A novel library-independent approach based on high-throughput cultivation in Bioscreen and fingerprinting by FTIR spectroscopy for microbial source tracking in food industry

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Library-independent source tracking

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SIGNIFICANCE AND IMPACT OF THE STUDY

The source tracking of fungal contamination in food industry is an important aspect of food safety. Currently all available methods are time consuming and require use of reference library that may limit the accuracy of the identification. In this study we report, for the first time, a library-independent FTIR spectroscopic approach for microbial source tracking of fungal contamination along the food production line. It combines high-throughput micro-cultivation and FTIR spectroscopy and is specific on genus and species level. Therefore, such approach possesses great importance for food safety control in food industry.

ABSTRACT

Microbiological source tracking (MST) for food industry is a rapid growing area of research and technology development. In the paper at hand, a new library-independent approach for microbial source tracking is presented. It is based on a high-throughput liquid micro-cultivation and FTIR spectroscopy. In the approach, FTIR spectra obtained from microorganisms isolated along the production line and a product are compared to each other. We tested and evaluated the new source tracking approach by simulating a source tracking situation. In the simulation study, a selection of twenty spoilage moulds strains from in total of six genera (*Alternaria*, *Aspergillus, Mucor, Paecilomyces, Peyronellaea* and *Phoma*) was used. The simulation of the source tracking situation showed that 80 – 100% of the sources could be correctly identified with respect to genus/species level.

When performing source tracking simulations, the FTIR identification diverged for *Phoma glomerata* strain in the reference collection. When re-identifying the strain by sequencing, it turned out that the strain was a *Peyronellaea arachidicola* .

The obtained results demonstrated that the proposed approach is a versatile tool for identifying sources of microbial contamination. Thus, it has a high potential for routine control in the food industry due to low costs and analysis time.

KEYROWDS: filamentous fungi, FTIR spectroscopy, microbial source tracking, library-independent approach, high-throughput

INTRODUCTION

Moulds (filamentous fungi) are often present in food production environments. They enter the food production lines through the raw material, worker activity, and equipment operation and are present in wastewater, rinse water and the air (Kure et al. 2003; Kornacki 2010). Thus, there is always a high chance that they spoil food products (Kornacki 2010).

Although the list of food spoilage moulds is long, in each manufacturing environment a unique and limited mycobiota is present, since the local conditions tend to select for a particular assemblage of moulds. Due to a natural mycobiota in raw materials and in the environment, and the spreading of spores through air, it is nearly impossible to eradicate moulds from food processing areas completely. In addition, mould spores have the ability to survive heat treatment, the fact causing severe contamination problems in many food production lines. Several factors contribute to the spore proliferation and growth of the mycelium to unacceptable levels in the product and in the processing environment. Among these factors are improper storage conditions, cleaning, processing, and handling by the

workers (Samson 1995; Asefa et al. 2010). When mould growth is detected in the final product, the origin of food spoilage needs to be identified and eliminated in the process.

Microbial source tracking (MST) is the concept of tracing origins of food spoilage microorganisms and pathogens – "trouble makers". MST refers to a group of analytical protocols based on microbiological, genotypic and phenotypic methods (Kornacki 2010). It is applied when safety and quality systems fail and products or equipment are contaminated with fungal pathogens and/or spoilage organisms at unacceptable levels. Protocols used for MST must be applicable on an industrial scale and are usually separated into three interrelated steps: a) a source identifier, b) an identification method, and c) an analytical approach (Kornacki 2010). By the source identifier, the nature of primary contamination and its causes are identified together with the stage(s) in the entire production process, where possible contamination is likely to occur. While the identification method used depends on the type of "trouble maker" (bacteria, yeasts, moulds), the choice of analytical approaches depends on the identification method.

Analytical approaches can be divided into library-dependent and library-independent methods. Currently, there is no standard protocol or analytical approach for source tracking of mould contamination. In most cases, MST routines are only at the identification step that identifies the "trouble makers" at genus or species level using a limited number of samples taken from the prodcut or along the production line. Most identification methods are based on macro- and microscopy, or on growth patterns on different nutrient media. All these methods are time consuming and subjective. Genetic methods, as for example AFLP can be very powerful, however, the set up cost, additional consumables and high level of skills required, limits its potential use in routine screening applications (Jordan 2010). As a conclusion, there are no reliable, fast, reproducible and cheap methods available for the tracking of sources of moulds contamination in food production environments.

