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Application Note

Rapid detection of yeast, mold and bacterial contaminants in beverages using fluorescence-based technology

Many beverage manufacturing processes are susceptible to yeast, mold or bacteria contamination. Contamination can alter the odor, flavor or turbidity of a beverage, resulting in customer dissatisfaction and, in some cases, in product recall. For these microorganisms, traditional monitoring methods require up to 10 days to obtain microbiological results allowing the release of the product. A rapid microbiology system that can detect potential contamination 3 times faster than traditional monitoring methods would result in a significant cost saving and preserved company reputation. The fluorescence-based technology is a convenient and a sensitive platform for the quantitative detection of contaminants in filterable samples. This rapid microbiological method is based on a universal enzymatic fluorescent staining of viable and culturable microorganisms. The fluorescent staining procedure is non-destructive, allowing microorganism identification following a positive result.

The fluorescence-based technology offers a fast and reliable alternative for the rapid detection of spoilage microorganisms in beverages. Throughout the beverage manufacturing process the system enables a faster response and corrective action. It improves process control and product yield and permits the faster release of final product to market.

Materials

- Fluorescence reader systems (EZFKIT001WW,MXQUANK01)
- Filtration systems (EZFTIMIC01, MXPPLUS01)

Equipment

- Fluorescence reagent kit (EZFREAG57, MXQTV0KT1)
- 100 mL, 0.45 µm mixed cellulose ester funnels (MZHAWG101, MXHAWG124)

Media

- Sabouraud dextrose agar, SDA (1460280020
- Sabouraud media cassette, SDAK (MXSMCSD48)
- De Man, Rogosa & Sharpe broth, MRSK (MHA00MRS2
- MRS agar, MRS (Difco, 288130)
- Potato dextrose agar, PDA (Oxoid, CM0437)
- BAT agar, BAT (1.07994.0500)
- Orange serum broth, OSB (MHA000P20)
- Wallerstein nutrient broth, WLN (MHA000P2N)
- Yeast and mold agar (MXSMCYM48)
- Pectinase (Sigma P2611)

Matrices tested

Flavored mineral water, ice tea lemon, apple juice, energy drink, ice tea green grapefruit, ice tea peach, ice tea red, mango juice, ready-to-drink black tea, ready-to-drink green tea, strawberry juice, red wine without sorbate, red wine with sorbate, bag-in-box (BIB) red wine, sweet wine

Microorganisms

- *Aspergillus brasiliensis* ATCC 16404
- *Lactobacillus acidophilus* ATCC 4356
- *Saccharomyces cerevisiae* ATCC 7754
- Wild isolate of *Candida parapsilosis*
- Wild isolate of *Candida intermedia*
- *Zygosaccharomyces bailii* DSM 70492
- *Dekkera bruxellensis* DSM 70742
- Non-identified strain isolated from red wine

Principle of detection

The principle of the fluorescence detection is based on an enzymatic reaction. The fluorogenic substrate used is a non-fluorescent viability marker that is cleaved by non-specific ubiquitous intracellular enzymes, resulting in a fluorescent product. Natural amplification of fluorescence by intracellular accumulation is an indicator of microbial metabolism. The dye is diluted in a staining buffer enhancing cell membrane permeability and thus facilitating the introduction of dye into cells.

