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Rapid Methods in the Micro World of Beer

Implementation of a rapid method in a brewery quality analysis system based on a user-friendly validated platform

Company profile

Established in 1864 by the Heineken family, HEINEKEN has a long and proud history and heritage as an independent global brewer. We brew quality beers, build award-winning brands and are committed to enthusing consumers everywhere.
Four key factors make us unique:

1. Heineken® is a truly global beer brand, enjoyed in 178 countries around the world
2. We have a unique, worldwide footprint with operations in over 70 countries, which means we have a broad reach for our brands
3. We have an internationally diverse, dynamic, committed and entrepreneurial team of over 85,000 employees
4. The passion of the Heineken family remains as strong today as it was in 1864 when we first started brewing beer.

Today, HEINEKEN is the number one brewer in Europe and the number three brewer by volume in the world. With recent acquisitions in Africa, India, Asia and Latin America, we are continuing to increase our presence within emerging markets, which will contribute to our ongoing growth.

Beer spoilers & contamination

Beer is not a friendly environment for growth of microorganisms. Few nutrients and sugars remain after the yeast fermentation, the pH is relatively low (3.9-4.6), the levels of CO_2 are very high (typically 5g/l) and O_2 is almost absent. Moreover, a few microorganisms can survive in an environment containing high levels of alcohol (typically 5% for pilsner beer) or in presence of hop components that have antibacterial effects, especially against gram positive bacteria.
During the different stages of beer production, from raw materials to packaged beer, the characteristics of the product change and specific microorganisms can contaminate at each step of the process.
Wort is normally sterile when it leaves the brewhouse. Microorganisms that can grow in the wort and in the early stages of the fermentation may be picked up from the equipment, from the pitching yeast, or during aeration. These include Gram negative bacteria such as *Enterobacter*, *Gluconobacter* and *Acetobacter*, Gram positive Lactic Acid bacteria and wild (i.e. non-brewing) yeasts. The pH decrease and alcohol production during fermentation normally inhibits the growth of these microorganisms.
Most Gram positive bacteria are sensitive to hop constituents and do not grow in beer. Important exceptions are hop resistant species of *Lactobacillus* and *Pediococcus*, both of which can cause serious damage, especially during fermentation. In case of a high level contamination during fermentation (>10^4 cells/mL), these microbes can produce off-flavour compounds such as lactic acid and diacetyl. In some cases, the contamination can cause premature yeast flocculation and stuck fermentations. Wild yeasts are also capable of growing during fermentation and can contribute phenolic off flavours. Some wild yeasts can also degrade the residual dextrins in the beer/wort and bring about over attenuation.
Secondary contamination by beer spoiling microorganisms can occur during the filling operations. This is especially dangerous in hygienic filling operations where the beer is not pasteurized in the package (e.g. kegs, PET containers and sometimes glass bottles and cans). Microorganisms that are capable of growing in packaged beer are summarized in the table below.

Traditionally, contaminants of packaged beer included acetic acid bacteria, lactic acid bacteria, and wild yeasts. Because current brewing operations are very effective in excluding oxygen, acetic acid bacteria are now rarely a problem except in draught beer outlets (because of the interface with air).
The extremely low levels of oxygen in packaged beer has enabled the emergence of the beer spoiling anaerobic Gram negative bacterial species, *Pectinatus* and *Megasphaera,* which were not detected in breweries before 1970. These microorganisms are of concern because they can produce particularly unpleasant sulfidic off-flavours as a result of their anaerobic metabolism. In addition they are difficult to detect using traditional brewing plating techniques. They are very heat sensitive and are easily eliminated during pasteurization, but are a particular risk in hygienic filling operations.

Most common beer spoiler species in final product

Hop resistant lactic acid bacteria (LABs)	*Lactobacillus brevis* *Lb lindneri, Lb backii* *Lb collinoides, Lb rossiae* *Pediococcus damnosus*
Strictly anaerobic Gram negative bacteria	*Pectinatus* *Megasphaera*
Beer spoiling wild yeasts	*Saccharomyces ceraviseae* subsp *diastaticus*

Controlling microbiology in the brewery

In breweries, microbiology is controlled by (i) Choosing equipment that is hygienically designed and maintained (ii) rigorous external and internal cleaning and disinfection programmes (ii) maintaining a pure yeast, free of contamination by unwanted micro-organisms (iv) sterilizing wort by boiling and (v) eliminating microorganisms from the final product by pasteurization or sterile filtration. In order to ensure these conditions are met, we also have rigorous microbiological quality control. It is important to be aware that excellent microbiological methods do not in themselves guarantee the hygienic status of the brewery. In practice hygiene and microbiology are controlled on a daily basis by monitoring the process indicators that influence microbiology. When these deviate from the specified norms we can anticipate microbiological issues which are verified by our QC analysis. In addition, it is essential to have processes in place that describe what actions to take in response to deviations from the microbiological norms.

Traditional microbiological methods

A typical brewery microbiology control plan, summarises what samples must be taken from brewhouse wort to packaged product and defines the frequency and time of sampling and what volumes must be analysed. The sample and control plan also indicates the test media and methods for each sample type and the accepted microbiological norms.
An example of a typical microbiological sample and control plan is presented below.