During recent years a biophysical technique, called FTIR spectroscopy emerged as an universal technique for the identification of microorganisms on genus, species and sometimes even on strain level (Naumann et al 1991; Gordon et al 1999; Wenning et al. 2002; Sandt et al. 2003; Essendoubi et al. 2005; Kummerle et al. 2005; Naumann et al. 2005; Fisher et al. 2006; Naumann 2009). The development of highly sensitive spectrometers as well as data-analytic methodology advanced FTIR spectroscopy as an attractive method for low-cost and high-throughput identification and characterization of microorganisms. FTIR spectra provide a precise fingerprint of the overall biochemical composition (lipids, proteins, carbohydrates and nucleic acids) of microbial cells (Bastert et al. 1999; Gordon et al 1999; Hahn 2002; Fisher et al. 2006; Santos et al. 2010).

Recently, our group established a protocol for high-throughput identification and differentiation of moulds based on high-throughput liquid micro-cultivation, FTIR spectroscopy and library-dependent identification using multivariate analysis (Shapaval et al. 2010; Shapaval et al. 2013). It has been shown that 94% of the fungal samples were correctly identified at the genus and species level (Shapaval et al. 2013). While currently all FTIR spectroscopic methods for the identification of microorganisms depend on spectral libraries, the purpose of the present study was to establish a new library-independent approach. It is worth noting, that a library-independent approach for identification of fungi can be used in combination with a library-dependent approach. The advantage of the additional library-independent approach is that even if a "trouble maker" is not represented by strains of the same species or genus in a spectral library, its contamination source can potentially be identified by the comparison of IR fingerprints of "trouble maker" (samples taken from the product or contamination point) and "point samples" (samples taken along the production line). In order to evaluate the new library-independent approach, we study in this paper the similarity of FTIR fingerprints for a set of food-related filamentous fungal strains. The

protocol used in the present study is based on the previously developed high-throughput micro-cultivation FTIR spectroscopy protocol. A selection of twenty filamentous fungal strains from a total of six different fungal genera was used for simulating a MST situation in order to validate the concept. Every strain is alternately used as 'trouble maker' while the remaining strains are used as 'point samples' in order to evaluate if the fingerprint of a "trouble maker" matches the fingerprint of the same strain from a respective contamination source.

RESULTS AND DISCUSSION

The main challenge of the source tracking in food industry is to identify the spreading path and the source of the given fungal strain, which is considered as "trouble maker". In order simulate a situation, which is close to an industrial source tracking situation we used independent agar cultures of 20 typical food spoilage fungi - "trouble makers". In our simulation, in total 240 independent "trouble maker" samples were compared with 360 independent "point samples". The scaled and EMSC baseline corrected IR spectra of *Alternaria alternata* 4889, *Mucor circinelloides* 4878 and *Aspergillus fumigatus* 2917 for the visualization are shown on the figure 2. The success of the source tracking by FTIR spectroscopy was evaluated as percentage of correct identification of "trouble makers" with resolution on genus, species and strain levels (Table 2). For identification, the Pearson correlation coefficient (PCC) was used as a measure of the similarity between the IR spectra of the "trouble makers" and the IR spectra of "point samples". In total, all simulated "trouble makers" were tracked correctly and 80-100% correctly identified at the right genus/species. All "trouble makers" were tracked and identified correctly at genus level (88.8-100% correct identification) and ten were correctly identified at the species level (80.5-100% correct

identification) (Table 2). Tracking of "trouble makers" and identification on the strain level showed a significantly lower number of correctly tracked spectra. In less than 80.0% of the cases with the exception for *Aspergillus fumigatus* MF4914, *Aspergillus fumigatus* MF4915, *Paecilomyces variotii* MF4903, *Peyronellaea arachidicola MF*4900 and *Phoma glomerata* MF4899 which were identified with 94,4%, 83,3%, 100%, 100% and 88,8% correct identification, respectively on the strain level. Initially, the strain MF4900 had previously been identified as *Phoma glomerata*. However, when we used this species information in the analysis, low identification results both on genus and species level were observed indicating that this strain may have been wrongly identified by reference analysis. In order to confirm this, sequencing of ITS region of MF4900 was performed, resulting in a re-identification as *Peyronellaea arachidicola.* After correcting the phylogeny of MF4900 to *Peyronellaea arachidicola* it was correctly identified by the source tracking algorithm.

