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Optimization of peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) for the detection of bacteria: the effect of pH, dextran sulfate and probe concentration

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Abstract

Fluorescence *in situ* hybridization (FISH) is a molecular technique widely used for the detection and characterization of microbial populations. FISH is affected by a wide variety of abiotic and biotic variables and the way they interact with each other. This is translated into a wide variability of FISH procedures found in the literature. The aim of this work is to systematically study the effects of pH, dextran sulfate and probe concentration in the FISH protocol, using a general peptide nucleic acid (PNA) probe for the *Eubacteria* domain. For this, response surface methodology was used to optimize these 3 PNA-FISH parameters for Gram-negative (*Escherichia coli* and *Pseudomonas fluorescens*) and Gram-positive species (*Listeria innocua*, *Staphylococcus epidermidis* and *Bacillus cereus*). The obtained results show that a probe concentration higher than 300 nM is favorable for both groups. Interestingly, a clear distinction between the two groups regarding the optimal pH and dextran sulfate concentration was found: a high pH (approx. 10), combined with lower dextran sulfate concentration (approx. 2% [w/v]) for Gram-negative species and near-neutral pH (approx. 8), together with higher dextran sulfate concentrations (approx. 10% [w/v]) for Gram-positive species. This behavior seems to result from an interplay between pH and dextran sulfate and their ability to influence probe concentration and diffusion towards the rRNA target. This study shows that, for an optimum hybridization protocol, dextran sulfate and pH should be adjusted according to the target bacteria.

Keywords: PNA-FISH, *Eubacteria*, dextran sulfate, pH, PNA EUB338

1. Introduction

In situ hybridization (ISH) consists of an array of methodologies that ultimately allow the specific detection of nucleic acid sequences in biological samples (Jin and Lloyd, 1997). At the present moment, most ISH techniques use fluorescent dyes as reporter molecules, in a process called Fluorescence *in situ* Hybridization (FISH) (Speicher and Carter, 2005; Trask, 2002). On its original form, FISH consists essentially on hybridizing an oligonucleotide probe to its complementary sequence in previously fixed samples, obeying to the Watson-Crick hydrogen-bonding rules (Cerqueira *et al*., 2008; Volpi and Bridger, 2008). FISH is widely used in the field of microbiology (Amann and Fuchs, 2008), namely in the identification, quantification and characterization of phylogenetically defined microbial populations in complex environments (Wagner *et al*., 2003).

Since the first application of FISH to microorganisms by DeLong *et al*. (1989), diverse FISH-based diagnostic assays have been developed (see review from Volpi and Bridger, 2008). These result from combinations of FISH with other techniques or improvements at the FISH procedure level, such as the use of other molecules, rather than standard DNA or RNA, as probes. A good example of this is the application of peptide nucleic acid (PNA), a nucleic acid mimic with recognized superior hybridization features (Cerqueira *et al*., 2008; Stender *et al*., 2000; Stender *et al*., 1999). PNA is comprised of a neutral polyamide backbone (Nielsen *et al*., 1991) with an identical chemical configuration to the DNA molecules that allows PNA to hybridize with complementary DNA or RNA sequences (Nielsen, 2001; Shakeel *et al*., 2006). Its superior hybridization features arise from the lack of electrostatic repulsion between the non-charged polyamide backbone and the charged DNA/RNA phosphodiester backbone. This is translated into an improved thermal stability of the duplex (Nielsen,

2001; Perry-O'Keefe *et al*., 2001) and allows the hybridization step to be performed under low salt concentrations (Orum *et al*., 1998), a condition that destabilizes the rRNA secondary structures and results in an improved access to target sequences (Azevedo *et al*., 2003; Fuchs *et al*., 1998; Yilmaz *et al*., 2006). The neutrally-charged PNA also diffuses well through the bacterial membrane (Drobniewski *et al*., 2000) and its synthetic nature leads to an increased resistance to nucleases and proteases (Demidov *et al*., 1994; Stender *et al*., 2002; Wagner *et al*., 2003).

In spite of PNA-FISH robustness, there is a considerable variability between the procedures described in the literature and its implementation usually requires an initial optimization to adjust the hybridization efficiency (Herzer and Englert, 2001), currently performed as a trial-and-error approach. This is a laborious and time-consuming step that could be greatly shortened if knowledge on how to develop a novel PNA-FISH method was at hand. In fact, variables such as type of fixative used (aldehyde or alcohol-based fixation), hybridization time, temperature, pH, concentration of probe, dextran sulfate (DS) and formamide, among others, are known to affect hybridization efficiency. Santos *et al*. (2014) recently assessed the effects of formamide, temperature and time on the hybridization efficiency, while successfully establishing an approach for FISH optimization, applying response surface methodology (RSM).

