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Surface Enhanced Raman Spectroscopy detection of *Brettanomyces bruxellensis* yeast on nanostructured ultrafine glass supports

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Abstract

Novel genosensors *(Brett DNA)* on nanostructured ultrafine coverslips are reported for the identification of *Brettanomyces bruxellensis* yeast that is well-known to create complex and difficult problems in aged red wine all over the word. Surface Enhanced Raman Scattering (SERS) and Localize Surface Plasmon Resonance (LSPR) are used as detection methods of target-ssDNA yeast. A specific detection is obtained in the presence of thiol-DNA probe. SERS technique made possible the detection of Brett-DNA with a limit of detection (LD) of 0,1 ng/µL((57,2 nM) and with a wide dynamic range (DR) , while LSPR revealed a DL of 0,5 ng/µL real DNA extracted from B*rettanomyces bruxellensis* DKA in the laboratory and a limited DR (0.5 ng/ µL to 5 ng/ µL). In this work, 14 yeasts that can be found in wine-growing environments were SERS tested to confirm the selectivity of the proposed *Brett*-DNA sensing schemes and used in the construction of the first SERS phylogenetic tree.

Keywords: annealed nanostructures, genosensors, *Brettanomyces bruxellensis,* phylogenetic tree*,* SERS and LSPR on coverslips

1. Introduction

Brettanomyces bruxellensis is an important spoilage yeast causing the "*Brett off-flavor*" of wine. It is considered one of the most complex and controversial issue of aged red wine. Although a "contaminated" red wine has a particular taste with "plastic" or smoke flavors or exotic aromas, it is generally undesirable as being responsible for horse blanket - persistence aromas [1-3].

Different approaches have been adopted in wineries around the world to identify the presence of *Brett* yeast. Some compounds presented in grape juice and/or wine, such as p-coumaric and ferulic acid were converted to 4-ethylphenol and 4-ethylguaiacol, the volatile phenols responsible for the depreciation of wine [4-7]. *B. bruxellensis* also produces volatile fatty acids, such as acetic acid and isovaleric acid, which contribute to negative aromas [8]. Another important compound produced by B. bruxellensis is the biogenic ammines [9].

To avoid the spread of *Brettanomyces* in wineries, fast, specific and sensitive detection methods must be developed. Traditional methods are based on plate microbiology, molecular biology and metabolite analysis [10]. These methods have disadvantages, either the long incubation period (about two weeks) and/or the need of specialized personnel or obtaining results in the presence of interfering molecules or a minimum of *Brett c*ells as 10 CFU/mL [11]. Advanced detection methods using fluorescence [12, 13], electrochemistry [14, 15], and localized surface plasmon resonance (LSPR) [16] have been employed for detection of *Brett* of different sources. Moreover, the optical technologies have proven strong biosensing advantages: easy operation, non-destructive detection, rapid diagnostics [17-19], multiplexing and miniaturization capacities [20, 21]. Genosensing is one of the most sensitive approach because of the nucleotide strands lined up with strong pairing between DNA bases and their complementary parts [22-24]. Thus, DNA biosensors apply immobilized DNA as diagnostic tools [25]. Unlike common biosensors based on enzyme or antibodies, DNA-based biosensors with high sensitivity and high assembly efficiency can be easily prepared. Moreover, the utilization of nanomaterials promotes the development of new generation

of DNA biosensors towards the goal of smart, simple and inexpensive detection of complex target analytes [26-28].

In the case of the LSPR technique, the noble metallic nanostructures interact with a beam of light when a part of the photons is absorbed while other is scattered. When LSPR occurs, an intense and confined electromagnetic fields provide a very sensitive technique for the detection of single molecule [29]. Practically, the LSPR spectrum is influenced by various parameters, including the metal dielectric function, the particle shape, size and size distribution, the interparticle distance and the local refractive index near the surface of the nanoparticles [30]. Despite silver displays sharper and more intense LSPR bands than gold, the higher chemical stability of gold nanostructures has favored its preferential application in the construction of biosensors [31]. Consequently, this type of simple and low-cost spectroscopy is widely used for the detection of the modifications of nanostructures with chemical species suspended either in alcoholic or aqueous solutions. Several LSPR metallic nanostructured solid substrates are reported [32-34]. For example, robust DNA-LSPR biosensors on annealed gold nanostructures on thick glasses were developed for the detection of 10 ng/µL DNA - *Brettanomyces bruxellensis* in aqueous SSPE buffered solution [16].

On the other hand, the SERS is intensively used as an ultrasensitive fingerprint method of whole microorganisms such as *E. coli* [35 - 37] and different categories of chemicals in either their native form or after interactions with additional molecules presented in a buffer solution and applied for a specific detection of (bio)molecules: DNA or RNA [38-44]. Rough solid supports such as gold grating [45], nanocubes [46], artificial neural networks for complex (bio) samples [47] are experimentally required for the excitation of surface plasmon by a light source of a wavelength that resonates with an electronic absorption band, when the intensities of the Raman lines are mainly determined by the properties of the excited electronic state. SERS technology is thus suitable for multiplex detection and recommended for (bio)sensing with lower limit of detection [48, 49].

With this in mind, the present work reports on the construction of the first SERS phylogenic

tree for rapid and specific detection of *Brettanomyces bruxellensis* yeast in wine using home-designed gold nanostructures on ultrafine solid and transparent supports. Plasmonic LSPR DNA biosensors are also developed. The analytical performances of SERS and LSPR biosensors are further discussed. It is expected, that due to the outstanding sensitivity of nanostructured ultrafine glasses in SERS (enhancement factor of 2.71 x 10^7 in the presence of 10^{-3} M 1,2-bis-(4-pyridyl)-ethene (BPE), [34])an attractive and low cost solution for rapid bioidentification of DNA in complex biological media is possible.

2. Materials and methods

2.1. Instruments

The concentration of extracted DNA was measured with a NanoDrop 2000c instrument ((Thermo Scientific, Wilmington, USA). All reagents were sterilized in a Tuttnauer Autoclave Steam Sterilizer 2540ML (Tuttnauer, Villenoy, France). The coverslips samples were dried in the oven provided by VWR company (DRY-Line drying oven DL 53). The (bio)functionalization of glasses (Figure 1, i) was made under a biological hood provided by Thermo-Scientific MSC 1.2 ADV (Illkirch Cedex, France).