As demonstrated above, FTIR spectroscopy may correctly track the contamination source with the resolution on genus and species level, while tracking on the strain level is difficult. This shows that the variability of the spectral signals of biological replicates of the same strain is of the same order as the variability of the spectral signal of different strains of the same species. Further, the variability of the spectral signal of strains from different species is much higher than the within species variability. This is a big advantage for the library-independent approach, since strains may slightly change from the source of contamination through the spreading path until they reach the final product through adaption to different environments. Since spectral signals of strains from the same species are similar, the source of contamination can still be identified. Thus, in order to identify all possible origins of the microbial contamination in a typical industrial scenario, the source of contamination has to be identified on genus and species level. If a strain from a given specie is found in the final product, it is therefore important to know all sources that are inhabited by

this specie. In order to identify strains that inhabit the production line, selected fungi may be subjected to more detailed analysis after the source of contamination is identified.

Current analytical approaches applied in source tracking of microbial contamination in food industry are library-dependent. The approach presented in this work is library-independent and it based on the use of mono-cultures, meaning that multi-cultures of fungi obtained on agar right after sampling along the production line need to be separated into clean mono-cultures by additional agar cultivation. The library-independent approach can be used in addition to a library-dependent approach or alone and it has clear advantages. If a spectral library is not available for strains that are typically present in a special food production environment or if a respective "trouble maker" is not represented by strains of the same species in a spectral library used in a library-dependent approach, the source of contamination can still be identified with the library-independent approach presented in this paper. The source tracking approach can give an immediate indication of the possible sources of contamination, an important prerequisite for taking the right decision, which may involve the adjustment of equipment or cleaning and disinfection without halting the production process for a long time. Thus, the library-independent approach may be considered as a valuable and complementary tool, in addition to the library-dependent approach. Together with the library-independent approach, a spectral library could be used to identify strains that are isolated from the food production environment. The information obtained about the possible contamination sources may then be used together with the identification results obtained from the library-dependent approach to analyze existing HACCP routines and to further improve them.

The need of strictly controlling cultivation conditions such as temperature, cultivation time and media in the library-dependent approach has been discussed in the respective literature (Oust et al. 2004). Spectra obtained of "point samples" and "trouble makers" from a

production line need to be obtained by employing strictly the same cultivation conditions as used for establishing the spectral library. By changing for example growth media composition, spectral fingerprints may vary considerably. In the library-independent approach introduced in this paper, "point samples" and "trouble makers" are cultivated in the same cultivation run. Thus, the comparison of "trouble maker" spectra with "point sample" spectra is not hampered by variations in cultivation parameters. In addition, for the library-independent approach different media may be used. This may in future provide the possibility to use more tailored media that for example favor the growth of the target microorganisms.

The library-independent approach as presented in this paper as well as the library-dependent approach presented in our previous work (Shapaval et al. 2010; Shapaval et al. 2013) involves high-throughput micro-cultivation which has several advantages. The micro-cultivation of 200 samples in the Bioscreen C allows to analyze all samples of one source tracking incidence in one experimental run under exactly the same conditions, decreasing the time for analysis and spectral variability due to cultivation variations (Tauk-Tornisielo et al. 2007). Further, because 200 samples can be cultivated in parallel, there is a possibility of cultivating experimental replicates of every "point sample" and "trouble maker", allowing calculating the best estimate of the spectra of "point samples" and "trouble makers" as the average of several independent cultivation replicates.