Principle of fluorescent staining

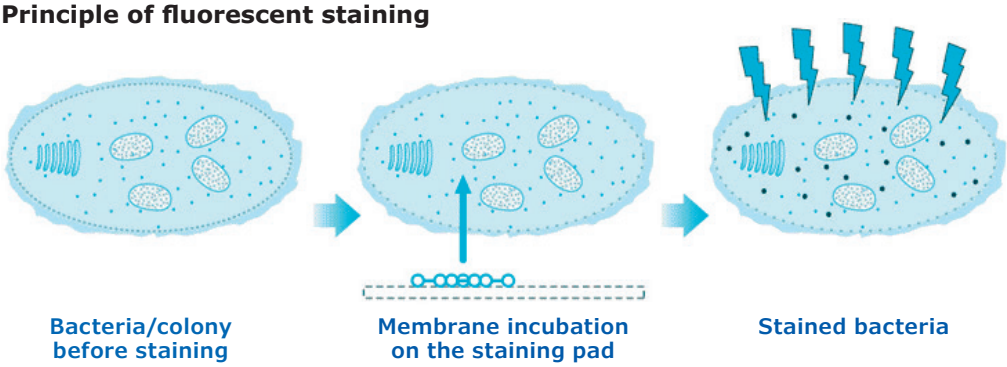


Fig. 1: Fluorescent staining

Note: Fluorescence detection is a non-destructive method that enables the microorganisms to continue to grow after they have been stained in order to identify them using standard ID technology.

Protocol for rapid detection

The procedure used was a standard protocol to detect spoilage microorganisms in samples of interest with the fluorescence detection:

1. A filtration unit is installed onto the filtration system.
2. The appropriate volume of sample is poured into the filtration unit.
3. After filtration, the membrane is disconnected from the device and aseptically transferred onto a media cassette.
4. The incubation is performed according to the specifications.
5. After the incubation, the membrane is stained with the fluorogenic reagent for 30 min at 32.5 °C +/- 2.5 °C.
6. The fluorescent microcolonies are counted using the fluorescence reader.
7. After detection, the stained membrane can be re-incubated on fresh media for traditional plate count and identification if required.

Definition of a rapid incubation time

An appropriate incubation time is defined as the minimum time necessary to achieve a recovery rate higher than 70 % compared to the traditional method. The calculation is based on both formulas:

- The fluorescence recovery is the fluorescent count compared to the traditional method count. Fluorescence recovery (%) = (average of fluorescence counts / average of traditional method count) x 100.
- The viability recovery is the colony count on stained membranes after re-incubation compared to the traditional method count. Viability recovery (%) = (average of CFU counts after re-incubation / average of traditional method counts) x 100.

An optimal incubation time should allow a sufficient fluorescent signal intensity and fluorescence and viability recoveries above 70 %.

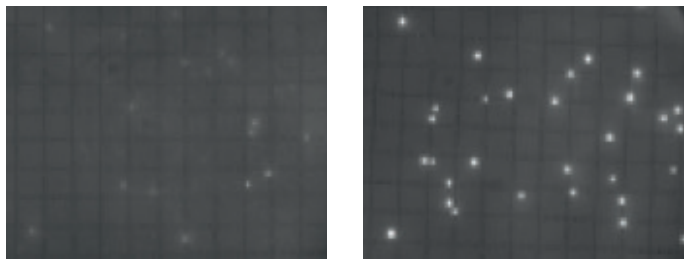


Fig. 2

The image on the right illustrates a sufficient fluorescent signal intensity translating into an appropriate incubation time. The picture on the left shows that an accurate count is not possible if the intensity of fluorescence is too low due to an insufficient incubation time.

Results

Beverage compatibility with the fluorescence technology

A wide range of beverages susceptible to microbial contamination were tested. Beverages are compatible with the fluorescence technology when showing a good filterability, an absence of a fluorescence background, and an absence of a fluorescence inhibition.

Beverage	Compatibility
Aromatized water	**OK**
Ice tea lemon	**OK**
Apple juice	**OK**
Energetic drink	**OK**
Ice tea green grapefruit	**OK**
Ice tea peach	**OK**
Ice tea red	**OK**
Mango juice	**OK with enzymatic pretreatment to improve filterability**
Ready-to-drink black tea	**OK**
Ready-to-drink green tea	**OK**
Strawberry juice	**OK with enzymatic pretreatment to improve filterability**
Red wine without sorbate	**OK**
Red wine with sorbate	**OK**
Bag-in-box (BIB) red wine	**OK**
Sweet wine	**OK**

All filterable beverages tested were compatible with the fluorescence-based technology, as no background was observed. Thick fruit juices required pretreatment to improve filterability and a rinsing protocol to reduce the fluorescence background.

