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Electrochemical biosensing of 16s rRNA gene sequence of *Enterococcus faecalis*

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ABSTRACT

Some of microorganisms are potential pathogens that can be infectious agents under some circumstances, and development of new detection methods of the pathogens is of high interest. In the present study, an *Enterococcus faecalis* (*E. faecalis*) DNA biosensor (ef-biosensor) was fabricated to quantify the bacterium genome. A specific *E. faecalis* DNA probe was selected from 16S rRNA sequence of *E. faecalis* and immobilized on a gold electrode surface in an optimized time to fabricate the ef-biosensor. The ef-biosensor detected a synthetic target of the probe with a detection limit of 3.3 amol L^{-1} and with a nice selectivity to resolve from one-, two- and three-base mismatched sequences. In addition, the bacterium genomic DNA was quantified with a detection limit of 7.1×10^{-9} ng mL^{-1} in a concentration range of 1.1×10^{-7} to 1.1 ng mL^{-1}. The ef-biosensor had a long time stability, good fabrication reproducibility and good regeneration ability. The ef-biosensor was successfully applied for *E. faecalis* detection in human samples.

1. Introduction

Enterococci are gram-positive cocci and catalase-negative bacteria that can occur singly, in pair, or as short chains (Cai et al., 2012). They have the ability to survive in harsh conditions including alkaline pH and high ionic strength. Also, they can grow in a temperature range of 10–45 °C, and survive up to 60 °C for 30 min (Endo et al., 2015). In healthy individuals and under certain circumstances, they can expand dramatically, given appropriate physiological perturbations such as drug regimens, and cause different types of infections in humans (Christopher et al., 2014). The bacteria source in these infections has been reported to originate from clinical instruments, health care hand workers or from patient to patient (Higuita and Huycke, 2014; Siegel et al., 2007). From *enterococcal* species, only *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* commonly colonize and infect human in detectable numbers (Kim et al., 2018). They are a cause of bacteremia, bacterial meningitis, urinary tract infections, endocarditis, wound infections, and various other infections in human (Lucena et al., 2013; Oesterle et al., 2019). *Enterococcal* infections now roughly account for 12% of nosocomial infections in US with a major cause by *E. faecalis* (> 80%) and *Enterococcus faecium* being major reason of the remaining infections (Gordana et al., 2018; Zhang et al., 2016).

Pathogen diagnosis is of utmost importance for disease prevention and proper treatment (Agashe et al., 2009). Established methods in clinical pathogen diagnosis are normally PCR for nucleic acid testing, culture and biochemical analysis, and immunology-based methods (Yen et al., 2014). These methods are exclusively performed in well-equipped laboratories; demand high-end instrumentations and trained personnel; and are time-consuming. There have also limitations such as needing complicated sample preparation.

New technologies have been developed in recent years for improving the clinical diagnostics and therapy (Abkar et al., 2019; Gorgizadeh et al. 2018, 2019; Negahdary et al., 2018; Sattarahmady et al. 2017b, 2018). In this regard, biosensors and analytical devices can be alternatives to the routine methods with great advantages of simple, direct and rapid determination using a minimum volume of sample (Heli et al., 2013; Sattarahmady et al., 2015; Tondro et al., 2018). Electrochemical biosensors detect a wide range of targets from small molecules to large pathogens by coupling a biological recognition element and an electrochemical transducer (Heli et al., 2016; Mohammadi et al., 2017; Nazari-Vanani et al., 2018; Rahi et al., 2016; Sattarahmady et al., 2017a). These compacted size devices work using relatively low-

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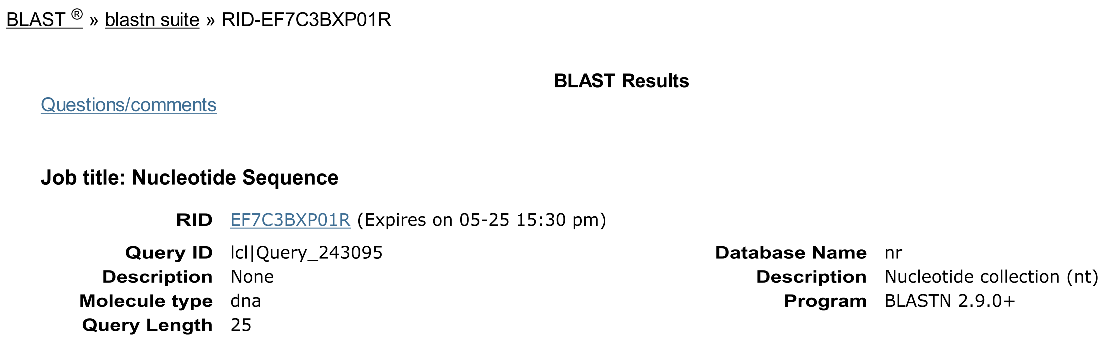


BLAST ® » blastn suite » RID-EF7C3BXP01R

BLAST Results

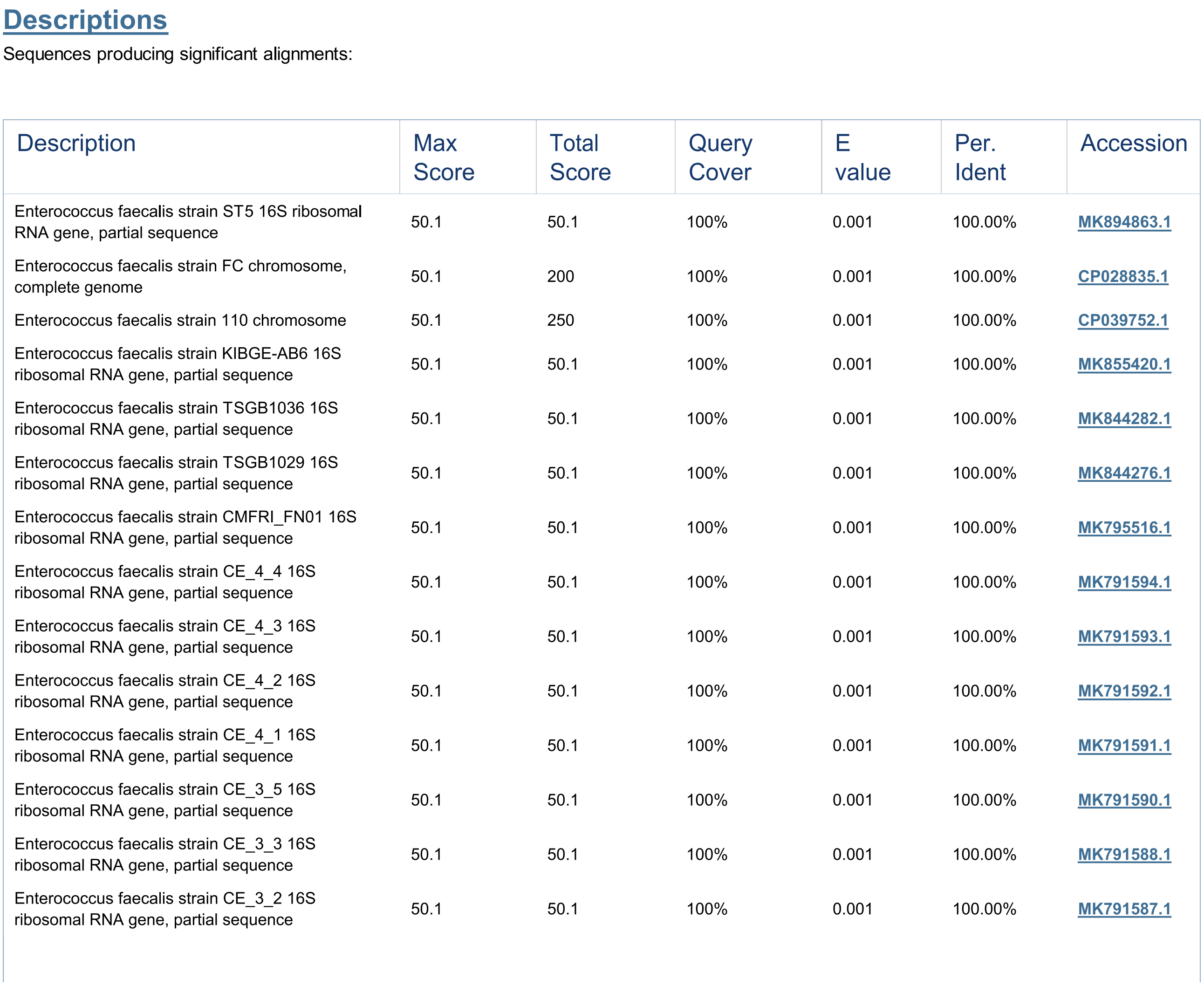
Questions/comments

Job title: Nucleotide Sequence

RID EF7C3BXP01R (Expires on 05-25 15:30 pm)