In this study, for the first time, FTIR spectroscopy was proposed and its potential tested for source tracking of fungal contamination along the food production line. The main novelty of the proposed FTIR-based source tracking algorithm is the direct identification of contamination source(s) by simple comparison of IR spectra of "trouble maker" and "point sample(s)", a complementary tool to the library-dependent identification analysis. The developed source tracking approach is more flexible than library-dependent FTIR analysis, since it allows the use of several media including tailored media favoring the growth of target

microorganisms. The method is also an excellent tool to be used in routine control. The daily routine control in food industry involves analysis of high number of samples obtained from the product and along the production line. Currently, the main analytical tool in the routine control is agar cultivation and estimation of colony forming unites (cfu), only limited number of samples subjected to the identification analysis. The number of samples subjected to the identification analysis could significantly be increased by using library-independent approach based on FTIR spectroscopy. Due to the high-throughput possibility and robustness of FTIR spectroscopy when "trouble maker" samples can be compared to "point samples" and the "point samples" may also be compared among each other, the spreading path of microorganisms could be monitored. Consequently, possible problems may be detected before potential contaminant(s) reach the final product. The high identification resolution of the library-independent approach on genus and species level in combination with the high-throughput characteristics of the developed protocol, render FTIR spectroscopy as an attractive tool in microbial routine analysis.

MATERIALS AND METHODS

Model of microbial source tracking based on FTIR spectroscopy

The proposed simulation of a source tracking situation in food industry by FTIR spectroscopy is shown in figure 1 and has the following steps: 1). Isolation and purification of microorganisms from samples taken from different control points along the food production line ("point samples") and from the contaminated product ("trouble maker"); 2). A first cultivation step: the cultivation of all isolated microorganisms on agar medium; 3). A second cultivation step: broth high-throughput micro-cultivation of all isolated microorganisms together in the same cultivation run; 4). Preparation of samples for FTIR spectroscopy; 5). FTIR spectroscopy; 6). Identification of "trouble maker" source(s) using a library-independent source tracking algorithm.

Filamentous fungi strains

Twenty mould strains, representing six genera and eight species, obtained from Norwegian Veterinary Institute (Oslo, Norway) and Elopak (Spikkestad, Norway), were used in the study. All moulds strains originated from source tracking incidences in juice/dairy industry or spoiled food and had been identified by microscopy and sequencing of ITS regions. Detailed information about strains is presented in Table 1. During the validation of the library-independent source tracking concept, each fungal strain was considered as a "point sample" and a "trouble maker".

Cultivation of moulds

Moulds were grown on Malt Extract Agar (MEA) (Oxoid, Basingstoke, England) and Malt Extract Broth (MEB) (Oxoid, Basingstoke, England). The first cultivation step was done on MEA at 25 °C for 5-6 days. Each fungal strain was cultivated on five MEA plates. By that, we obtained five independent replicates for "trouble makers" and "point samples". Spore suspensions were prepared by collecting spores from the MEA plates with cotton tips followed by re-suspension in 1 mL of MEB. The second high-throughput micro-cultivation step was done in the automated Bioscreen C system (Oy Growth Curves AB, Helsinki, Finland), using MEB as cultivation medium. The working volume of the spore suspension in the wells of the Bioscreen honeycomb microtiter plate (Oy Growth Curves AB, Helsinki, Finland) was 300µl. From each of the five MEA plates referring to the same strain, one inoculum was obtained resulting in five wells on the microtiter plate. The isolates were distributed randomly in the microtiter plates. The micro-cultivation was carried out at 25°C, with automatically continuous shaking, and the optical density of the fungal cultures was measured automatically at 540 nm every other hour, for five days.

Sample preparation and FTIR measurements

Before FTIR measurements each sample was prepared in the following way: (1) Mycelium was transferred from the Bioscreen honeycomb microtiter plates by a bacterial loop into Eppendorf tubes and washed three times with 400µl of deionized H_2O. (2) After the last washing step 30-50 µl of deionized H_2O was added to the suspension and sonicated with a tip-sonicator (Qsonica LLC, Newtown, USA) for 10-20 seconds to make the suspension of the fungal mycelium homogenous. From each suspension, 8 µl was transferred to an IR-light-transparent Silicon 384-well microtiter plate (Bruker Optik GmbH, Ettlingen, Germany). The samples were dried at room temperature for 45 minutes to form films suitable for FTIR analysis. FTIR measurements were performed using a High Throughput Screening eXTension (HTS-XT) unit coupled to a Tensor 27 spectrometer (both Bruker Optik GmbH). Spectra were recorded in the region between 4000 and 500 cm^{-1} with a spectral resolution of 6 cm^{-1} and an aperture of 5.0 mm. For each spectrum, 64 scans were averaged.