Filterability pretreatment:

- Mix 1 mL of beverage with 150 µL of pectinase.
- Incubate at 37 °C for 30 min.
- Filter with 10 mL of Fluid K (containing 1 % of Tween 80).

Rinsing protocol:

- Rinse with 100 mL of Fluid K (containing 1 % of Tween 80).
- Rinse with 100 mL of physiological water.

Strains

Strains of microorganisms frequently responsible for beverage spoilage were tested. The objective was to determine the time to result for the detection of the strains with the fluorescence-based technology in various media and temperature conditions, as well as the ability of the strains to grow and produce visible colonies after staining. Incubation time was determined using three replicates.

Strain (medium, temperature)	Rapid fluorescence detection incubation time	Compendial method incubation time
Aspergillus brasiliensis (SDA, 30°C)	**16 h**	**48 h**
Lactobacillus acidophilus (MRS, 37°C)	**48 h**	**72 h**
Saccharomyces cerevisiae (orange serum, 30°C)	**16 h**	**48 h**
Zygosaccharomyces bailii (ZBA, 30°C)	**40 h**	**72 h**
Candida parapsilosis (SDA, 30°C)	**16 h**	**48 h**
Candida intermedia (SDA, 28°C)	**30 h**	**7 days**
Zygosaccharomyces bailii (ZBA, 30°C)	**32 h**	**5 days**
Dekkera bruxellensis (SDA, 30°C)	**72 h**	**7 days**

Our fluorescence-based technology detected spoilage microorganisms 2 to 4 times faster than the compendial method, while maintaining the viability of the colonies and the ability to produce colonies visible to the naked eye after staining.

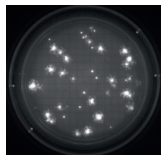
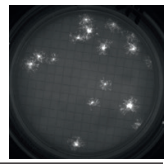
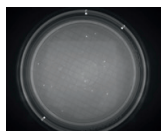
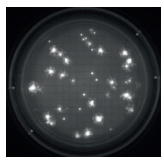
Media

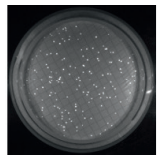
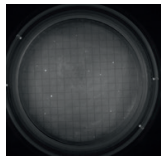
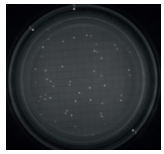
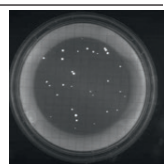
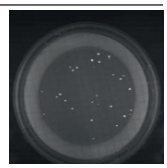
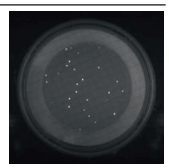
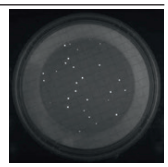
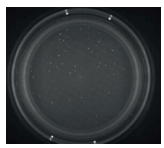
A variety of media commonly used in the monitoring of beverages were tested. A medium is compatible when it shows no fluorescence background. Sterile water was filtered and incubated overnight on the different media tested. Membranes were then stained and observed in the reader.

Medium	Compatibility
Sabouraud dextrose agar (SDA)	**OK**
Sabouraud media cassette (SDAK)	**OK**
De Man, Rogosa & Sharpe broth (MRSK)	**OK**
MRS agar (MRS)	**OK**
Potato dextrose agar (PDA)	**OK**
BAT agar (BAT)	**OK**
Orange serum broth (OSB)	**OK**
Wallerstein nutrient broth (WLN)	**OK**
Yeast and mold agar (Y&M)	**OK**
Pectinase (ZBA)	**OK**

Fluorescence and re-incubation recoveries

Various assays, using different beverages, strains and media, were performed. Each assay was repeated with three replicates for staining and re-incubation, and three replicates for the compendial method. All assays showed fluorescence and viability recoveries fulfilling the acceptance criteria described on the Definition of a rapid incubation time section.

