122

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True sample-to-results molecular analysis of multiple species associated with beer spoilage

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Abstract:

Despite the inhospitable environment of beer towards the growth of microorganisms, beer remain susceptible to various spoilage organisms that have developed mechanisms enabling growth in the presence of hops, alcohol, and acidic conditions. The standard detection strategy for these spoilage organisms has traditionally involved culturing, a process that may take up to a week to obtain results. This wait is problematic for brewers and may result in delayed release or release of untested and potentially spoiled product to market. In contrast to culture, molecular detection provides a rapid, sensitive, and specific method of identifying the presence of beer spoilage organisms. Available kits on the market identify many species of spoilers, regardless of whether the specific strain contains genes associated with spoilage, or focus on a limited number of beer spoilage genes, risking the lack of detection of other relevant spoilers. Most available tests require pre-enrichment and other lengthy preparative steps, in addition to sophisticated molecular lab equipment that may not be present in a craft brewery. This study describes the development of the Beer SpoilerAlert™ assay; a robust sample-to-results molecular detection system for beer spoilage organisms, including various species of lactic acid bacteria (LAB), and four genes associated with beer spoilage in LAB. Concurrently, the assay detects the presence of the wild yeast *Brettanomyces bruxellensis* and brewer's yeast. Samples taken at any point during the brewing process are loaded into sample tubes and placed in the sample rack. The sample rack, cartridges and reagent kit are placed in the Encompass *Optimum*™ workstation and all processes required for lysing organisms, extracting nucleic acids, amplifying and detecting target genes, and analyzing results, are automatically performed without user intervention on the Rheonix CARD® (Chemistry and Reagent Device) cartridges in the workstation. Reagents are dispensed by an onboard robot and liquid is moved via microfluidic pumps and channels within the cartridge. Amplification occurs via the onboard thermocycler and endpoint detection occurs through hybridization to a low-density capture array. Captured targets are detected and analyzed by an onboard camera and imaging software, which provides the user with a report of which genes and/or organisms are present. Four individual samples are analyzed per cartridge, with 6 cartridges per run, resulting in 24 independent samples analyzed in 5 hours, with minimal hands on time. Due to the sensitivity of the assay, a pre-enrichment step is not required in most instances. However, if the user prefers to do so, a pre-enrichment step is compatible with this assay with no further modifications. The results described demonstrate validation of the assay using beer samples spiked with known microbes and actual beer samples suspected or known to contain spoilage organisms. Results were verified through conventional culturing methods.

Materials and Methods

Bacterial strains, media and culture conditions

Microorganisms were obtained from BRI, ATCC or USDA and maintained as stock cultures in 20% glycerol at -70˚C and propagated as per provider instructions. LAB were grown in liquid or solid MRS media (DIFCO) prepared as 55g/L MRS powder and 16 g/L Agar (Sigma) and incubated at 28˚C, under a 10% CO_2 atmosphere. *Brettanomyces bruxellensis* was grown in YM media (Difco) and incubated at 25˚C, aerobically.

Sample preparation and analysis on Encompass *Optimum*™ workstation

All experiments were performed with 2 biological replicates and 4 technical replicates. Cultures were started from a frozen stock on solid media first, from which one colony was grown overnight (O/N) in 5 mL of liquid MRS. Next, 100 µL from the O/N liquid culture was transferred to 5 mL fresh MRS and grown O/N under the same conditions to obtain a working culture in logarithmic growth stage (Figure 1). Limit of Detection (LOD) was determined as the most diluted sample that was detected on the instrument. The cultures were analyzed on the Encompass *Optimum*™ workstation using the Rheonix CARD® cartridge and reagent kit following the flow described in Figure 2.

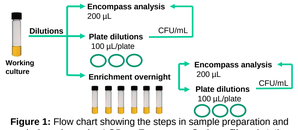

Figure 1: Flow chart showing the steps in sample preparation and analysis to determine LOD on Encompass *Optimum*™ workstation

Control DNA testing

Purified DNA, 100 pg, from individual strains of various LABS were subjected to analysis on the Encompass *Optimum*™ workstation.