Experimental set-up

For the simulation of source tracking, each fungal strain was cultivated in five MEA plates. Each single MEA plate can be considered as an independent "point sample" or "trouble maker". Thus, for each of the 20 strains, five independent "point samples" or "trouble makers" were obtained, resulting in 100 MEA plates. The 100 MEA plates simulate a set of 100 isolates that were sampled along the food production chain. For this set of 100 MEA plates, three independent experiments were performed. In each experiment, high-throughput micro-cultivation in liquid broth was performed for subsequent FTIR spectroscopy. In each high-throughput micro-cultivation run, samples from the 100 MEA plates were used to inoculate two different wells, one in each of the two Bioscreen C microtiter plates used in one run. In total this resulted in 1200 FTIR spectra, (20 strains x 5 independent agar plates x 2 wells per bioscreen run x 2 technical replicates x 3 independent

Bioscreen runs). Finally, for the analysis we used 1180 spectra. We removed 20 spectra since we observed contamination of 4879 *Mucor circinelloides* in one run. All spectra were subjected to Opus spectral quality control test (Bruker® Analytik GmbH).

Pre-processing of the FTIR spectra and data analysis

The infrared spectra were pre-processed by taking second derivative spectra using a nine-point Savitsky-Golay algorithm and subsequently applying extended multiplicative signal correction (EMSC) (Kohler et al. 2009). For further processing of spectra the spectral region between 1400 and 700 cm^{-1} was used. For the EMSC correction, a replicate model was constructed as described by Kohler et al. (Kohler et al. 2009). In order to estimate the replicate variation only technical replicates were used, i.e. variation between spectra obtained from the same micro-cultivation was estimated and built into the replicate correction model. Technical replicate spectra were available in all source tracking situations. In the EMSC replicate model, five principal components were used. The number of components was defined with respect to the explained variance; explained variance was 90% using five components. The average of the technical replicates was calculated.

The data analysis was performed using Unscrambler 9.6 (Camo, Oslo, Norway), for pre-processing of spectra, and in-house developed algorithms in Matlab (The MathWorks Inc., Natick, United States) for source-tracking simulations.

Algorithm for source tracking of fungal contamination by FTIR spectroscopy

For the set of 100 MEA plates simulating a set of 100 isolates that were sampled along the food production chain, three independent Bioscreen experiments were performed. Each Bioscreen experiment resulted in two microtiter plates, where each plate contained 100 cultivations inoculated with samples from the 100 MEA plates. Thus, each microplate contained five cultivations referring to the same strain while each cultivation was derived

from a different MEA plate. From each of the 100 cultivations from each microtiter plate two technical replicate spectra were obtained. After preprocessing according to the protocol described above, the technical replicates were averaged, resulting in 100 spectra per microtiter plate, i.e. one spectrum per cultivation. Spectra of each microtiter plate were then divided into a "trouble maker" data set and a "point sample" data set. In order to estimate the percentage of correct identification of sources of contamination, we used two spectra of each strain for a "trouble maker" data set and three spectra of each strain for a "point sample" set resulting in a set of 40 spectra for "trouble makers" and 60 spectra for "point samples" for each plate. Since in total 6 microtiter plates were used, in total 360 "point samples" and 240 "trouble makers" were obtained. For each microtiter plate, the spectra of the "trouble maker" data set were then compared with the spectra of the "point sample" set, and the best matches were considered as the source of contamination. Alternately, two spectra from the "trouble maker" data set referring to the same strain were compared to all spectra of the "point sample" set for each microplate separately. Since there were three spectra in the "point sample" set (and two spectra in the "trouble maker" data set) referring to the same strain, we could as a maximum find three correct identifications. Thus, we considered the three best matches for all "point sample" spectrum when comparing them to one "trouble maker" spectrum. The best matches were identified by calculating the Pearson correlation coefficient between the "point sample" spectra and the "trouble maker" spectrum. These three best matches were evaluated on genus, species and strain level and the number of correctly identified samples determined. The number of correctly identified samples was calculated for all point samples and all microplates and correctly identified samples are given in percentage.