Gold evaporation on coverslips was performed with Plassys MEB 400 (Plassys, Bestek, France) A hotplate (Thermo Fisher Scientific, Waltham, MA, USA) for annealing the Au-coverslips under the clean room conditions and an ultrasonic bath provided by Elmasonic S30H (Elma Schmidbauer GmbH, Singen, Germany) for cleaning the coverslips before (bio)functionalization were systematically used. The absorbance (A) of gold modified glass substrate was characterized before and after annealing at 500 °C using a UV-visible spectrometer (Carry 300 Agilent). Prior the measurements, the baseline has been measured without any sample between the source and the detector.

The nanostructured surfaces were characterized with a Scanning Electron Microscope (SEM) (FEG-SU8030, Tokyo, Japan) (Figure 1, ii).

SERS measurements were performed within a backscattering geometry using a modified Jobin-Yvon LabRAM (Horiba scientific, Longjumeau, France) with an excitation He-Ne laser wavelength of 632.8 nm (11 mW). All spectra were recorded with a 10 x objective Olympus MPlanFl and 5.5 μm^2 laser spot. The acquisition time varied from 10 s to 120 s, and all spectra were recorded 3 times with a density filter between 0 - 0.3.

The LSPR system used a white light source and an optical fiber placed on top of the optical objective to collect the transmitted light. The LSPR signal is collected by a spectrometer Maya 2000 Pro provided by Ocean optics (EW Duiven, Netherlands).

2.2. Chemicals

Ultrapure water (18.2 MΩ·cm) was produced by a Millipore Milli-Q water purification system (Molsheim, France) and used after autoclave sterilization at 121°C (1.5 bar) for 15 min. Ethanol 70% (Sigma-Aldrich, USA) was used for rinsing steps. The glass coverslips were cleaned with Decon 90 (Decon Laboratories™ Decon 90™ Liquid Detergent, Fisher Scientific, Goteborg, Sweden).

Various types of buffers were tested for biofunctionalization, such as phosphate buffer saline (PBS) (sodium chloride 1.5 M, sodium phosphate dibasic 81 mM, sodium phosphate monobasic 19 mM, pH 7.4), SSPE buffer (sodium chloride 3 M, sodium phosphate dibasic 0.23 M, ethylenediaminetetraacetic acid 25 mM, pH 7.4), TE buffer (Tris(hydroxymethyl)aminomethane hydrochloride 0.5 M, ethylenediaminetetraacetic acid 0.1 mM, sodium chloride 1 M, pH 8.0), and polyethylene glycol buffer (PEG buffer) (PEG 6000 12.5 mM, sodium chloride 0.5 M, sodium phosphate dibasic 0.2 M, ethylenediaminetetraacetic acid 5 mM, sodium dodecyl sulfate (0.5 %

(w/v)). All reagents were provided by Sigma-Aldrich (USA).

Cleaned coverslips were biofunctionalized with a *Brettanomyces bruxellensis* Thiol-probe (*Thiol-probe*), provided by Eurofins Genomic (Tubingen, Germany) subsequently treated with a solution of TCEP at 10 mM and sodium acetate at 3 M for deprotection of Thiol group. Then the Thiol-probe was diluted in Sodium Chloride-Sodium Phosphate-EDTA (1xSSPE, pH 7.4), previously sterilized in autoclave at 121 °C (1.5 bar) for 15 min and stored at -20 °C prior to be used. For SERS and LSPR investigations, the Thiol-Probe was used at 10 ng/µL (0.56 µM) suspended in 1 x SSPE buffer.

The oligonucleotides (complementary and non-complementary sequences) were also provided by Eurofins Genomic (Tubingen, Germany) and diluted as follows: 670 µL for the complementary and 652 µL for non-complementary to obtain 100 pmol/µL. Further, several *Brett-DNA* concentrations were prepared: 100 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL and 1 pg/µL.

In table 1 are described the Thiol-probe, complementary and non-complementary DNA sequences. To evaluate the specificity of the Thiol- DNA probe, different yeast strains that can be found in winery or vineyard environments were tested (Table 2). Thus, a SERS phylogenetic *Brett-tree* is obtained.

Pure yeast colonies were obtained by growing the yeast on Malt Extract Agar (Oxoid, Milan, Italy) and *Brettanomyces bruxellensis* spp at 30 °C for 2 days and 5 days, respectively. The obtained strains were streaked on malt agar and/or WL Differential agar (8 % (w/v)) (Oxoid, Milan, Italy) for purification accordingly to standard procedures. Thus, one colony was collected from agar plates and placed in a tube containing 200 µL of breaking buffer and 0.3 g of glass beads (5 mm diameter) for DNA extraction [10]. The extracted DNA was standardized at 100 ng/µL using a NanoDrop 2000c instrument.

2.3. Substrate preparation and biofunctionalization

Round glass coverslip (22 mm diameter) and square glass coverslips (22 x 22 mm) with a thickness of 0.13 - 0.16 mm (Carl Roth GmbH + Co.KG, Germany), were used as solid supports. Before gold evaporation, the glass coverslips were cleaned with ultrapure water and detergent (Deacon 90) solution (2:8, (v/v)) at 50 °C for 15 min in an ultrasonic bath [32]. After, the glasses were rinsed and washed with ultrapure water in an ultrasonic bath at 50 °C for 5 min. After an additional washing step with ultrapure water the glass coverslips were dried with nitrogen stream and deposited on a hot plate at 100 °C for 10 min to completely dry the surface. The metal evaporation conditions were set at 10^{-5} Torr pressure, 25 °C and 0.03 nm/s as evaporation rate. After evaporation, the glass coverslips were subjected to annealing on a hot plate at 550 °C for 3 h according to [34], and finally cleaned [32]. After ultrasonic bath with sterile water for 10 min at 30 °C, coverslips were allowed to dry in oven at 50 °C for 20 min. The cleaned glass coverslips were biofunctionalized with 10 µL of Thiol - DNA probe (10 ng/µL in 1 x SSPE buffer) and stored overnight at 4 °C (Figure 1).

Hybridization tests: The thiol-functionalized coverslips were used for hybridization experiments with the complementary *Brett DNA*-sequence (for specific experiments) and with non-complementary sequence (for control experiments) of different concentrations 100 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL and 1 pg/µL suspended in 1x SSPE buffer. The hybridization was performed overnight at 4 °C followed by washing with sterile water and drying at RT. SERS tests using genomic DNA extracted from *Dekkera bruxellensis* DKA were also conducted using the optimized conditions for the complementary and not- complementary sequences.