Cross-reactivity testing

DNA, $5x10^4$-$5x10^6$ copies/ml from *Enterococcus faecium* and *Bacillus cereus* were subjected to analysis with the Beer SpoilerAlert™ assay on Encompass *Optimum*™ workstation. Furthermore, an aliquot of the purified DNA was amplified with species specific primers using a BioRad MJ thermocycler. Resulting amplified products were analyzed via gel electrophoresis. Additional cross-reactivity studies are ongoing.

Brewery sample testing

Brewery samples were tested directly with the Beer SpoilerAlert™ assay on the Encompass *Optimum*™ workstation. Greater than 300 individual samples were analyzed multiple times. Samples were either colonies from culture plates analyzed directly, or in-process samples analyzed directly and/or subjected to filtration onto 0.45 micron mixed cellulose filters followed by overnight enrichment of the filter in MRS or NBB growth media. Enriched cultures, 200 µL, were subjected to analysis.

Table 1: Targets detected with the Beer SpoilerAlert™ assay with key

RS	Assay reference spot
MM1	Control for PCR master mix 1
MM2	Control for PCR master mix 2
SC	*Saccharomyces cerevisiae*
BR	*Brettanomyces bruxellensis*
LB	*Lactobacillus brevis*
PED	Target found in all currently sequenced *Pediococcus* species
PC	Target found specifically in *P. claussenii* & *P. acidilactici*
LABS	Plasmid biomarker present in strains of various LABS
HA	Hops resistant gene, *horA*, found on plasmids in various LABs
HC	Hops resistant gene, *horC*, found on plasmids in various LABs
BA	Hops resistant gene, *bsrA*, found in *P. claussenii*
BB	Hops resistant gene, *bsrB*, found in *P. claussenii*

Beer SpoilerAlert™ Assay Workflow

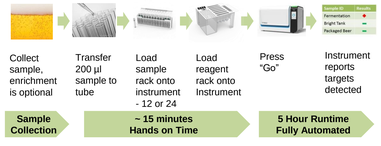

Figure 2: Encompass *Optimum*™ workstation and Beer SpoilerAlert™ assay workflow

Results

Table 2: LOD with and without enrichment

LIMITS OF DETECTION*		
Microorganism tested	**Direct detection CFU/mL**	**Enriched O/N CFU/3 mL**
Lactobacillus brevis	~8,000	1 to 5
Pediococcus damnosus	~1,000	1 to 5
Pediococcus claussenii	~1,000	1 to 5

***** Studies to define LODs for *S. cerevisiae* and *B. bruxellensis* are ongoing.

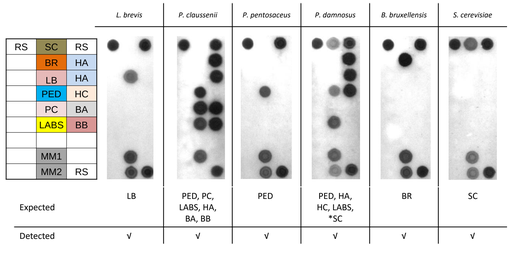

Figure 3: Purified DNA from specified organisms was subjected to amplification with master mixes from the Beer SpoilerAlert™ assay. Resulting amplified products were analyzed via reverse dot blot and end point detection on the Encompass *Optimum*™ workstation. The filter key indicates the location of the capture probe of the specific targets (refer to target description in Table 1). The expected targets indicate which targets are expected to be amplified and detected in each organism. The filter images confirm detection of the appropriate targets in each organism. For this experiment, a non-spoilage *L. brevis,* was analyzed. No beer spoilage genes are present in this strain. RS: reference spot. MM1 and MM2: Control spots for the two PCR master mixes used in the Beer SpoilerAlert™ assay.

* The lot of this commercially available *P. damnosus* was contaminated with *S. cerevisiae* DNA.