Beverage	Strain	Media	Incubation temperature	Fluorescence incubation time	Compendial incubation time	Fluorescence recovery (%)	Re-incubation recovery (%)	Fluorescence incubation time
Aromatized water	*Aspergillus brasiliensis*	SDA	22.5 °C	47 h 30	5 days	100.6	92.1	
			28 °C	30 h	5 days	114.0	111.0	
	Lacto-bacillus acidophilus	MRS	37 °C	48 h	72 h	103	121	
	Candida intermedia	SDA	28 °C	30 h	7 days	105	105	
Apple juice	*Saccharomyces cerevisiae*	Orange serum	30 °C	16 h	48 h	97.9	95.7	
Energy drink	*Aspergillus brasiliensis* + *Candida par-apsilosis*	SDA	30 °C	20 h	48 h	A.b: 114.3 C.p: 108.1	A.b: 108.1 C.p: 98.1	
Ice tea grapefruit	*Candida parapsilosis*	SDA	30 °C	16 h	48 h	130.7	135	No image available
Ice tea peach	*Aspergillus brasiliensis*	SDA	30 °C	16 h	48 h	117.1	94.3	No image available
Ice tea red	*Saccharomyces cerevisiae*	SDA	30 °C	16 h	48 h	102.2	107.6	No image available

Beverage	Strain	Media	Incubation temperature	Fluorescence incubation time	Compendial incubation time	Fluorescence recovery (%)	Re-incubation recovery (%)	Fluorescence incubation time
Mango juice	*Candida parapsilosis*	SDA	30 °C	20 h	48 h	98.1	90.5	
Ready-to-drink black tea	*Saccharomyces cerevisiae*	Orange serum	30 °C	16 h	48 h	90.6	101.9	
Ready-to-drink green tea	*Zygosaccharomyces bailii*	SDA	30 °C	40 h	72 h	95.8	89.1	
Strawberry juice	*Candida parapsilosis*	Orange serum	30 °C	20 h	48 h	83.9	88.6	No image available
Red wine with sorbate	*Dekkera bruxellensis*	SDA	30 °C	72 h	7 days	78.8	75.4	
	Saccharomyces cerevisiae	WLN	30 °C	22 h 45	3 days	96.3	91.9	
Red wine with sorbate	*Saccharomyces cerevisiae*	SDA	30 °C	21 h	3 days	93.3	110.7	
		Y&M	30 °C	24 h	3 days	91.0	95.5	
Red wine without sorbate	*Non-identified strain (natural contamination)*	Y&M	28.5 °C	24 h	5 days	115.0	122.5	

Conclusion:

Using our fluorescence-based technology as a microbiology quality-control tool dramatically reduces the time needed to detect yeast, mold and bacterial contaminations in beverages. This study demonstrated that this technology could easily replace the compendial microbiological method with a 2 to 4 times faster time to result and a full compatibility with the standard culture media traditionally used for the detection of spoilage organisms in beverages. Moreover, as the method is non-destructive, each fluorescent microcolony detected will continue to grow to yield visible colonies allowing the identification of the contaminants using conventional identification methods.

By using our fluorescence-based technology, beverage manufacturers can improve their quality-control procedures by detecting contaminations earlier and implementing corrective actions faster. This early response creates savings of raw materials and manufacturing capacities. It can also help in the root-cause analysis of a process failure, yielding a better understanding of and confidence in the process and an enhanced quality control. Most importantly, the product release can be accelerated and storage time decreased, resulting in a financial saving for the manufacturer.

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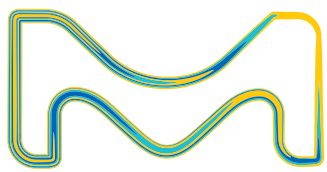

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