SERS Phylogenetic tree: DNA extracted from 16 yeast strains (Table 2) were SERS analyzed by DNA drop procedure (2 µL of 100 ng/µL) onto AuNPs coated coverslips using an acquisition time of 10 sec for 3 times, with the filter fixed to 0.

3. Results and discussion

Here is reported the first *Saccharomyces Phylogenetic SERS/DNA-tree* (Figure 2). Such SERS tree shows the possibility to simply evaluate the presence or absence of *Brettanomices bruxellensis* yeast *species* in large varieties of wines.

3.1. SERS spectra of different species of Saccharomyces genus

Various yeast strains were SERS investigated using tiny drops of DNA-strains ((2 µL of 100 ng/µL, concentration tested with NanoDrop) on gold nanostructured coverslips. The acquisition time was 10 sec x 3 times, and the filter was set to 0.

The chosen yeasts belong to *yeast* class and *Saccharomycetales* order that include 4 genus (*Pichiaceae, Debaryomycetaceae, Saccharomycodaceae and Saccharomycetaceae)* (Figure 2*)*. Each genus contains specific microorganisms that were either partially or totally investigated with the SERS technique. The recorded spectra are mapped with colored squares to facilitate visualization of the shape differences of peaks (Table 3). For example, in the case of *Brettanomyces bruxellensis* (*Pichiaceae* genus), 3 species were spectroscopically investigated: *Candida ethanolica*, *Dekkera bruxelensis* DKA and *Dekkera bruxelensis DSMZ*. Here is compared the evolution of SERS peak intensity and shape at 4 wavenumber zones: 350-550 cm^{-1} (square blue), 600-1000 cm^{-1} (square green), 1250 -1450 cm^{-1} (square navy) and 1600-1800 cm^{-1} (square orange). Experimentally, the SERS spectra of *Brettanomyces bruxellensis* DKA and *Brettanomyces bruxellensis* DSMZ 70726 present strong modifications at 1250 and 1450 cm^{-1} (Table 3). In the supplementary content, the SERS spectra of *Saccharomycodaceae genus (*Figure S1) and *Debaryomycetaceae* (*Millerozyma farinosa)* and *Saccharomycodaceae* (*Hansenula uvarum*) (Figure S2) are also recorded and

highlighted the most relevant peaks at 1250 - 1500 cm^{-1}.

In conclusion, the yeasts of *Saccharomycetes* class present major modifications of the SERS spectra either at 1205-1500 cm^{-1} or/and 1550-1800 cm^{-1}.

3.2. SERS spectrum of different buffers

For thiol DNA probe biofunctionalization of AuNPs coverslips, different aqueous buffers were SERS tested: PBS, SSPE, TE and PEG. The protocol consists on drop deposition (2 µL buffer) on gold nanostructured coverslip initially coated with 4 nm Au and annealed at 550 °C for 3 h on a hotplate [34]. For each buffer a SERS spectrum was recorded from three drop' zones; (1) external, (2) medium crystal formation near the external zone as a result of water evaporation, (3) internal. The four buffers were SERS tested over 10 sec x 3 times and with a filter D 0.3 (Figures S3-S6).

After analysis, the SSPE was chosen for the biofunctionalization steps due to its ability to create a friendly environment to the attachment of the probe to nanoparticles and the subsequent attachment of the complementary Brett-DNA sample. Other buffers (PBS, TE and PEG) induced visible modifications of gold nanoparticles despite their low intensity of SERS spectrum (Figure S7, influence of PBS). For example, Figure S8 shows the SERS graph of the DNA thiol probe deposited overnight at 4 °C and the effect of TE buffer on the nanoparticles after biofunctionalization with the DNA-probe and washing with water. The PEG buffer was problematic because it created a hydrophilic environment and therefore it was not possible for DNA micro-spotting on supports. Interesting, it has also been noticed that distilled deionized water (dd water) destabilized the AuNPs over time (Figure S9).

3.3 SERS spectrum of Thiol-DNA probe - buffer influence after an overnight at 4 °C - on "solid" supports

SERS spectrum of Thiol-DNA probe (100 pmol/µL) in water and 1xSSPE aqueous buffer was obtained from a tiny drop of 2 µL deposed on gold nanostructured coverslips. The acquisition time was 10 sec x 3 times, with a D 0.3 filter (Figure S10). Thus, in the case of water as buffer, the most significant increase of SERS intensity (6500 a.u.) was obtained at 1604,87 cm^{-1} while for the 1xSSPE, a very intense peak (25000 a.u.) was obtained at almost the same wavenumber (1600.07 cm^{-1}). This may be attribute to the complex composition of 1xSSPE buffer and confirmed also by the selected zone for SERS investigations in a drop (Figure S5). Evolution of another SERS peaks characteristic for Thiol-probe are reported in Table 4. Moreover, the SERS spectrum of thiol-probe on "*solid*" AuNPs coverslips was also investigated over five weeks. The strongest signal was obtained after two weeks [34].

3.4 SERS sensing configurations in "tubes" and their signal stability over three days

Three SERS sensing configurations are obtained after mixing in *Eppendorf tubes*: (i) aqueous solution of Thiol-probe (10 ng/µL) and complementary sequence (100 ng/µL); (ii) aqueous solution of Thiol-probe (10 ng/µL) and genomic DNA of *Brettanomyces bruxellensis* DKA (100 ng/µL); (iii) aqueous solution of Thiol-probe (10 ng/µL) and complementary sequence (100 ng/µL) suspended in PEG buffer (Figure 3). The SERS spectrum of each configuration was obtained from 2 µL droplet on gold nanostructured coverslips and repeated over three consecutive days. Between measurements, the tubes were kept at room temperature. Interestingly, in the third day, (ii) sensing configuration, showed the strongest SERS spectra modifications when comparing to (i) and (iii). This led us to carry out the SERS tests over several days and evaluated the signal stability and the robustness of the DNA-DNA biorecognition events and/or biofunctionalization protocol.

3.5 Calibration curves for specific and non-specific Brett-genosensors based on SERS spectra

In the construction of the first SERS phylogenic tree for rapid biosensing on gold nanostructures and characterization of *Brett*-DNA (*specific tests*) and of large-game of DNAs from interfering yeasts in wine (*non-specific* tests) were chosen five concentrations of complementary *Brett*-DNA and of non-complementary sequence: 1 pg/µL, 10 pg/µL, 100 pg/µL, 1 ng/µL and 10 ng/µL (Figure 4).