Sample type & number	Colony (97)	Colony (99)	Colony (100)	Membrane (91)	Membrane (148)	Beer (107)
Plate reading	Cocci/rods	Cocci/rods	Rods	Cocci/rods/ mold	3 rods	Cocci
Beer SpoilerAlert™ assay result	SC, PED, **HA**, LABS	SC, PED, **HA, HC**	SC, **LB, HA**	SC, LB, PED, PC, LABS, **HA, HC, BA, BB**	SC	SC, PED, **HA,** LABS

Filter key: RS SC RS; LB BR HA; LB MM1 HA; LB PEDS HC; LB PC BA; LB LABS BB; LB MM2 LB; LB LB LB; LB LB RS

Figure 4: Testing of brewery samples with the Beer SpoilerAlert™ assay. Greater than 300 samples were tested side-by-side with standard microbiological methods and the Beer SpoilerAlert™ assay on the Encompass *Optimum*™ workstation. The figure shows selected data from 6 positive samples from a variety of sample types. Colonies were picked from a plate, suspended in a buffer and analyzed. Membrane samples were from 50 mL of beer sample from either a tank or bottle, vacuum filtered, and the filter was inoculated into media broth. Beer samples were analyzed directly.

Species	Targets predicted	Targets detected
L. backii	HA, HC, LABS	HA, HC, LABS
L. brevis	LB, HA, HC	LB, HC
L. buchneri	HA	HA
L. casei	none	none
L. lindneri	HA, HC	HA
L. plantarum	LABS	none
P. acidilactici	PED, PC	PED, PC, HC
P. claussenii	PED, PC, BA, BB, HA, LABS	PED, PC, BA, BB, LABS
P. damnosus	PED, HA, HC, LABS	PED, HA, HC, LABS
P. inoptinatus	PED. HA	PED, HA, LABS
P. parvulus	PED	PED, LABS

Figure 5: Analysis of purified DNA of individual strains of different LABs. All predicted genomic targets were detected. Discrepancies between predicted and detected targets correspond to plasmid markers that may not be present in a particular strain.

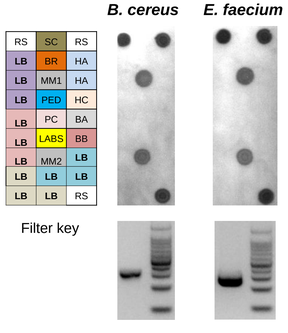

Figure 6: Testing of cross-reactivity with Beer SpoilerAlert™ assay. The filters demonstrate that no SpoilerAlert™ targets were detected (upper filters), despite the presence of the *B. cereus* and *E. faecium* specific DNA confirmed by organism specific amplification (lower gels).

Workflow Comparison

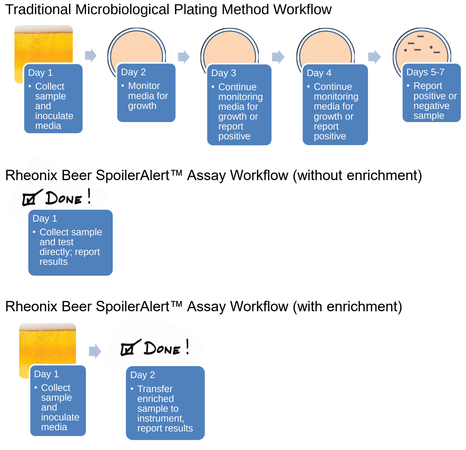

Figure 7: The schematic demonstrates traditional microbiological methods to test for the presence of beer spoiling organisms compared to the workflow using Beer SpoilerAlert™ Assay and Encompass *Optimum*™ workstation, highlighting the significantly reduced time to actionable results.

Summary

We have developed a fully automated sample to results assay for detecting the presence of beer spoilage organisms and genes associated with their ability to grow in the beer environment. We performed feasibility with commercially available organisms and/or purified genomic DNA. In addition, using known organisms, we performed LOD and cross-reactivity studies. Furthermore, the assay was validated using in process beer samples and organisms previously isolated from beer in an active brewery. The Beer SpoilerAlert™ assay successfully detects LABs, *B. bruxellensis*, *S. cerevisiae*, and 4 hops resistance genes in 5 hours.

Conclusions

Here we demonstrate the development and evaluation of the first commercially available, fully automated molecular assay for detecting beer spoilage organisms. The Beer SpoilerAlert™ assay detects not only beer spoilage organisms, but also hops resistance genes that allow these organisms to propagate in beer. The ability to detect both contaminating organisms and hops resistance genes allows brewers to make timely, informed decisions about the spoilage potential of the sample. The entire procedure can be completed in 1 (without enrichment) or 2 days (with enrichment), either way that is 3-5 days faster than traditional microbiological methods.