The present work aimed to understand the effect of hybridization pH, DS and probe concentration (and their interplay) on PNA-FISH efficiency for different bacteria. To this end, Response Surface Methodology was used to model the hybridization of an universal *Eubacteria* PNA probe (EUB338) (Amann *et al*., 1990; Santos *et al*., 2014), and signal quantification was assessed by flow cytometry.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains selected for this study were *Escherichia coli* CECT 434, *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* CECT 910, *Staphylococcus epidermidis* RP61A and *Bacillus cereus* isolated from a disinfectant solution and identified by 16S rRNA gene sequencing (Simões *et al.*, 2007). *E. coli* and *L. innocua* were grown on tryptic soy agar (TSA) [3% (w/v) tryptic soy broth and 1.5% (w/v) agar] (Oxoid, Basingstoke, England and Merck, Darmstadt, Germany). *B. cereus*, *P. fluorescens* and *S. epidermidis* were grown in plate count agar (Merck). All cultures were grown overnight at 30ºC and streaked onto fresh plates every 2 or 3 days.

2.2. PNA-FISH method

In order to evaluate the influence of pH, DS and probe concentration in the fluorescent signal outcome, a PNA-FISH protocol similar to the one described by Santos *et al*. (2014) was implemented, followed by signal quantification using flow cytometry. A universal PNA probe EUB338 (5'-TGCCTCCCGTAGGA-3'), based on the work of Amann *et al*. (1990), which recognizes a conserved region of the 16S rRNA in the domain *Eubacteria*, was used. The probe was synthesized and labelled at the N terminus with AlexaFluor488 via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker (Panagene, Daejeon, South Korea).

Bacterial cells were harvested from plates and suspended in sterile water to a final concentration of 10^8 to 10^9 cells.mL^{-1}. For sample fixation, the cell suspension was pelleted by centrifugation at 10,000 × *g* for 5 min, resuspended in 400 µL of 4% (w/v) paraformaldehyde (Acros Organics, New Jersey, USA) and incubated for 1 h at room temperature. After centrifugation at 10,000 × *g* for 5 min, the pellet was resuspended in

500 µL of 50% (v/v) ethanol and incubated at -20°C for at least 30 min. For hybridization, 100 µL of the fixed-cell aliquot were pelleted by centrifugation (10,000 × *g* for 5 min) and resuspended in 100 µL of hybridization solution. With the exception of the parameters under study, the composition of the hybridization solution was the same as the one reported by Santos *et al*. (2014), with the optimum formamide concentration obtained on that study. Consequently, formamide (Acros Organics) at 5.5% (v/v) was used for *E. coli*, *P. fluorescens, L. innocua* and *S. epidermidis* and at 49.5% (v/v) for *B. cereus.* Regarding the 3 parameters under study, the ranges selected are presented in Table 1. The conditions for assay 1 were selected to cover the values commonly described in the literature (Table S1 of the Supplemental material). Based on the results obtained in assay 1, new ranges were selected for assay 2, to achieve a suitable model for *E. coli* and *P. fluorescens*. Ranges defined in assay 3 and 4 were used to further evaluate the influence of DS molecular weight (MW) and pH on the signal outcome of Gram-positive bacteria. Different buffers were used at a concentration of 50 mM to control the pH of the hybridization solution, specifically citrate-phosphate (for pH 4 to 6); Tris-HCl (pH 7 to 8); Glycine-NaOH (pH 9 to 10); Sodium bicarbonate-NaOH (pH 11.2 and 11.3) and potassium chloride-NaOH (for pH above 12). Samples were hybridized at 60ºC for 55 min, except for *B. cereus* samples that were incubated for 110 min, based on the optimum conditions found by Santos *et al*. (2014). As a negative control, all procedures described above were repeated for each condition, but the PNA probe was not added to the hybridization solution. After hybridization, cells were centrifuged (10,000 × *g* for 5 min), resuspended in 500 µL of washing solution containing 5 mM Tris base (pH 10; Fisher Scientific, New Jersey, USA), 15 mM NaCl (Panreac, Barcelona, Spain) and 0.1% (v/v) Triton X-100 (Panreac) and incubated for 30 min at 60ºC. After centrifugation (10,000 × *g* for 5 min), the pellet was resuspended