The resulted SERS spectra were analyzed and the intensity values of more representative peaks at 1294 cm^{-1} in the case of specific tests were used to plot the calibration or dose-response curves. To keep the symmetry of SERS interpretation, in the case of non-specific tests the attenuated intensity was also collected at 1294 cm^{-1} (Figure 5). For concentrations lower than 0,1 ng/µL, it is observed a slight increase of the SERS intensity with no significant differences of spectra between the non-specific and the specific DNA-target. For concentration higher than 0,1 ng/µL, the SERS intensity measured in the presence of the specific target increases rapidly and proportionally to the logarithm of the concentration whereas it keeps stable in the case of the non-specific non-complementary DNA-target. The limit of detection is consequently estimated to 0,1 ng/µL and the dynamic range of the genosensor extends from the LD value to the highest concentration (10 ng/ µL) tested in this experiment.

3.6 Calibration curves for specific and non-specific Brett-DNA genosensors based on LSPR spectra

An additional surface characterization technique -LSPR was used for complementary investigations of proposed yeast selective biosensors. Strong absorbance (A) values in the ultraviolet range were recorded for both, clean glass and gold modified substrates and compared with A values obtained in the visible range when the evaporated gold film exhibits a broad peak of absorption at 637 nm

whereas the annealed gold film exhibits a narrow peak at 566 nm. This results confirm the possibility to use such annealed substrates for plasmonic biodetection. Further, the spectroscopic data were collected to evaluate the specific and non-specific *Brett*-DNA (bio)functionalization of gold nanostructured coverslips. It was found that in the case of specific *Brett*-DNA (Figure S11, A) and real-DNA from *Brettanomyces bruxellensis* DKA biosensors (Figure S11, C) the LSPR evolution is logical with the increase of target-DNA concentrations, while random LSPR peak increases are recorded for non-specific (control) DNA-yeasts biosensors (Fig. S11,B). Calibration curves are constructed using the wavelength shift values specific to maximum of absorbance recorded by LSPR measurements for each selected *Brett*-DNA concentration (Figure 6B).

Specifically, in the case of specific DNA-biosensing configuration, an increase of DNA concentration corresponds to increase of wavelengths that produces red shifts from 557.35 nm (1 pg/µL) to 574.89 nm (10 ng/µL) when compared to plasmonic evolution of naked-nanoparticles (λ_{max} = 553.55 nm). Except for the nonspecific-control sensing configuration, the two others for specific and the identification of real-DNA Brett-DKA can be reasonably fitted with the help of a logarithm curve and are in good agreement with the obtained results also observed with the SERS measurements.

Interestingly, in the case of non-specific experiments using a non-complementary *Brett*-DNA sequence, for concentrations lower than 0.5 ng/µL or greater than 5.5 ng/µL, are noticed strong shifts of maximum wavelength even stronger that in the case of positive control and real-DNA from *Brettanomyces bruxellensis* DKA. This reduces the capability of the genosensor to efficiently detect the DNA-target traces by increasing the limit of detection to 0,5 ng/ µL (compared to the 0.1 ng/ µL value obtained in SERS) and by limiting its dynamic range to an interval of concentrations between 0.5ng/ µL - 5ng/ µL.

4. Conclusions

The specific and fast identification of DNA sequences is essential in various fields: medicine, environment, food and beverage industry. In this context, the SERS genosensors have a promising future thanks to their simplicity and accessibility, the biofunctionalization protocol easily transferable to the food industry for rapid identification of pathogenic microorganisms, and thus, elegantly replacing/complementing the classical microbiology, ELISA techniques or the need of detection of the 4-ethylphenol yeast-product.

Herein, *Brett*-DNA biosensors have been successfully developed on gold nanostructured coverslips and showed excellent specificity and selectivity based on SERS and LSPR detections of *Brettanomyces bruxellensis* yeast in wine. More specifically, SERS technique made possible the detection of Brett-DNA in pM range, while LSPR revealed detection of a real DNA extracted in the laboratory (0.1 ng/µL).

In the near future, the authors intend to create specific DNA-SERS databases for monitoring the presence of different categories of pathogens and their interfering products.

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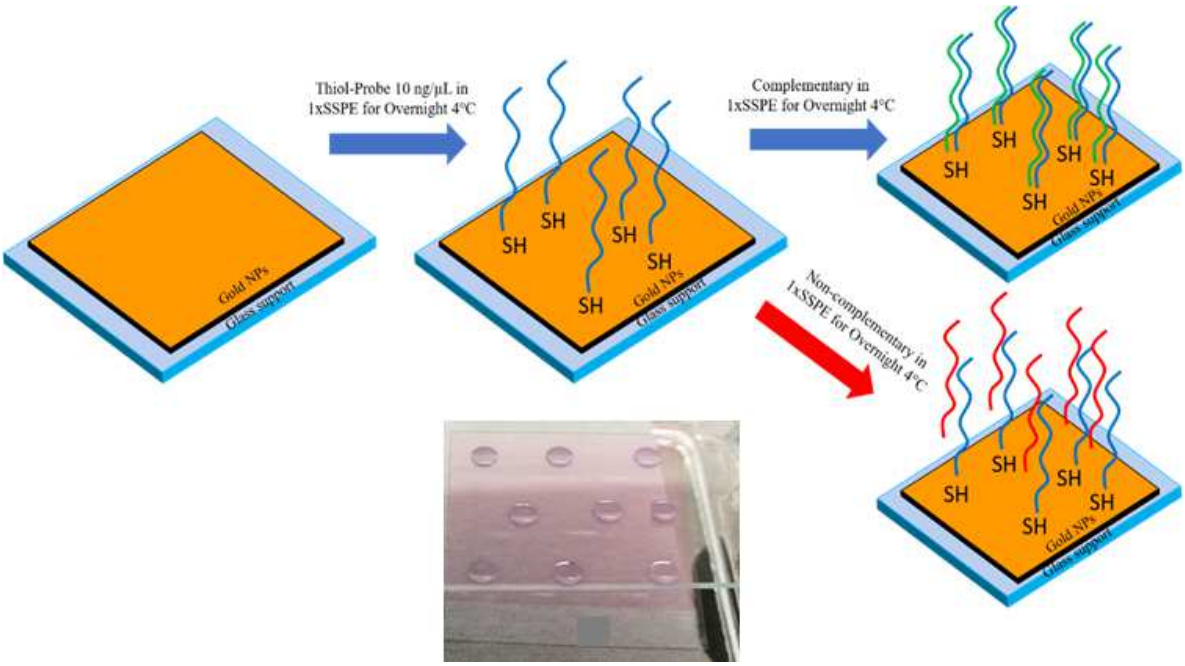
Figure captions