Routine control protocol for different brewery samples

Sample type	*Volume*	*Method*	*Medium*	*Incubation*
Fermentation tank Lager tanks Rest beer tanks	1 mL	Poured plate	Raka Ray	Anaerobic 5 days - 30°C
	1 mL	Poured plate	WLD	Aerobic 3 days - 30°C
Bright beer and packaged beer	/	Enrichment	NBBC 7%	Anaerobic 11 days - 30°C
	100 mL	Membrane filtration	Raka Ray	Anaerobic 5 days - 30°C
	100 mL	Membrane filtration	WLN	Aerobic 3 days - 30°C

Traditional brewery microbiological plating methods have a long history and brewery production and quality managers are very familiar with them. They generate results in the form of CFU per volume tested to which brewery staff can quickly relate. Because they rely on growing single cells into colonies, only viable microorganisms are detected. However, there is no single medium that can detect all beer spoiling species (e.g. gram negative anaerobic bacteria cannot be detected in this manner), it takes time (up to 7 days) to develop colonies on agar media and these methods do not identify or give information about the beer spoiling potential of the detected colonies. This often leads to false positive results and leaves brewery managers wondering how to interpret such results and in doubt about what, if any, RCI and CAPA actions to take. Broth enrichment methods can take up to 21 days to detect anaerobic species, which can result in unacceptable delays in product release.
For these reasons it is of interest to develop and implement rapid microbiological methods that can save time and add value.

Rapid Microbiological Platform

There are various existing fast detection method for food and beverage contaminants that could be adapted for detecting beer spoiling microbes. Some rapid detection kits, usually based on PCR are already available. For our purposes, the ideal rapid method should:

- Be sensitive to detect low levels of infection
- Be easy to perform
- Be easy to interpretet
- Be robust and reproducible
- Predict beer spoilage potential
- Have low capital investment cost
- Have low cost per test
- Can be used on filterable samples
- Can be used in yeast containing samples

Heineken chose to implement a DNA based method: real time PCR. The platform used is called GeneDisc and is provided by PALL Corporation (Port Washington, NY, USA). This platform has been chosen because it fits most of the above criteria, especially in terms of its user friendliness, ease of implementation and its low investment cost relative to competitor technologies. In this system, 6 samples can be loaded into a disc. Each sample is then distributed across 6 wells and simultaneously analysed by PCR for different target beer spoiling bacteria and indicator species.

Bacterial species detected by PCR with the Beer Spoiler GeneDisc plate

Well number	FAM Fluorescence Dye Detection	ROX Fluorescence Dye Detection
1	*L. collinoides* & *paracollinoides*	Inhibition control
2	*L. backii*	
3	*L. brevis*	*Pectinatus* spp.
4	*L. lindneri*	
5	*Pediococcus* spp.	*Megasphaera* spp.
6	*Lactobacillus* group [1]	

[1] *L. casei, L. paracasei, L. coryniformis, L. rossiae, L. parabuchneri (=frigidus), L. perolens* and *L. plantarum*

This PCR platform was first validated by Heineken research group. Software, hardware, test conditions and settings were adjusted. A lab scale feasibility study was performed to establish specificity, limits of detection, reproducibility and robustness. A pilot trial was then performed in one brewery for six months, followed by a final round of hardware and software adjustments. Finally full scale trails were performed in 4 breweries (Poland, UK, Mexico, and Austria) for 3 months.
Currently, PCR is used in multiple breweries to control bright beer and packaged product. The sample is enriched and incubated at 30°C. This enables the enrichment phase to be reduced from 11 to 4 days. This

protocol was developed to simultaneously detect and identify all known beer spoiling bacteria, including the strictly anaerobic species *Pectinatus* and *Megasphaera* that are not detectable by traditional plating methods. The benefits of the method are: (i) quarantine time for hygienically filled product can be reduced, (ii) product performance in the market can be predicted, (iii) rapid tracking and tracing of infection in the brewery is enabled and (iv) early detection is facilitated, enabling timely intervention to prevent serious incidents. In general the risk of product recalls due to market complaints, along with their associated costs is reduced. Ultimately, incorporating the new method into the sample and control plan enables rationalization of the plan and further cost savings.

In this case the key steps in getting a new method accepted and incorporated can be summarised as follows:

1. Choose a platform that meets the desired criteria (investment cost, cost per test, user frienliness, ease of interpretation, simplicity etc)
2. Adapt this platform to the specific needs of HEINEKEN (target microbes and enrichment method)
3. Perform a laboratory scale evaluation and comparison with the standard method. Follow the ISO norm for comparison of new methods
4. Perform a pilot scale feasability study in one brewery followed by a critical evaluation of the outcome
5. Perform full scale brewery trials with agreed success criteria
6. Evaluate the outcome and conclude the business case.
7. Establish new standards (analytical method and norms) and incorporate the method into the sample and control plan
8. Roll out as an approved standard for analysis of packaged prduct and communicate this to the breweries
9. Rationalise the sample and control plan
10. Define how to respond to microbiological failures including RCI and CAPA activities

Incorporating a new method into the sample and control plan

Developing and optimizing a rapid detection method is one step, but getting brewery acceptance and incorporating it into the sample and control plan and into the product release plan presents additional challenges.
Traditional microbiological methods (counting colony forming unit) have a long history and, despite their shortcomings, are widely accepted. Making predictions about the spoilage risk of a product by using DNA based techniques requires a dramatic shift in mental attitude from brewery management. This can only be accomplished by demonstrating and communicating the benefits of the new technologies clearly and by agreeing success criteria with all relevant stakeholders in advance of performing full scale trials. Breweries that have successfully adopted this methodology are powerful champions for the new method.

At Heineken the developed rapid microbiological detection test was incorporated in the Sample and Control plan for hygienically packaged products. As a result, some traditional plating tests could be eliminated and, eventually, sample and control plans could be rationalised as breweries established improved microbiological control. The next phase will be incorporating the rapid microbiological method for the analysis of fermentation, lagering and yeast storage samples. This will lead to further rationalisation of the sample and control plan.

Bibliography

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