding corroborates the right characterization of the microbial species in tower extractors and completes further microbiological knowledge in the sugar industry. The characteristic UV maximum of uracil at 258 nm, together with the retention time, permits a detection of Clostridial activity by HPLC. This could be helpful in case of troubles caused by Clostridia in the future.

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Détection et contrôle des bactéries sporifères, anaérobies strictes dans les tours d'extraction des betteraves sucrières (Résumé)

La fermentation par des bactéries du genre *Clostridum* au cours de l'extraction contribue au taux élevé d'acides gras volatils dans le processus de fabrication du sucre et, par conséquent, à la détérioration de la qualité des mélasses. De plus, des émissions d'odeurs désagréables et de TOC* élevé en sont les effets secondaires.

Des produits dérivés du houblon, introduits dans l'industrie sucrière par l'Institut Sucrier de Recherche de Tulln (ZFT) semblent être efficaces dans la lutte contre ces fermentations indésirables. Un dosage peut être déclenché par un signal donné par un détecteur d'hydrogène en ligne lors de l'apparition de Clostridium. Avec des applications périodiques de produits dérivés du houblon, on a observé un parallélisme entre l'apparition d'acide butyrique et une substance inconnue, absorbant les UV, qui a été identifiée comme étant l'uracile. L'uracile n'a pas encore été décrit dans le contexte de la microbiologie de la production du sucre, mais contrairement à ce qui se passe avec les acides gras volatils, on n'a pas observé d'inhibition dans la croissance des levures. Toutefois, grâce à sa caractéristique d'absorber les rayons UV à 258 nm, il peut servir comme indicateur en HPLC pour détecter une fermentation due au Clostridium dans les tours d'extraction.

Detección y control de bacterias estrictamente anaerobias que forman esporas en torres de extracción en la industria del azúcar de remolacha (Resumen)

Fermentaciones clostridiales durante la extracción de remolachas azucareras pueden contribuir a un contenido elevado de ácidos grasos volátiles y, de este modo, a una alteración de la calidad de la melaza así como mostrar efectos desfavorables como molestias por olores o emisiones elevadas de TOC. Los productos de lúpulo, introducidos en la industria azucarera por Zuckerforschung Tulln (ZFT), parecen ser apropiados para el control de tales fermentaciones erróneas. En el caso que se presenten clostridias será posible producir una dosificación en base de una detección online de hidrógeno. Por el empleo periódico de productos de lúpulo se observó un paralelismo entre ácido butírico y una sustancia con absorción ultravioleta, identificada como uracil. Hasta entonces no se había descrito uracil en combinación con la microbiología de la producción de azúcar, y comparado con ácidos volátiles no mostró una acción inhibidora de levadura. Por su absorción ultravioleta característica a 258 nm, sin embargo, sirve como indicador de HPLC para fermentaciones clostridiales en torres de extracción.

Authors addresses: Dr. *Günter Pollach*, Dr. *Walter Hein*, Zuckerforschung Tulln Gesellschaft m.b.H. (ZFT), Reitherstraße 21–23, A-3430 Tulln; Mag. *Alexander Leitner*, Dr. *Peter Zöllner*, Institut für Analytische Chemie der Universität Wien (IAC), Währinger Straße 38, A-1090 Wien. Correspondence: *G. Pollach*, e-mail: guenter.pollach@zuckerforschung.at