Thiol-Probe 10 ng/µL in
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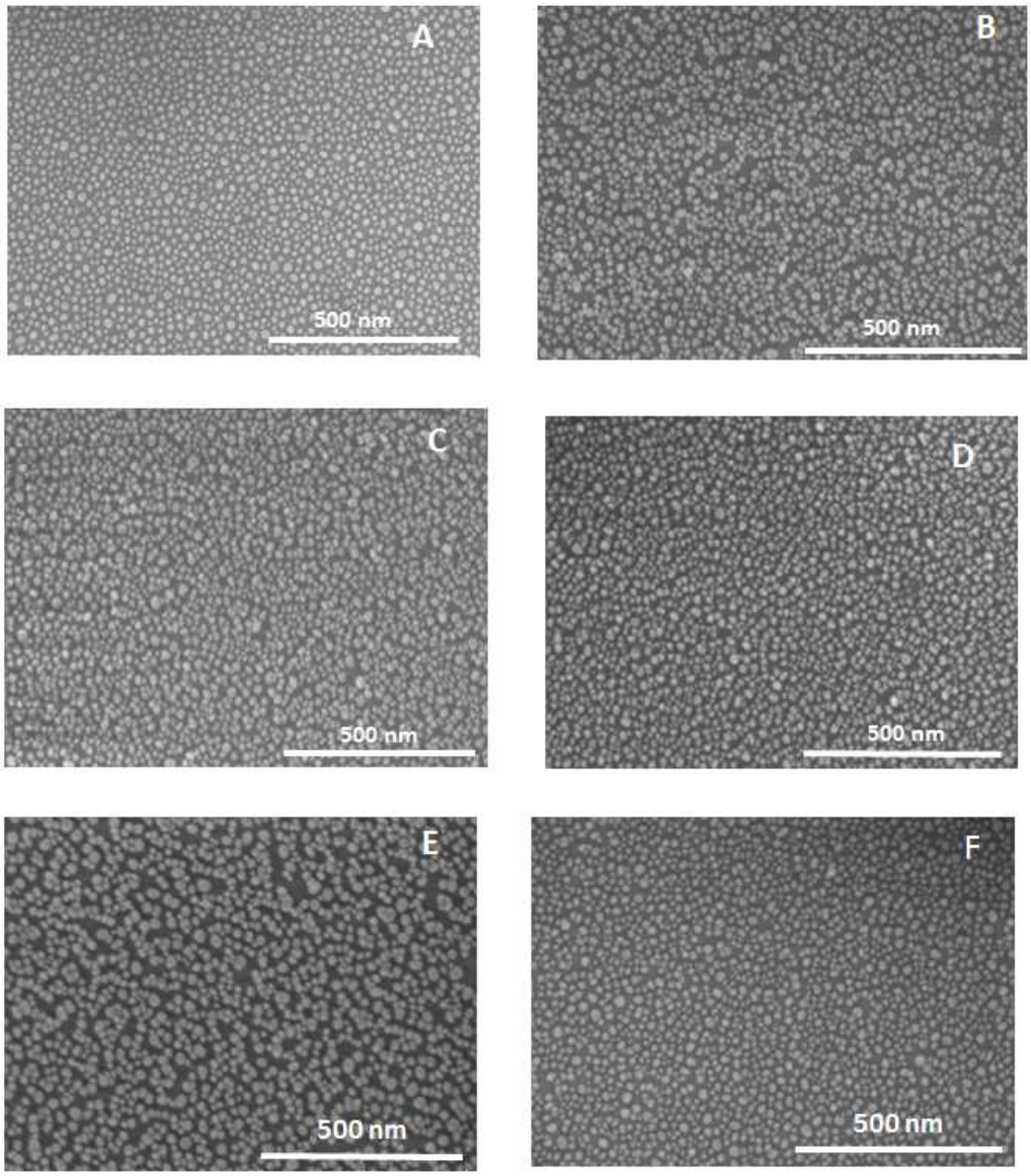
Complementary in
1xSSPE for Overnight 4°C
Non-complementary in
1xSSPE for Overnight 4°C
Gold NPs
Glass support
SH



(i)

(ii)

Fig. 1. (Bio)functionalization (overnight at 4 °C) of AuNPs on coverslips with biomolecules after incubation with complementary/non-complementary DNA sequences (real image of gold nanostructured coverslip with DNA aliquots of 2 µL) (i) SEM images after each modification step, (A) bare gold nanoparticles on glass coverslip, (B) with Thiol-DNA probe 10 ng/µL in 1xSSPE, (C), (D) with Brett-DNA complementary sequence of 10 ng/µL and 1 pg/µL while (E), (F) with Brett-DNA non-complementary of 10 ng/µL and 1 pg/µL.