Query ID	lcl|Query_243095	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	dna	**Program**	BLASTN 2.9.0+
Query Length	25		

Scheme 1. Similarity of he probe ssDNA against a database of nucleotide using the nucleotide BLAST.



Descriptions

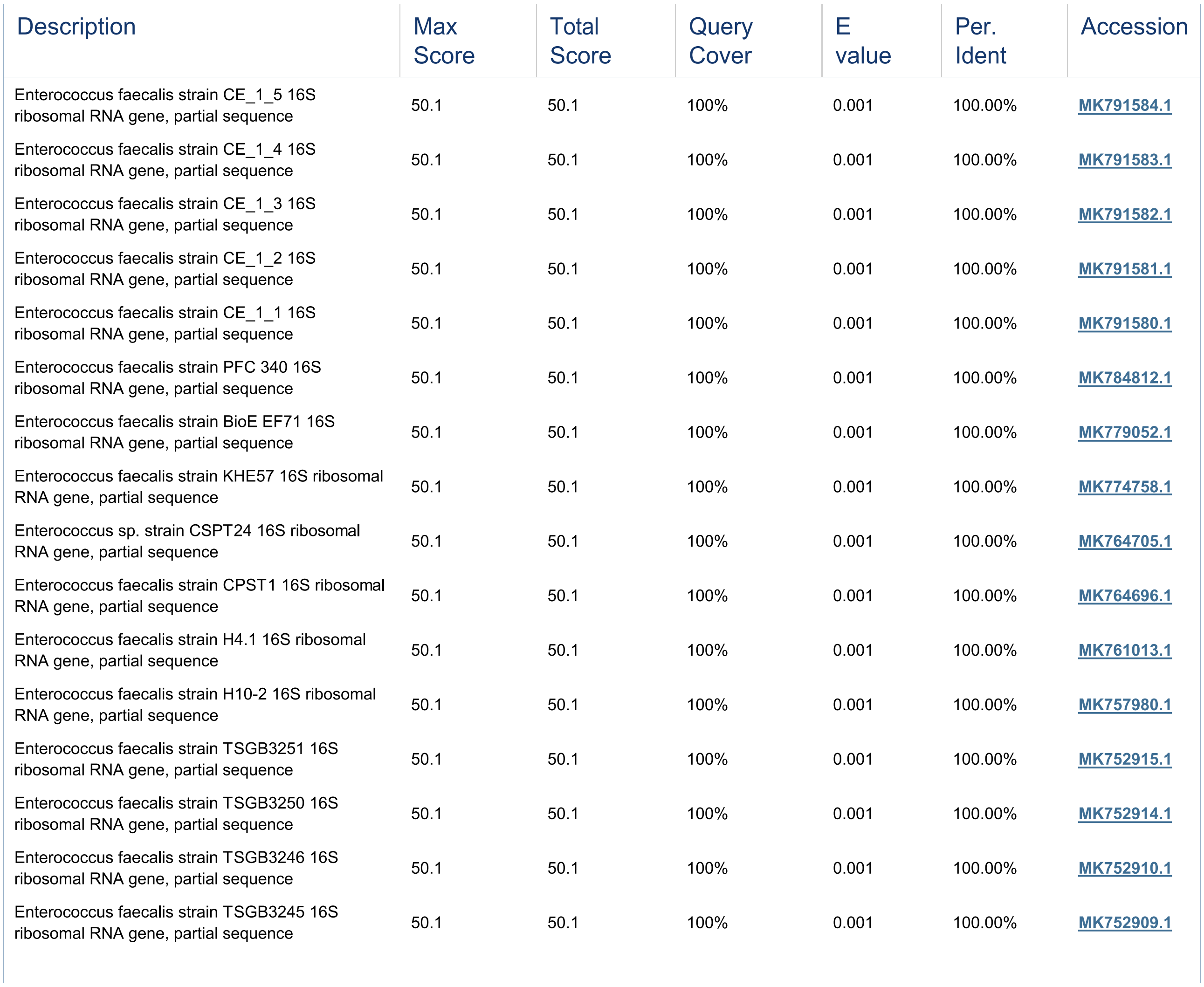
Sequences producing significant alignments:

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Enterococcus faecalis strain ST5 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK894863.1
Enterococcus faecalis strain FC chromosome, complete genome	50.1	200	100%	0.001	100.00%	CP028835.1
Enterococcus faecalis strain 110 chromosome	50.1	250	100%	0.001	100.00%	CP039752.1
Enterococcus faecalis strain KIBGE-AB6 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK855420.1
Enterococcus faecalis strain TSGB1036 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK844282.1
Enterococcus faecalis strain TSGB1029 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK844276.1
Enterococcus faecalis strain CMFRI_FN01 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK795516.1
Enterococcus faecalis strain CE_4_4 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791594.1
Enterococcus faecalis strain CE_4_3 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791593.1
Enterococcus faecalis strain CE_4_2 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791592.1
Enterococcus faecalis strain CE_4_1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791591.1
Enterococcus faecalis strain CE_3_5 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791590.1
Enterococcus faecalis strain CE_3_3 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791588.1
Enterococcus faecalis strain CE_3_2 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791587.1

Scheme 1. (*continued*)

cost instruments without requiring highly trained personnel to apply them (Heli et al., 2016; Mohammadi et al., 2017; Nazari-Vanani et al., 2018; Rahi et al., 2016; Sattarahmady et al., 2017a), and represent high sensitivity and selectivity in a short determination time (near real-time assay) (Lazcka et al., 2007). These devices are categorized based on the output signal into current, potential or impedance (Pohanka and Skladal, 2008).