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CONFLICT OF INTEREST

No conflict of interest declared.

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Table 1: Fungal strains included in the study

MF № *	Fungal strains	Other Designation	Origin
4889	*Alternaria alternata*[**]		Juice production
4887	*Alternaria alternata*[***]	VI 03120	Food
4888	*Alternaria alternata*[**]	VI 04067	Food
4917	*Aspergillus fumigatus*[****]		Apple juice
4914	*Aspergillus fumigatus*[**]	VI 02980	Food
4915	*Aspergillus fumigatus*[**]	VI 03680	Food
4878	*Mucor circinelloides*[*]	21	Juice production
4879	*Mucor circinelloides*[*]	49	Juice production
4876	*Mucor circinelloides*[**]	VI 01914	Food

4877	*Mucor circinelloides* [**]	VI 04473	Food
4872	*Mucor plumbeus* [*]	4	Juice production
4873	*Mucor plumbeus* [*]	5	Juice production
4870	*Mucor plumbeus* [**]	VI 02022	Food
4874	*Mucor plumbeus bonord* [***]	22	Juice production
4875	*Mucor plumbeus bonord* [***]	24	Juice production
4902	*Paecilomyces variotii* [*]	20	Juice production
4903	*Paecilomyces variotii* [*]	48	Juice production
4901	*Paecilomyces variotii* [**]	VI 04645	Food
4900	*Peyronellaea arachidicola* [*****]		Milk
4899	*Phoma glomerata* [***]	VI 03583	Food

[*]MF numbers for fungal strains in microbiological collection at Nofima (Ås, Norway)

[**] Fungal strains characterised and identified by Microbiological Laboratory at Elopak (Spikkestad, Norway)

[***] Fungal strains obtained from Norwegian Veterinary Institute (Oslo, Norway)

[****] Fungal strains characterised and identified by Mycoteam AS (Oslo, Norway)

[*****]Fungal strain initially wrongly identified (Elopak) and re-identified by sequencing and at Nofima AS (Ås, Norway)

Table 2: Identification of simulated "trouble makers" by FTIR spectroscopy using library-independent source tracking algorithm (Identification results are presented in percentage)

MF №	Mould strains	Strain level	Species level	Genus level
4889	*Alternaria alternata*	58.3	94.4	94.4
4887	*Alternaria alternata*	52.7	100.0	100.0
4888	*Alternaria alternata*	61.1	88.8	88.8
4917	*Aspergillus fumigatus*	50.0	100.0	100.0
4914	*Aspergillus fumigatus*	94.4	100.0	100.0

4915	*Aspergillus fumigatus*	83.3	100.0	100.0
4878	*Mucor circinelloides*	41.6	94.4	100.0
4879	*Mucor circinelloides*	45.5	94.4	100.0
4876	*Mucor circinelloides*	33.3	94.4	100.0
4877	*Mucor circinelloides*	22.2	86.1	100.0
4872	*Mucor plumbeus*	30.5	58.3	94.4
4873	*Mucor plumbeus*	30.5	80.5	100.0
4870	*Mucor plumbeus*	27.7	100.0	100.0
4874	*Mucor plumbeus bonord*	22.2	100.0	100.0
4875	*Mucor plumbeus bonord*	47.2	97.2	100.0
4902	*Paecilomyces variotii*	55.5	100.0	100.0
4903	*Paecilomyces variotii*	100.0	100.0	100.0
4901	*Paecilomyces variotii*	50.0	100.0	100.0
4900	*Peyronellaea arachidicola*	100.0	100.0	100.0
4899	*Phoma glomerata*	88.8	88.8	88.8

Food production line

Final product

"Point samples"

"Trouble maker"

Isolation and purification of microorganisms

Cultivation on agar

Broth high-throughput micro-cultivation

Preparation of samples for FTIR spectroscopy

FTIR spectroscopy analysis

Library-Independent Source Tracking