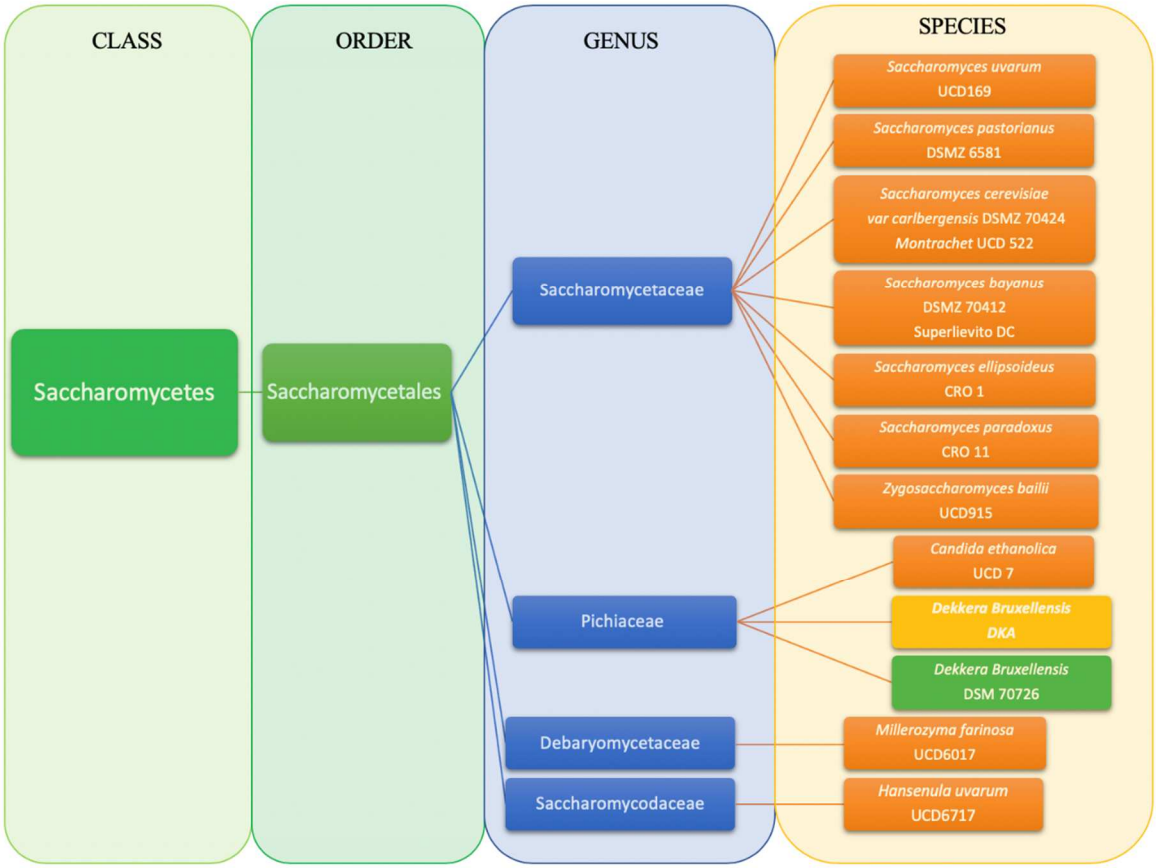


Fig. 2.

Fig. 2. Phylogenetic *Saccharomycetes* tree used for specific (DNA from *Dekkera Bruxellensis-DKA*) and non-specific experiments (DNA from other listed microorganisms) for validation of SERS genosensors.

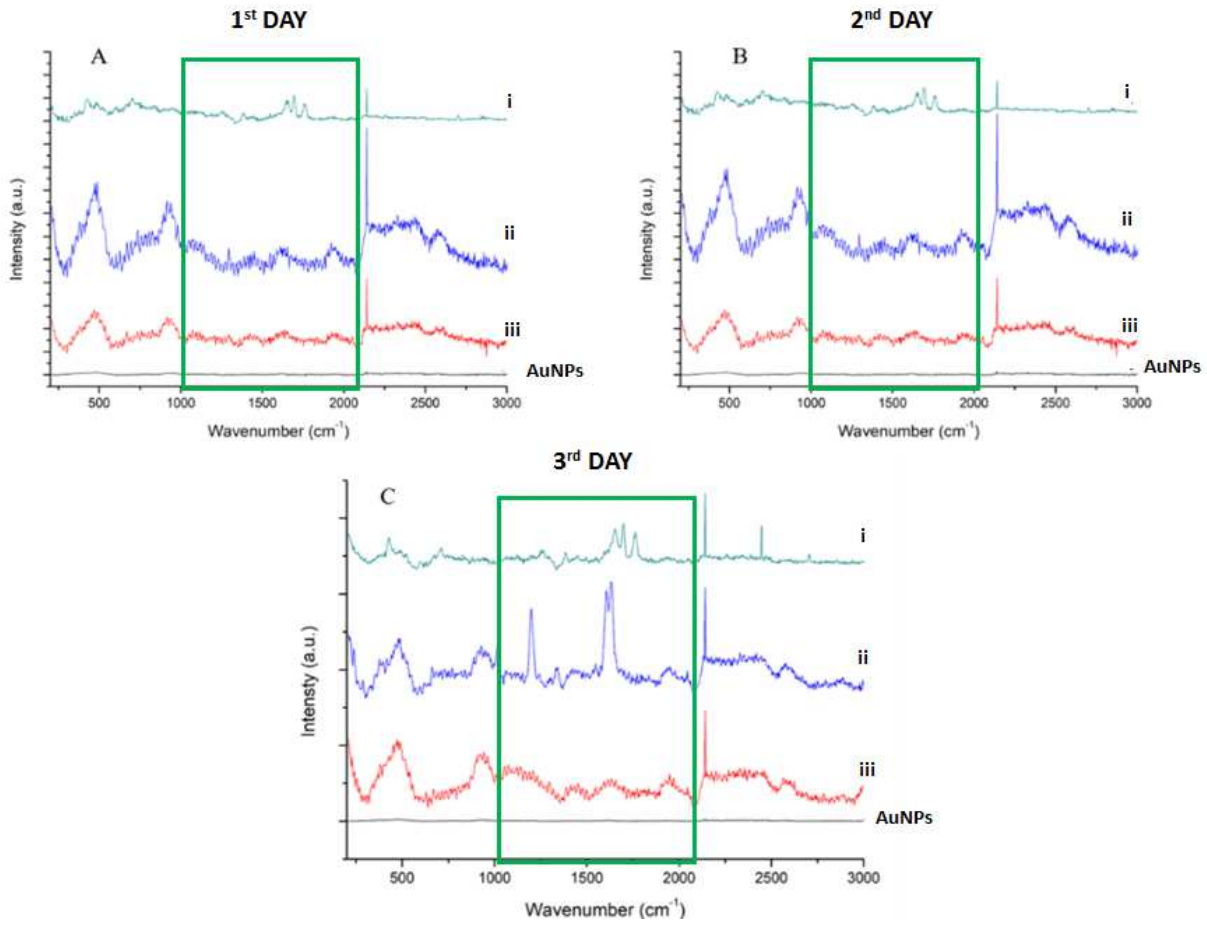


Fig. 3

Fig. 3. SERS spectra of three sensing *"in tube"* configurations (i, ii, iii) over three days (A, B, C). These configurations are based on mixing the Thiol-probe with DNA-analyte as the following: (i) DNA probe (10 ng/µL) and DNA complementary (100 ng/µL) in water, (ii) DNA- probe (10 ng/µL) and the whole genomic *Brettanomyces bruxellensis DKA* (100 ng/µL) in water, (iii) DNA-probe (10 ng/µL) and DNA - complementary (100 ng/µL) in PEG buffer. Each SERS spectrum was recorded from 2 µL of each configuration and deposited on AuNPs coverslips using an acquisition time of 60 sec x 3 times and a filter D 0.