Self-assembly as an autonomous process of components into ordered patterns has received particular attention as a platform for biosensor fabrication (Heli, 2014; Whitesides and Grzybowski, 2002). This has advantages including easy formation of stable monolayers, reduction in the amount of biomolecule for immobilization on surfaces and flexibility to design the head group of the monolayers with various functional groups (Martelet et al., 2007). Self-assembled monolayers (SAMs) usually consist of a head group, a spacer chain and a free end functional group. The head group strongly binds into a substrate, and the spacer chain (typically 1–3 nm) determines the SAMs thickness and provides flexibility for the immobilized species. The end functional group remains available and free for any modifications. They carry out an important role in determining the physical and chemical properties of the modified surfaces (DAcunto, 2006; Sattarahmady et al., 2017a). Thiol-gold binding system has been vastly used for SAMs formation due to the



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Enterococcus faecalis strain CE_1_5 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791584.1
Enterococcus faecalis strain CE_1_4 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791583.1
Enterococcus faecalis strain CE_1_3 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791582.1
Enterococcus faecalis strain CE_1_2 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791581.1
Enterococcus faecalis strain CE_1_1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK791580.1
Enterococcus faecalis strain PFC 340 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK784812.1
Enterococcus faecalis strain BioE EF71 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK779052.1
Enterococcus faecalis strain KHE57 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK774758.1
Enterococcus sp. strain CSPT24 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK764705.1
Enterococcus faecalis strain CPST1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK764696.1
Enterococcus faecalis strain H4.1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK761013.1
Enterococcus faecalis strain H10-2 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK757980.1
Enterococcus faecalis strain TSGB3251 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK752915.1
Enterococcus faecalis strain TSGB3250 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK752914.1
Enterococcus faecalis strain TSGB3246 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK752910.1
Enterococcus faecalis strain TSGB3245 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK752909.1

Scheme 1. (*continued*)

strength bond, inertness, biocompatibility, and easy formation (Hasan and Pandey, 2018). It has been widely employed for fabrication of biosensors (Nazari-Vanani et al., 2018; Negahdary et al., 2018; Rahi et al., 2016; Sattarahmady et al. 2015, 2017a; Tondro et al., 2018).

Detection of *E. faecalis* has been performed by polymerase chain reaction (PCR) assay and conventional culture procedure. However, in the last two decades, some limited detection methods for *E. faecalis* have been reported. Real-time PCR assay was developed for the detection and quantification of this pathogen in clinical samples with a limit of detection (LOD) of 5 CFU/reaction (He and Jiang, 2005). A PCR based on 16S rRNA gene could detect *E. faecalis* with a diagnostic sensitivity of 80%, while the culture assay attains only 16% (Zoletti et al., 2006). Also, a quantitative PCR assay detected this bacterium in a concentration range of 30–240 CFU mL^{-1} (Haugland et al., 2005). Other diagnostic methods have also been explored such as a localized surface plasmon resonance-based optical biosensor that was connected to a multispot copper-capped nanoparticles array chip (Kim et al., 2011). This chip displayed a LOD of 10 fmol L^{-1}.

Due to the high rate of prevalence and the difficulties involved in effective diagnosis of *E. faecalis* infection, and limited available biosensors for detection of this pathogen, in the present study, a new, PCR-free, label-free and electrochemical DNA biosensor (ef-biosensor), as a new approach, was fabricated as a rapid, simple and quantitative detection device for *E. faecalis* genome. The ef-biosensor was successfully tested for the *E. faecalis* genome in human samples. The detection limit, linear range, selectivity, regeneration, and stability of the ef-biosensor was analyzed. To the best of our knowledge, ef-biosensor is one of the unique tools for *E. faecalis* genome detection.

2. Experimental section

2.1. Chemicals and reagents

All reagents were obtained from Scharlau (Spain), Merck (Germany) or Sigma-Aldrich (USA). They were of reagent grade and used without further purification. Deionized water was used for preparation of the solutions. All DNA oligonucleotides were purchased from Bioneer (Korea). These nucleotide sequences were as follows:

Sequence of probe single-stranded DNA (ssDNA):

5′ TTT TTT TTT TCA ATT GGA AAG AGG AGT GGC GGA CG 3′

Sequence of target ssDNA:

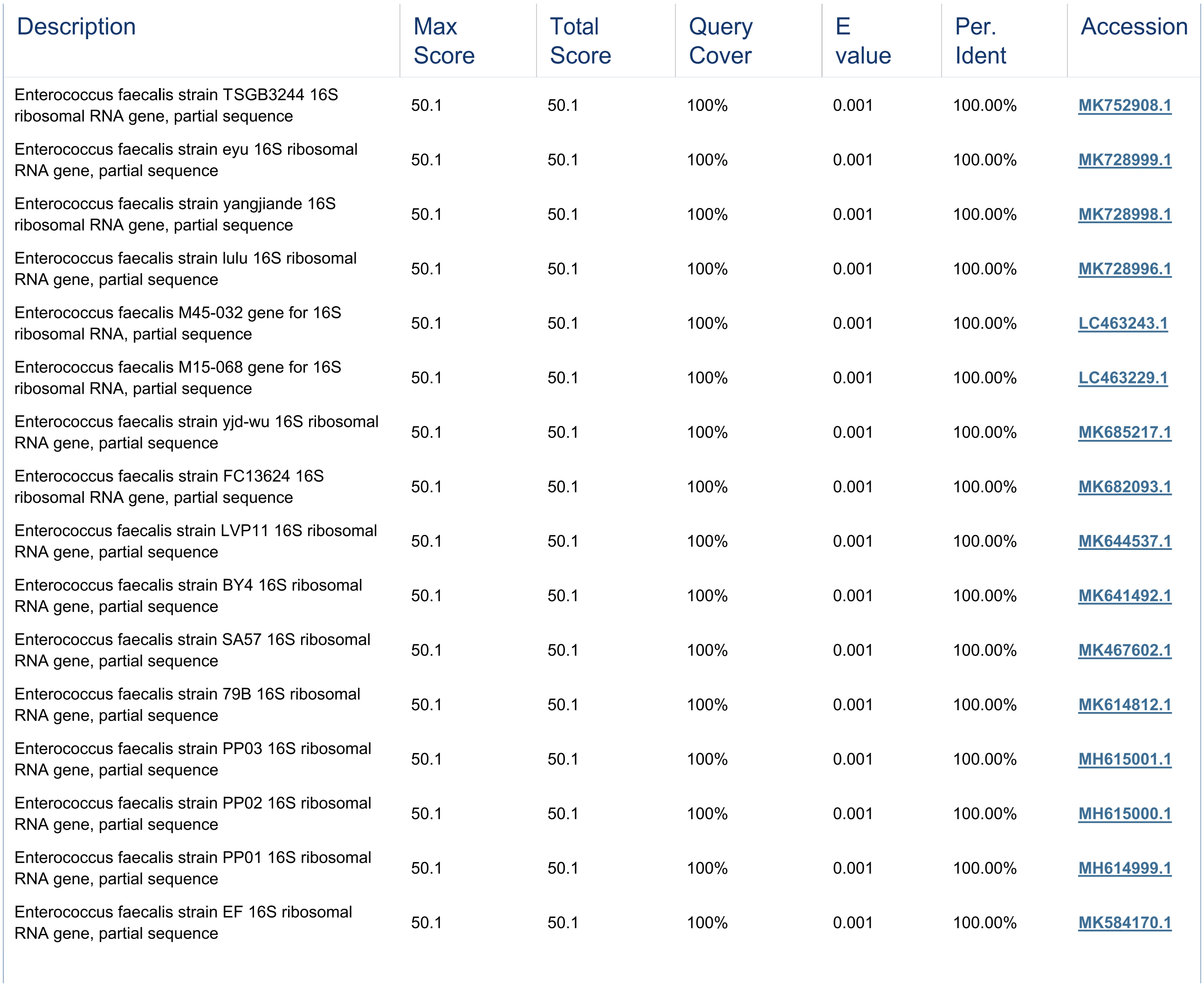
5′ CGT CCG CCA CTC CTC TTT CCA ATT G 3′

Sequence of non-complementary ssDNA:

5′ CAG AAT TTC AGA AGA CCC AAA CGA T 3′

Sequence of 1-base mismatched ssDNA:

5′ CGT CCG CCA CTC CTC TTT C**T**A ATT G 3′



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Enterococcus faecalis strain TSGB3244 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK752908.1
Enterococcus faecalis strain eyu 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK728999.1
Enterococcus faecalis strain yangjiande 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK728998.1
Enterococcus faecalis strain lulu 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK728996.1
Enterococcus faecalis M45-032 gene for 16S ribosomal RNA, partial sequence	50.1	50.1	100%	0.001	100.00%	LC463243.1
Enterococcus faecalis M15-068 gene for 16S ribosomal RNA, partial sequence	50.1	50.1	100%	0.001	100.00%	LC463229.1
Enterococcus faecalis strain yjd-wu 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK685217.1
Enterococcus faecalis strain FC13624 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK682093.1
Enterococcus faecalis strain LVP11 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK644537.1
Enterococcus faecalis strain BY4 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK641492.1
Enterococcus faecalis strain SA57 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK467602.1
Enterococcus faecalis strain 79B 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK614812.1
Enterococcus faecalis strain PP03 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MH615001.1
Enterococcus faecalis strain PP02 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MH615000.1
Enterococcus faecalis strain PP01 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MH614999.1
Enterococcus faecalis strain EF 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK584170.1

Scheme 1. (*continued*)

Sequence of 2-base mismatched ssDNA:

5′ CGT CCG CCA CTC CTC TTT **AT**A ATT G 3′

Sequence of 3-base mismatched ssDNA:

5′ CGT CCG CCA CTC CT**A** TTT **AT**A ATT G 3′

Sequence of forward PCR primer: 5′ CGC TTC TTT CCT CCC GAG T 3’

Sequence of reverse PCR primer: 5′ GCC ATG CGG CAT AAA CTG 3’

The probe ssDNA was selected from 16s rRNA gene sequence of *E. faecalis*. All oligonucleotides stock solutions were prepared with 20 mmol L^{-1} tris-HCl buffer, pH = 7.4 (Tris buffer) and stored at -20 °C.

2.2. Equipments

Electrochemical measurements were performed with a three-electrode system composed of an Ag/AgCl, a platinum wire and a gold disk of 2 mm in diameter as the reference, counter and working electrodes, respectively, powered by a μ-Autolab potentiostat/galvanostat (the Netherlands).

PCR was carried out in a thermal cycler of Bio-Rad T100 (Hungary). DNA concentrations were determined by a NanoDrop Lite (USA) spectrophotometer.

Field emission scanning electron microscopy (FESEM) images were recorded using a MIRA3 TESCAN-XMU (Czech Republic) microscope.

2.3. Preparation of the gold electrode and electrolytes

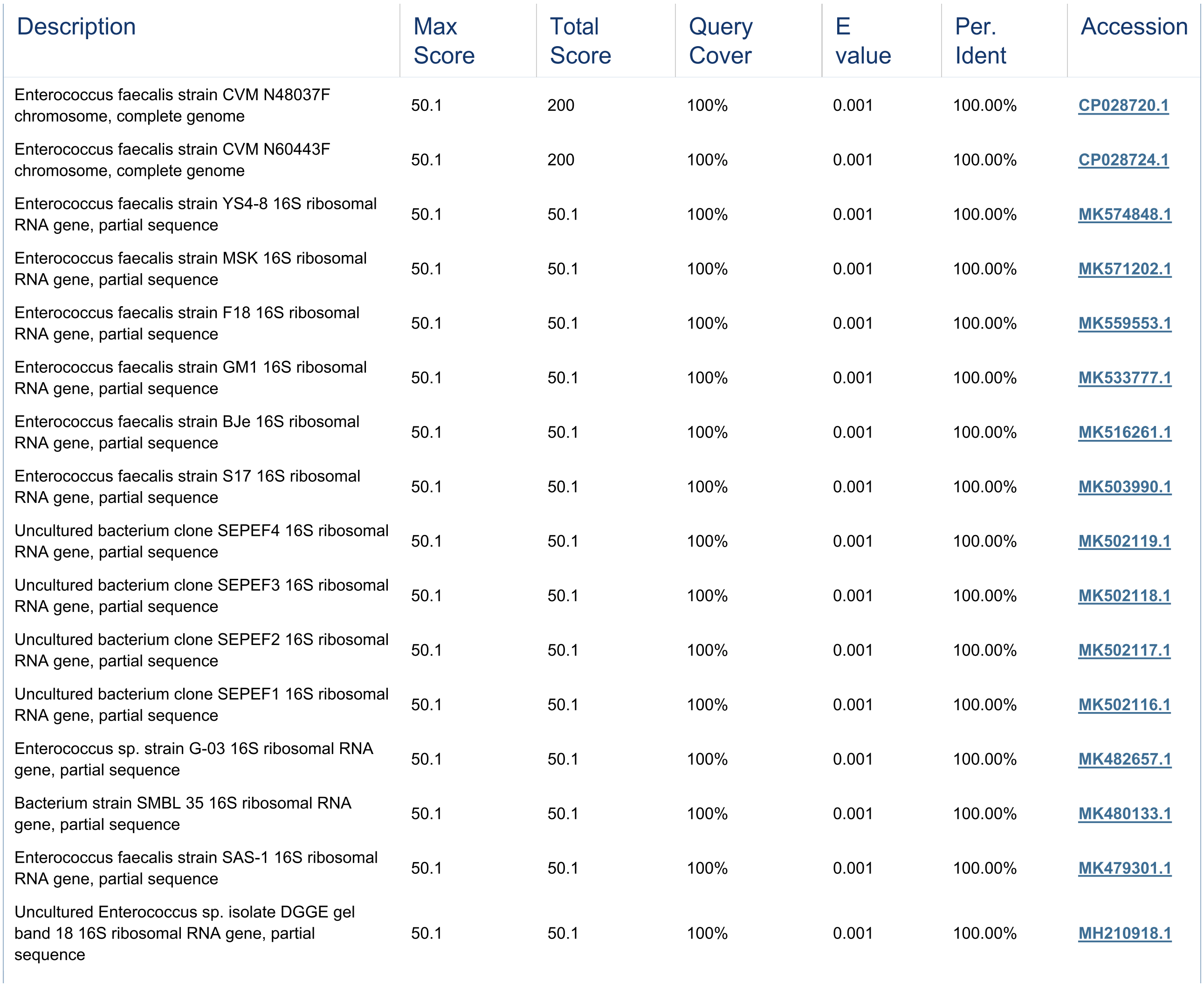
Prior to use, the gold disk electrode was polished with Al_2O_3 nanopowder to attain a mirror-like surface. Then, it was ultrasonically treated in an ethanol:water 3:1 (v/v) mixture for 5 min to remove the adhered substances. The pretreated electrode was then rinsed with distilled water and allowed to dry at room temperature.

A ferricyanide/ferrocyanide mixed solution containing 0.5 mol L^{-1} KCl and 0.5 mmol L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) was employed as a redox marker. Piranha solution was prepared by mixing the concentrated hydrogen peroxide and sulfuric acid in a 1:2 (v/v) ratio. All of the above solutions were maintained at 4 °C until used.

A dithiothreitol (DTT) solution containing 10 mmol L^{-1} sodium acetate, pH = 5.2 and 500 mmol L^{-1} DTT was prepared and kept at -20 °C.

2.4. Evaluation of the best time of probe ssDNA immobilization

Open-circuit potential (OCP) measurements were carried out to obtain an optimized immobilization time of the probe ssDNA on the gold electrode surface. Screen printed electrodes from Dropsens (Spain) were employed in this part of the study. 10 μL of 10 μmol L^{-1} solution of the probe ssDNA was dropped onto the electrode surface and refrigerated at 4 °C for 14 h. OCP was continuously measured over time using a digital multimeter of Mastech Inc. (China).