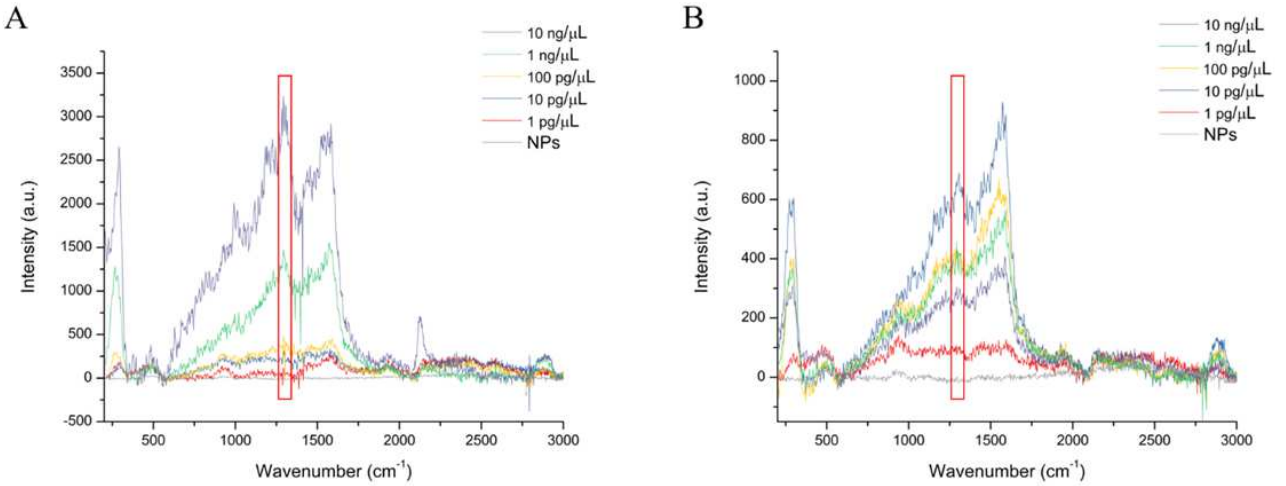


Fig. 4

Fig. 4. SERS spectra of (A) specific and (B) non-specific *Brett*-genosensors. The acquisition time was set at 10 sec x 3 times using a D 0.3 filter. The red box indicates the maximum peak wavenumber position used in the construction of the calibration curves.

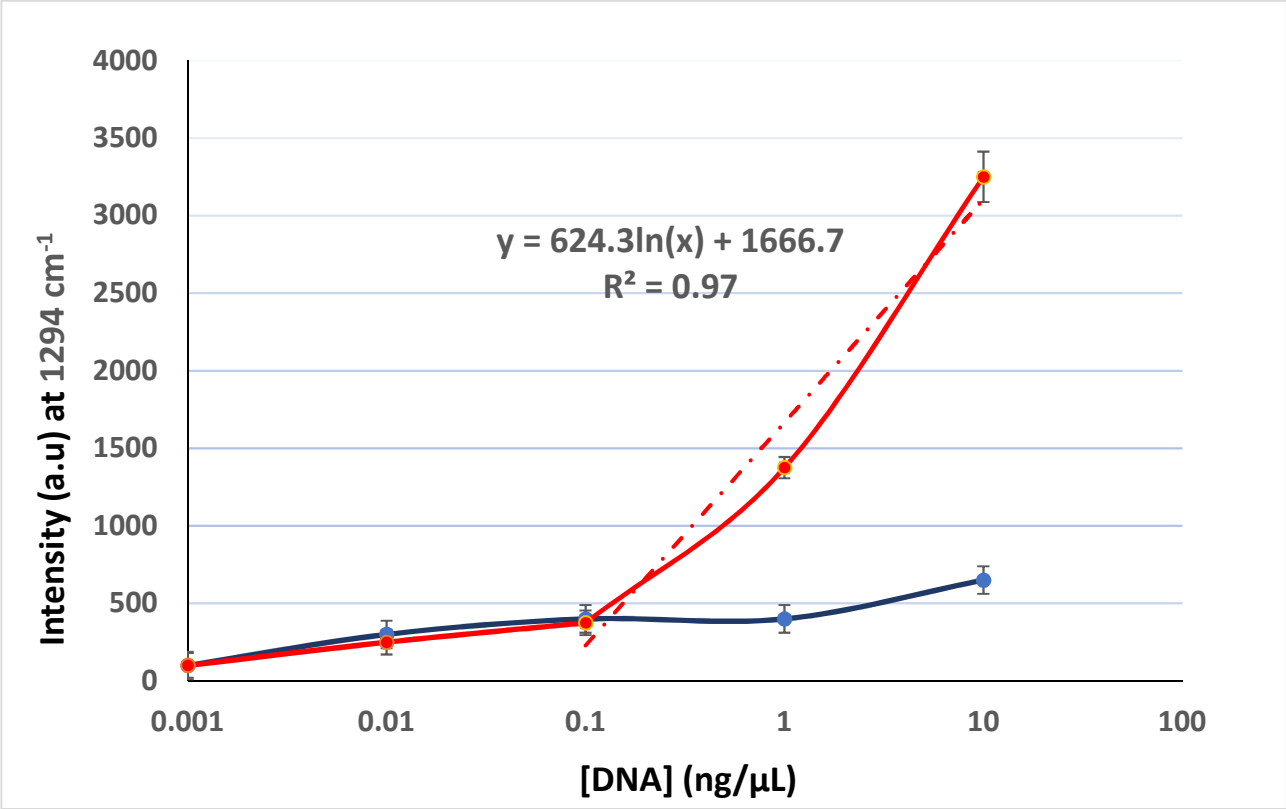
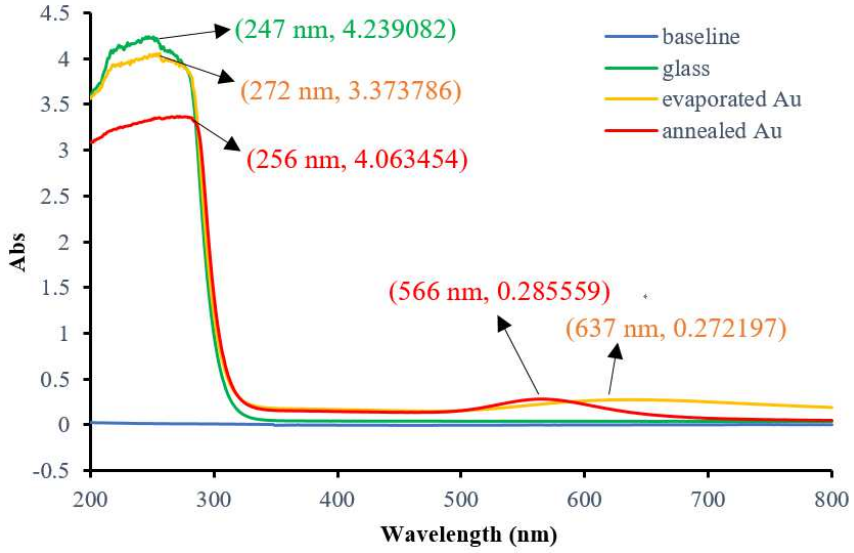
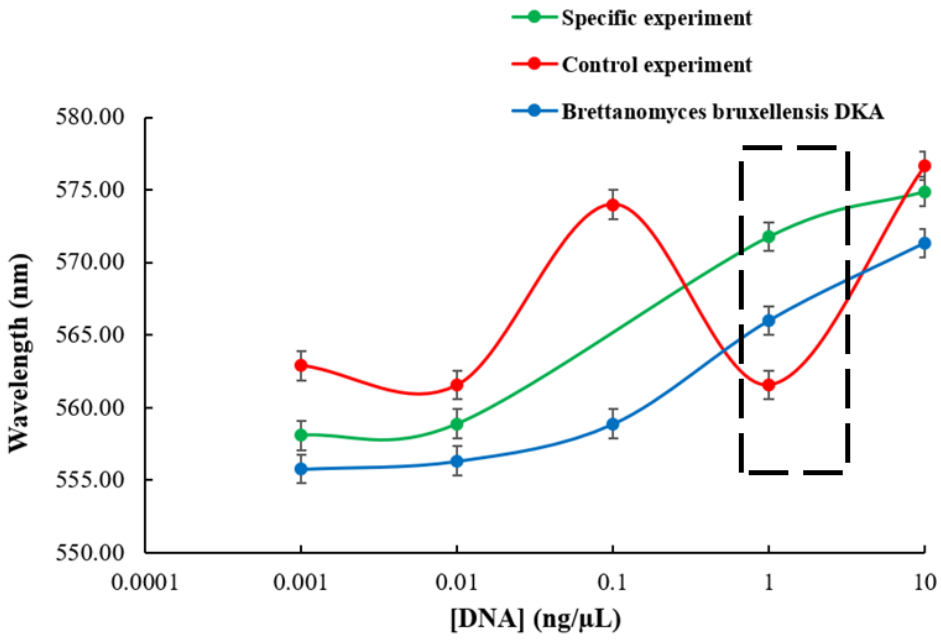


Fig. 5. Calibration curves of specific and control *Brett*-genosensors based on SERS measurements.



(A)



(B)

Fig. 6

Fig. 6. (A) UV-VIS spectra of glass samples modified with evaporated gold (Au) and annealed Au, (B) Calibration curves of specific, non-specific (control) and real DNA strains using the maximum the wavelength shifts of LSPR peaks.

Table 1. Sequences of Thiol-Probe, complementary and non-complementary DNA. In the non-complementary DNA sequence, the non-specific bases are highlighted in red.

Thiol-Probe	[ThiolC6]TGTTTGAGCGTCATTTCCTTCTCACTATTTAGTGGTT ATGAGATTACACGAGG (**53 bp**).
Complementary *(Brettanomyces bruxellensis)*	CCTCGTGTAATCTCATAACCACTAAATAGTGAGAAGGAAAT GACGCTCAAACA (**53 bp**)
Non-complementary	CCTAAGGTAATAGCATAAGTACTAAATAACCAGAATCAAAG AACGCTCAACTT (**53 bp**).

Table 2. List of yeast strains tested in SERS to prove the specificity of *Brett*-DNA sequence in the presence of Thiol-probe.

Saccharomyces paradoxus DI4A[c] 11
Saccharomyces ellipsoideus DI4A[c] 1
Saccharomyces pastorianus DSMZ[a] 6581
Saccharomyces bayanus DSMZ[a] 70412
Saccharomyces cerevisiae var carlbergensis DSMZ[a] 70424
Saccharomyces cerevisiae Montrachet UCD[b] 522
Saccharomyces ellipsoideus CRO[e] 2
Saccharomyces bayanus Superlievito DC[d]
Saccharomyces uvarum UCD[b] 169
Candida ethanolica UCD[b] 37
Torulaspora delbrueckii DSMZ[a] 70607
Millerozyma farinosa UCD[b] 6017
Zygosaccharomyces bailii UCD[b] 915
Hansenula uvarum UCD[b] 6717
Dekkera bruxellensis DSMZ[a] 70726
Dekkera bruxellensis DKA[e]

a) Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbM, Braunschweig, Germany.

b) University of Davis, CA, USA.

c) University of Udine, UD, Italy

d) Commercial yeast for wine

e) University of Udine, UD, Italy

Table 3. SERS spectra of microorganism from *Saccharomycetes* class.

		SERS peaks					
Genus	**Species**	**350 -550 cm^{-1} (blue)**	**600 -1000 cm^{-1} (green)**	**1250 – 1450 cm^{-1} (navy)**	**1600 - 1800 cm^{-1} (orange)**	**1850 - 2000 cm^{-1} (brown)**	**2700 - 2900 cm^{-1} (navy)**
Saccharomycetaceae	All listed in Table 2	450-550	850-1000	Different shapes of peaks for each type of yeast (few differences but recognizable)	One peak in 1600	Some differences	Some differences and different shape of peaks
Pichiaceae	*Dekkera Bruxellensis* DKA	417, 485	698	Some differences	1644, 1688, 1757	x	x
	Dekkera Bruxellensis DSMZ 70726	Some differences	Some differences	**Strong differences**	Some differences	x	x
Debaryomycetaceae	*Millerozyma farinosa*	Min: 300	850-1050	Max 1500	1500-1750	Max 2050	Max 2850
Saccharomycodaceae	*Hansenula uvarum*	Min: 300	x	Different shapes of peaks	x	x	x

Table 4. Position of SERS peaks of Thiol-probe suspended either in water or 1xSSPE on gold nanostructured coverslips after an overnight incubation at 4 °C (Figure S5).

Thiol-probe in water Wavenumber (cm^{-1})	Thiol-probe in 1xSSPE Wavenumber (cm^{-1})
658	656
925	964
1018	1012
1201	1192
1604	1600
1634	1629