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Enterococcus faecalis strain CVM N48037F chromosome, complete genome	50.1	200	100%	0.001	100.00%	CP028720.1
Enterococcus faecalis strain CVM N60443F chromosome, complete genome	50.1	200	100%	0.001	100.00%	CP028724.1
Enterococcus faecalis strain YS4-8 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK574848.1
Enterococcus faecalis strain MSK 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK571202.1
Enterococcus faecalis strain F18 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK559553.1
Enterococcus faecalis strain GM1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK533777.1
Enterococcus faecalis strain BJe 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK516261.1
Enterococcus faecalis strain S17 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK503990.1
Uncultured bacterium clone SEPEF4 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK502119.1
Uncultured bacterium clone SEPEF3 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK502118.1
Uncultured bacterium clone SEPEF2 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK502117.1
Uncultured bacterium clone SEPEF1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK502116.1
Enterococcus sp. strain G-03 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK482657.1
Bacterium strain SMBL 35 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK480133.1
Enterococcus faecalis strain SAS-1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK479301.1
Uncultured Enterococcus sp. isolate DGGE gel band 18 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MH210918.1

Scheme 1. (*continued*)

2.5. Immobilization of the probe ssDNA

10 µL of DTT solution was added into 10 µmol L^{-1} solution of the probe ssDNA, mixed by a vortex and placed at room temperature for 30 min. Then, triplicate extraction with 200 µL of ethyl acetate was carried out to remove the excess DTT. 10 µL of the resultant probe ssDNA was immediately dropped on the electrode surface and refrigerated at 4 °C at desired time (obtained from section 2.4 and equal to 7 h) to complete immobilization. Then, the electrode was further washed with distilled water and treated with 10 µL of 1.0 mmol L^{-1} 6-mercapto-1-hexanol for 30 min to obtain an immobilized, self-assembled and aligned monolayer of the probe ssDNA. The electrode was then rinsed thoroughly with distilled water to remove unadsorbed materials and denoted as the ef-biosensor.

2.6. Detection of DNA hybridization

Hybridization detection was done by dropping 10 µL of the hybridization solution (containing target, 1-base mismatched, 2-base mismatched, 2-base mismatched or non-complementary ssDNA sequences, or genomic DNA) on the surface of the ef-biosensor. For the target ssDNA, a concentration range of 1.0×10^{-17} to 1.0×10^{-12} mol L^{-1}; for the genomic DNA, a concentration range of 1.1×10^{-7} to 1.1 ng mL^{-1}; and for the other sequences, a concentration of 1.0×10^{-12} mol L^{-1} were employed. The hybridization process was carried out at 37 °C for 60 min (Nazari-Vanani et al., 2018). Next, the ef-biosensor was thoroughly rinsed with distilled water and DPVs were recorded in the redox marker. DPVs were recorded with a pulse width of 25 mV, a pulse time of 50 ms, and a potential sweep rate of 10 mV s^{-1}.

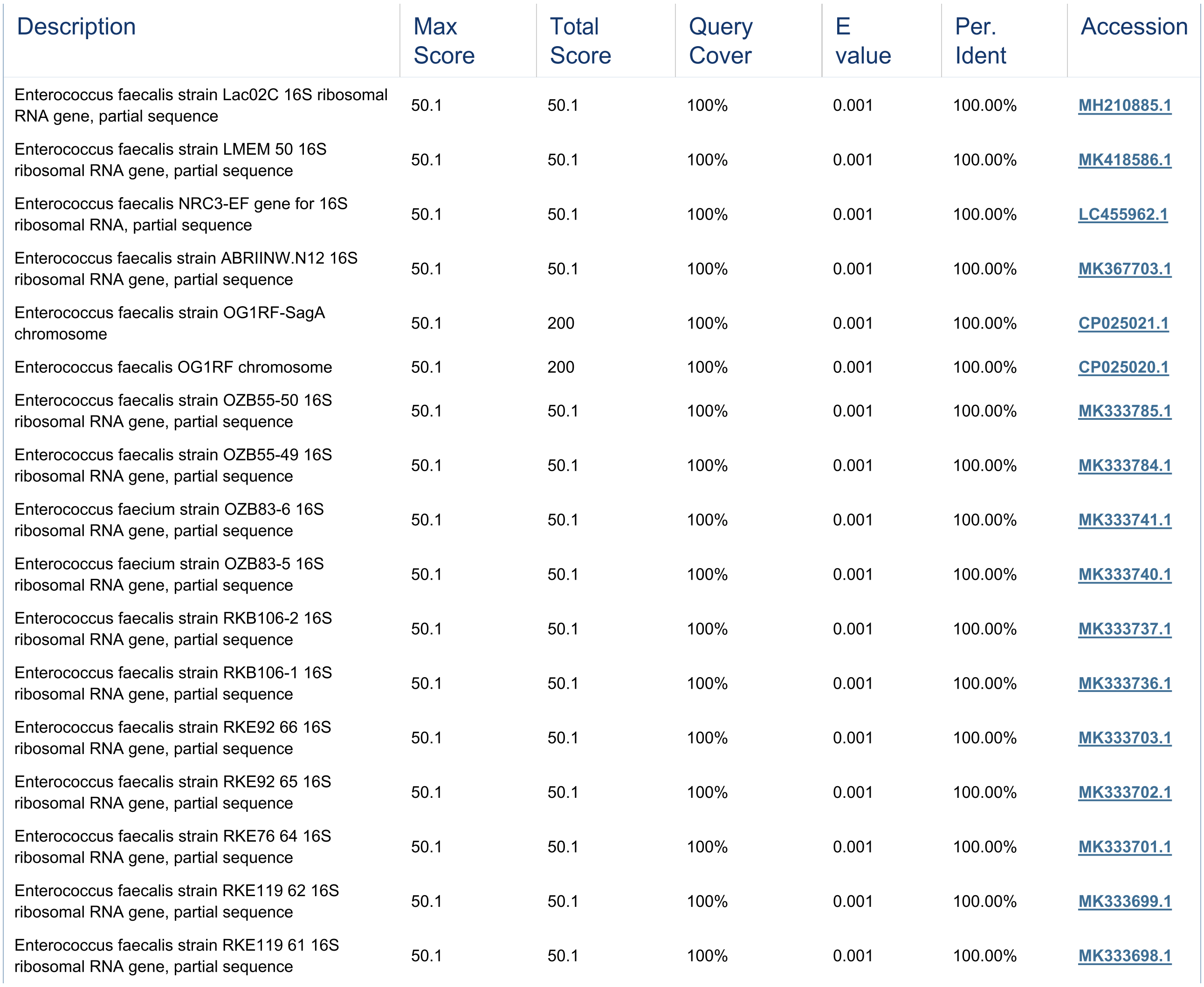
2.7. Reproducibility, regeneration, stability and selectivity of the ef-biosensor

To evaluate the fabrication reproducibility of the ef-biosensor, it was soaked in the Piranha solution for 30 s and then re-fabricated. This process was repeated five times. DPVs of the ef-biosensor for the repeating fabrications were recorded.

In order to evaluate the regeneration ability of the ef-biosensor, after its hybridization with 1.0×10^{-12} mol L^{-1} target ssDNA, we soaked it in hot water for 5 min at 90 °C to de-hybridize it. Then, the ef-biosensor was re-hybridized with the same concentration of the target ssDNA, and this process was repeated five times. At each step, DPVs were recorded.

In order to check stability, we hybridized the ef-biosensor with 1.0×10^{-13} mol L^{-1} target ssDNA. DPVs were recorded during consecutive days, while the ef-biosensor was stored in Tris buffer in a refrigerator at 4 °C and measured once a day in the same conditions.

For evaluation of the selectivity of the ef-biosensor, it was hybridized with a concentration of 1.0×10^{-12} mol L^{-1} of 1-base mismatched, 2-base mismatched, 3-base mismatched and non-



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
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Enterococcus faecalis strain LMEM 50 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK418586.1
Enterococcus faecalis NRC3-EF gene for 16S ribosomal RNA, partial sequence	50.1	50.1	100%	0.001	100.00%	LC455962.1
Enterococcus faecalis strain ABRIINW.N12 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK367703.1
Enterococcus faecalis strain OG1RF-SagA chromosome	50.1	200	100%	0.001	100.00%	CP025021.1
Enterococcus faecalis OG1RF chromosome	50.1	200	100%	0.001	100.00%	CP025020.1
Enterococcus faecalis strain OZB55-50 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333785.1
Enterococcus faecalis strain OZB55-49 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333784.1
Enterococcus faecium strain OZB83-6 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333741.1
Enterococcus faecium strain OZB83-5 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333740.1
Enterococcus faecalis strain RKB106-2 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333737.1
Enterococcus faecalis strain RKB106-1 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333736.1
Enterococcus faecalis strain RKE92 66 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333703.1
Enterococcus faecalis strain RKE92 65 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333702.1
Enterococcus faecalis strain RKE76 64 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333701.1
Enterococcus faecalis strain RKE119 62 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333699.1
Enterococcus faecalis strain RKE119 61 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333698.1

Scheme 1. (*continued*)

complementary ssDNA sequences. DPVs before and after hybridization with these sequences were recorded.

2.8. Patient samples

De-identified samples of urine, stool, and abdomen discarded from routine clinical practice were obtained from the clinical lab of Namazi Hospital (Shiraz, Iran). The samples had been submitted for routine clinical tests and confirmed. Genomic DNA was extracted by a DNA extraction kit from CinnaGen (Iran) according to the manufacturer's instructions. DNA concentrations were determined by a Thermo Scientific NanoDrop Lite (USA) spectrophotometer and stored at -20 °C until used.

2.9. PCR details

PCR was carried out to confirm the presence of bacterial genome in the real samples. PCR amplification was performed in a total volume of 50 μL, containing 0.4 μL of 10 μmol L^{-1} each primer, 10 μL of 2 × Taq PCR Master Mix, 2.0 μL of DNA template, and 7.2 μL of distilled water. 2.0 μL of each sample was added to the PCR master mixture. The cycling conditions were as follows: initial denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 4 min. The PCR steps are schematically shown in supplementary material S1.

2.10. Electrophoresis

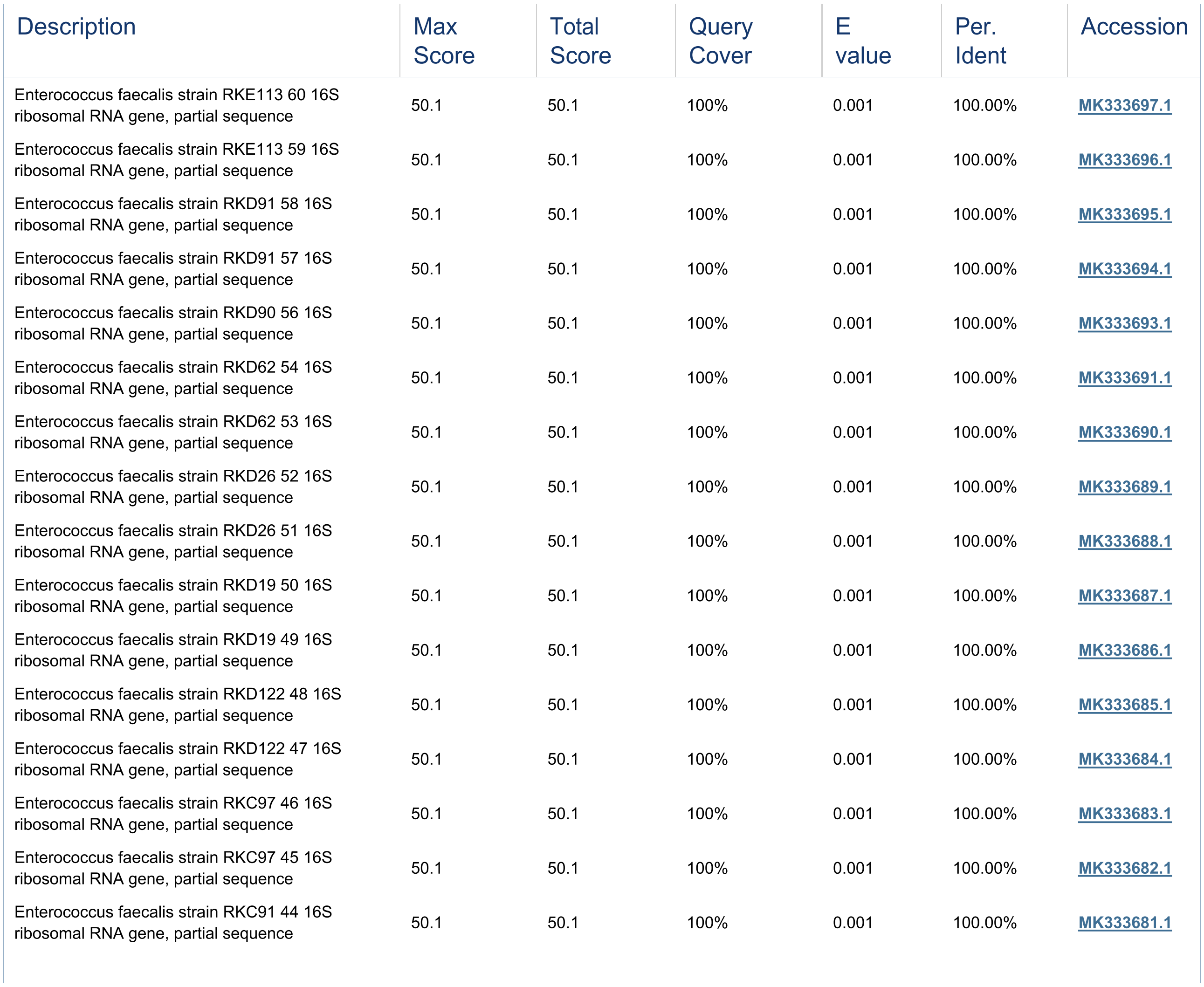
Electrophoresis with 2% agarose gel was conducted with 1 × TEA buffer (Tris-EDTA-acetate), pH = 8.3. The gel was incubated with 1 μL safe stain and polymerized for 40 min at room temperature. Then, it was soaked in 1 × TEA buffer for use. 7 μL of each sample was then subjected to agarose gel electrophoresis after addition of 1 μL of a loading buffer. Electrophoresis was run at 100 V for 2 h.

3. Results and discussion

3.1. Probe and transducer characterization and optimization of experimental variables

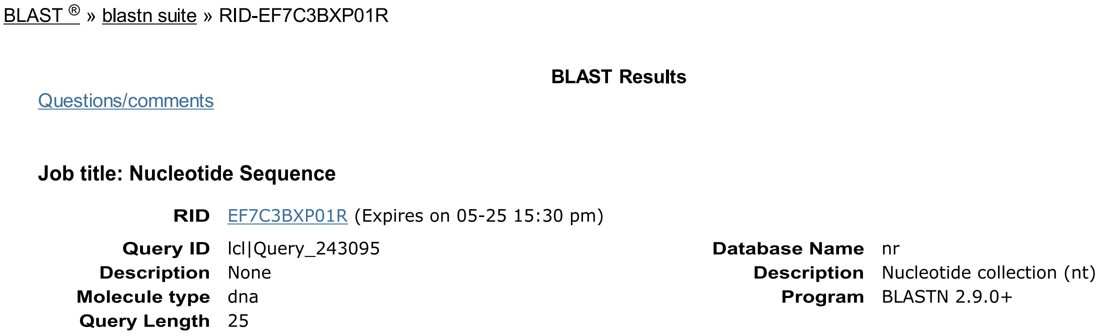
The probe ssDNA was explored as a query against a database of nucleotide using the nucleotide BLAST. All of the sequences similar to this query are presented in Scheme 1. In addition, the percent identity (a parameter that depicts how similar the query sequence is to the target sequence) and E-values (number of hits that can be expected by searching a database with a particular size) are presented. Based on the results, the probe ssDNA that was selected from 16S rRNA sequence of *E. faecalis* is conserved in the target region of all *E. faecalis* genomes.

FESEM images recorded at different magnifications from the gold disk electrode were presented in supplementary material S2. The images show a relatively uniform surface at lower magnifications, and



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Enterococcus faecalis strain RKE113 60 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333697.1
Enterococcus faecalis strain RKE113 59 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333696.1
Enterococcus faecalis strain RKD91 58 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333695.1
Enterococcus faecalis strain RKD91 57 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333694.1
Enterococcus faecalis strain RKD90 56 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333693.1
Enterococcus faecalis strain RKD62 54 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333691.1
Enterococcus faecalis strain RKD62 53 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333690.1
Enterococcus faecalis strain RKD26 52 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333689.1
Enterococcus faecalis strain RKD26 51 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333688.1
Enterococcus faecalis strain RKD19 50 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333687.1
Enterococcus faecalis strain RKD19 49 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333686.1
Enterococcus faecalis strain RKD122 48 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333685.1
Enterococcus faecalis strain RKD122 47 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333684.1
Enterococcus faecalis strain RKC97 46 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333683.1
Enterococcus faecalis strain RKC97 45 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333682.1
Enterococcus faecalis strain RKC91 44 16S ribosomal RNA gene, partial sequence	50.1	50.1	100%	0.001	100.00%	MK333681.1

Scheme 1. (*continued*)



BLAST ® » blastn suite » RID-EF7C3BXP01R

BLAST Results

Questions/comments

Job title: Nucleotide Sequence

RID EF7C3BXP01R (Expires on 05-25 15:30 pm)

Query ID lcl|Query_243095
Description None
Molecule type dna
Query Length 25

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.9.0+

Scheme 1. (*continued*)

at higher magnifications, some very thin tracks arising from abrasion by Al_2O_3 nanopowder are observed. The surface is clean from any adhered species.

In order to enhance the characteristics of the ef-biosensor, we optimized the time of immobilization of the probe ssDNA. To increase the sensitivity of detection, we should increase the surface concentration of the probe ssDNA on the gold electrode surface. Because OCP value during thiols self-assembling remains within the range of gold stability (Ma and Lennox, 2000), self-assembled monolayers of thiols are formed without needing application of external potential. On the other hand, probe ssDNA bears a net negative charge due to its sugar-phosphate backbone, and its immobilization on the surface has to cause change in the surface potential, and therefore, OCP of the electrode. OCP value of the gold electrode during immobilization of the probe ssDNA was monitored, and the data are shown in supplementary material S3. OCP rapidly increased within ~350 s, showed a bit decrement within ~700 s, and decreased again to reach a final and relatively stable value after ~7 h. Changes in the OCP value during the first 700 s can be related to fast formation of a disordered monolayer of probe ssDNA on the gold surface, and at longer times (up to 7 h), slow rearrangement occurred to produce an ordered monolayer (Dijksma et al., 2000). According to the results, an immobilization time of 7 h was considered to

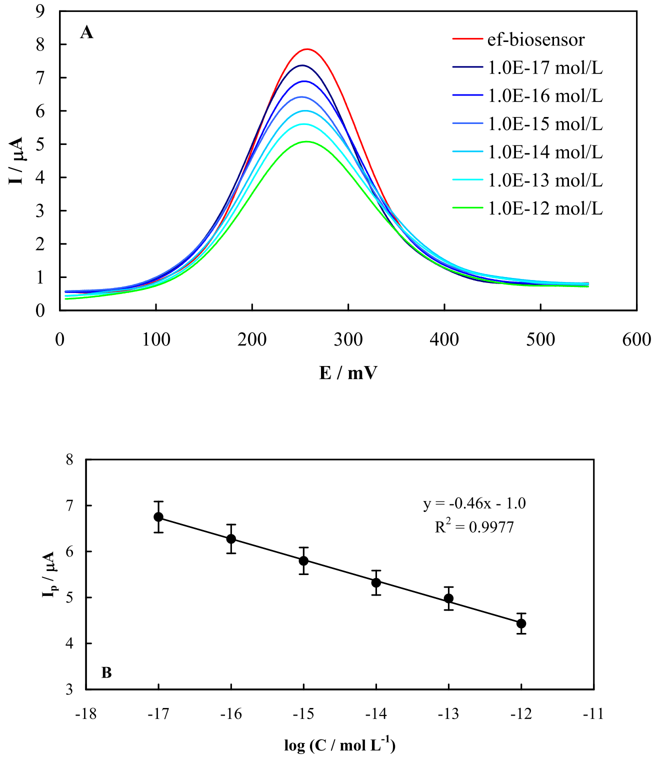


Fig. 1. DPVs recorded using the ef-biosensor after hybridization with different concentrations of the target ssDNA of 1.0 × 10^{-17}, 1.0 × 10^{-16}, 1.0 × 10^{-15}, 1.0 × 10^{-14}, 1.0 × 10^{-13} and 1.0 × 10^{-12}, mol L^{-1} (A), and dependency of the peak currents on the concentration of the target ssDNA (B).

fabricate the ef-biosensor.

3.2. *Ef-biosensor analytical performance*

DPVs recorded using the ef-biosensor after hybridization with different concentrations of the target ssDNA are presented in Fig. 1A. Upon increment in the concentration of the target ssDNA, peak currents in the voltammograms decreased. Decrement in the peak current upon increment in the concentration of the target ssDNA was due to different level of repulsion of the redox marker from ssDNA and double-stranded DNA (dsDNA). ssDNA bears a net negative charge mainly due to its phosphate backbone (and partly because of the bases). Upon hybridization and base stacking occurrence, the formed dsDNA bears more negative charge, excretes the marker, and the current decreases (Scheme 2). The values of the peak currents had a dependency on the concentration of the target ssDNA, a calibration curve, as shown in Fig. 1B. The calibration curve had a regression equation of I_p (μA) = -(0.46 ± 0.01) log (C (/mol L^{-1})) - (1.0 ± 0.16) (μA), R^2 = 0.9977 in a concentration range of 1.0 × 10^{-17} to 1.0 × 10^{-12} mol L^{-1} for the target ssDNA. Therefore, a calibration sensitivity of -14.52 μA cm^{-2} (log (C/mol L^{-1}))$^{-1}$ was attained for the ef-biosensor. Using the slope of the calibration curve, standard deviation (SD_b) of the peak current in the repeated voltammograms recorded for the ef-biosensor (in the absence of the target ssDNA, the blank signal, $I_{p,b}$), the value of $I_{p,b}$ - 3 × SD_b as the signal of LOD, and keeping in the mind that the ef-biosensor works signal-off, LOD was obtained as 3.3 amol L^{-1} or 25 ag mL^{-1}. The calculated LOD value is very low, and compared with other methods of *E. faecalis* detection in supplementary material S4, the ef-biosensor represented a good performance for *E. faecalis* detection with fabrication and the working steps are schematically shown in Scheme 2.

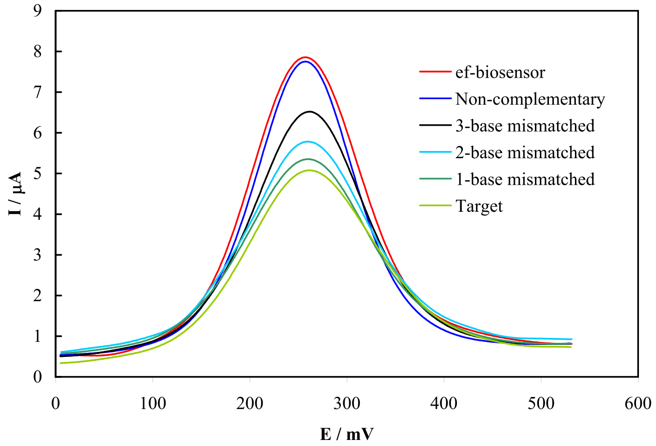
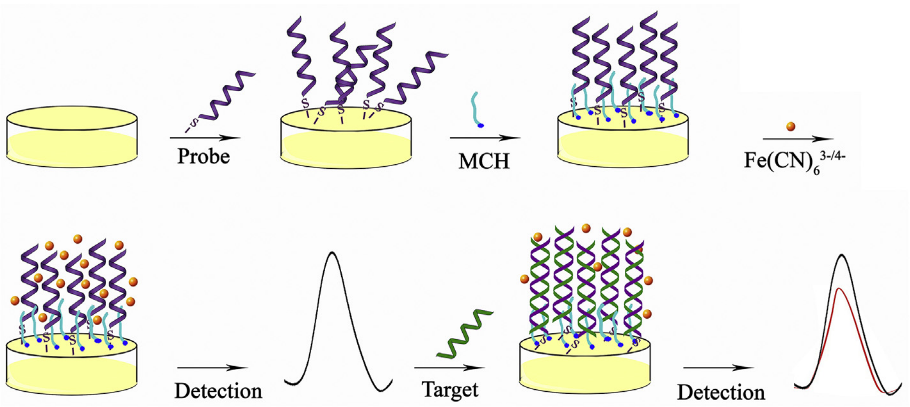


Fig. 2. DPVs recorded using the ef-biosensor before and after hybridization with target, 1-base mismatched, 2-base mismatched, 3-base mismatched and non-complementary ssDNA sequences of 1.0 × 10^{-12} mol L^{-1}.

3.3. *Reproducibility, regeneration, stability, and selectivity of the Ef-biosensor*

The important factors of fabrication reproducibility, regeneration ability and signal stability of the ef-biosensor were evaluated. The ef-biosensor was fabricated five times with a new pretreated gold electrode. Then, DPVs were recorded using each ef-biosensor (supplementary material S5). For changes in the peak current of these voltammograms, a relative standard deviation (RSD) of 2.8% was obtained, indicating that the fabrication procedure was highly reproducible. Regeneration ability of the ef-biosensor was inspected by performing five cycles of hybridization/de-hybridization. For each cycle, a DPV was recorded using the ef-biosensor; then, it was hybridized with 1.0 × 10-



Scheme 2. Fabrication and working steps of the ef-biosensor.

^{12} mol L^{-1} target ssDNA and another DPV was recorded. Afterwards, the ef-biosensor was placed in deionized water at 90 °C for 5 min, cooled rapidly on an ice bath, rinsed with Tris buffer, and employed for another cycle. The difference between the peak currents before and after hybridization had a RSD of 5.1%. As for evaluation of the stability, DPVs were recorded using the ef-biosensor after hybridization with 1.0×10^{-13} mol L^{-1} target ssDNA for several consecutive days. The ef-biosensor signal reached 90% of the initial value after more than two months (supplementary material S6), and this time it was considered as the stability time.

The ability to recognize similar DNA sequences is a significant step in the assessment of a proposed bioassay. The selectivity of the ef-biosensor was examined by measuring the change in the peak current in DPVs induced by hybridization with base mismatched ssDNA sequences. In Fig. 2, DPVs recorded using the ef-biosensor before and after hybridization with target, 1-base mismatched, 2-base mismatched, 3-base mismatched and non-complementary ssDNA sequences of the same concentrations of 1.0×10^{-12} mol L^{-1} are presented. Based on these DPVs, the more similarity in the hybridizing sequence with the target one resulted in a further decrement in the peak current wherein target ssDNA provided the most decrement and non-complementary ssDNA sequence provided fewer decrement in the peak current of the ef-biosensor. The order of peak current decrement was: target > 1-base mismatched > 2-base mismatched > 3-base mismatched > non-complementary ssDNA sequences. Therefore, the ef-biosensor represented a high selectivity and could discriminate the based-mismatched sequences.

3.4. Genomic DNA detection by the ef-biosensor

The ability of the ef-biosensor to quantify the genomic DNA, DPVs was recorded before and after hybridization with different concentrations of *E. faecalis* genome, as shown in supplementary material S7. Upon increment in the genome concentration, the peak current regularly decreased, and a calibration curve for genomic DNA quantitation is also depicted in supplementary material S7. The calibration curve had a regression equation of I_p (μA) = -(0.27 ± 0.01) log (C (/ng mL^{-1})) + (0.29 ± 0.04) (μA), $R^2 = 0.9912$ in a concentration range of 1.1×10^{-7} to 1.1 ng mL^{-1}, with a calibration sensitivity of -8.49 μA cm^{-2} (log (C/ng mL^{-1}))$^{-1}$. By the use of the slope of the calibration curve, SD_b of the blank signal, the value of $I_{p,b}$ - 3 × SD_b as the signal of LOD, LOD for genomic DNA was obtained as 7.1×10^{-9} ng mL^{-1}. The calculated LOD of the genomic DNA detection for ef-biosensor was very low as compared with other methods of *E. faecalis* detection in supplementary material S4.

3.5. Real sample analysis

In order to verify the applicability of ef-biosensor and its employment capability in complex matrices, we detected *E. faecalis* genome from human specimens. For this purpose, the genome was taken out from the human samples and divided into two parts; one part was amplified by PCR followed by gel electrophoresis to confirm or reject the presence of *E. faecalis* in the samples (supplementary material S8), and the other part was analyzed by the ef-biosensor. After recording ten replicated DPVs using the ef-biosensor and obtaining SD_b value, DPVs were recorded for the human samples. A sample was considered as positive when it produced a peak current lower than the $I_{p,b}$ - 10 × SD_b value (signal of the limit of quantitation of the ef-biosensor). The results of the human samples analysis are displayed in supplementary material S9, showing that the results obtained by the ef-biosensor were in accordance to those obtained by PCR-gel electrophoresis. It should be noted that a culture result was considered positive if there was either *E. faecalis* or *Enterococcus faecium* in the sample, and the PCR-gel electrophoresis was applied to confirm the presence of *E. faecalis*. Therefore, the ef-biosensor represented an excellent applicability in the analysis of clinical samples.

4. Conclusion

A specific probe ssDNA was selected from 16s rRNA gene sequence of *E. faecalis* followed by self-assembling on a gold electrode surface. Different ssDNA sequences accompanied by the bacterium genome were hybridized with the immobilized probe to be quantified. The selected sequence for the probe ssDNA provided a high selectivity to discriminate base-mismatched targets in one hand, and a high selectivity to detect genomic DNA. Beside, the ef-biosensor represented high calibration sensitivities and low LOD values (for target ssDNA as well as genomic DNA quantitation). The other advantages were low cost, long time stability, high reproducibility and easy fabrication. When the ef-biosensor was applied for analysis of human samples from the diagnosis point of view, it represented a very good diagnosis sensitivity and specificity. The ef-biosensor would have applied for *E. faecalis* detection in the samples with a low load of bacterium without pre-PCR amplification; this is not attainable by the other reported/conventional methods.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

R. Nazari-Vanani: Data curation, Formal analysis, Methodology, Writing - review & editing. **N. Sattarahmady:** Conceptualization, Formal analysis, Investigation, Project administration, Resources, Writing - review & editing. **H. Yadegari:** Formal analysis, Validation, Writing - review & editing. **M. Khatami:** Formal analysis, Funding acquisition, Writing - review & editing. **H. Heli:** Conceptualization, Formal analysis, Resources, Validation, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111541.

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