152 in 700 μL sterile saline solution, 0.9% (w/v) NaCl (Panreac). Each experiment was
153 performed in triplicate.
154
155 **2.3. Flow cytometry analysis**
156 The fluorescence intensity of hybridized samples and negative controls was
157 quantified by an Epics XL flow cytometer (Beckman Coulter, Florida, USA) equipped
158 with a 488 nm argon ion laser. Forward angle light scatter (FS), side angle light scatter
159 (SS), and green (FL1) fluorescence were detected at logarithmic scale. A minimum of
160 20,000 events falling into the bacterial gate defined on the FS-SS plot were acquired per
161 sample. The data was analysed with the Expo32 software (Beckman Coulter), and the
162 average fluorescence intensity was determined for each triplicate experiment.
163
164 **2.4. Response surface methodology (RSM)**
165 In order to model the effect of pH, DS and probe concentration in the
166 hybridization of PNA EUB338 probe in bacteria, RSM was employed according to the
167 procedure applied by Santos *et al.* (2014). The average fluorescence intensity obtained
168 after PNA-FISH was used as the dependent variable.
169 Central composite designs (CCD) were set up for *E. coli*, *B. cereus*, *P.*
170 *fluorescens*, *L. innocua* and *S. epidermidis*, using the statistical software Design Expert®
171 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA) to estimate the coefficients of the model.
172 The range and levels of all variables were defined according to previous studies (Table
173 S1 of the Supplemental material) and the results obtained within this study. Each CCD
174 for assays 1, 2 and 3 included 2^3 factorial points (coded at ± 1), 6 axial points (coded as
175 ± α) that represent extreme values used for the estimation of the model curvature and 6
176 centre points (all factors at coded level 0) repeated to take into account the experimental

177 error (Myers and Montgomery, 1995; Silva *et al*., 2011). Therefore, each design matrix
178 consisted of 20 PNA-FISH experiments. For the assay number 4 the CCD included 2^2
179 factorial points (coded as ± 1), 4 axial points (coded as ± α) and 5 centre points (all
180 factors at coded level 0). Therefore, this design matrix consisted of 13 PNA-FISH
181 experiments.
182
183 **2.5. Viscometer analysis**
184 Viscosity measurements of DS 500 kDa solutions at pH 6, pH 9 and pH 12 were
185 performed using a Cannon-Fenske viscometer size 100 (Hipex, Portugal). Different
186 buffers were used at a concentration of 50 mM to control de pH of the DS solutions,
187 specifically citrate-phosphate for pH 6, Glycine-NaOH for pH 9 and potassium
188 chloride-NaOH for pH 12. The viscometer was placed in a water bath at a constant
189 temperature of 25 ± 1ºC. The viscosity of DS solutions at different pH was determined
190 by the comparison of the flow time of DS solutions against the flow time of distilled
191 water in triplicate.
192
193 **2.6. Statistical analysis**
194 In order to find the optimum hybridization conditions for all five species in the
195 study, the average intensity fluorescence values obtained by flow cytometry were
196 introduced in the software Design Expert® 8.0.7.1 to fit a quadratic model and each
197 obtained model was analysed using analysis of variance (ANOVA). The interaction of
198 the three independent variables and their effect on the fluorescence intensity was
199 inspected by constructing the response surface and contour plots. The optimization
200 function of the software was then used to estimate the optimum conditions within the
201 experimental range that maximized the fluorescence intensity. A confirmation

experiment of the predicted optimum point was performed for each bacterium in triplicate.

3. Results and Discussion

3.1. PNA-FISH optimization in bacteria: pH, DS and probe concentration

In this work, the effect of three parameters (pH, DS and probe concentration) on the hybridization efficiency of PNA-FISH was studied. To model their effect, RSM was applied to the hybridization data obtained from 3 Gram-positive (*L. innocua*, *S. epidermidis* and *B. cereus*) and 2 Gram-negative species (*E. coli* and *P. fluorescens*). This setup was selected in order to include bacteria with different cell wall thicknesses, ranging from thin, *e. g.* Gram-negative *P. fluorescens*, to thick cell walls, *e. g.* Gram-positive *B. cereus* (Santos *et al*., 2014).

The first range of pH, DS and probe concentrations tested in the CCD were based on the values typically described in the literature for PNA-FISH methods (Table 1, assay 1 and Table S1 of the Supplemental material). After performing the CCD set of experiments, significant quadratic models (p-value <0.05), a non-significant lack of fit (p-value >0.05) and a satisfactory coefficient of determination (R^2) combined with an optimum on the response surface plots were obtained for all three Gram-positive species tested (Figure 1). However, for the Gram-negative species, an optimum value from the response surface plots was not obtained, although a general tendency for lower DS concentrations and higher pH values was observed (Figure 1).

In order to obtain a satisfactory model for the Gram-negative species, the range of pH and DS concentration on the CCD were redesigned for higher pH values and lower DS concentrations (Table 1, assay 2), while maintaining the probe concentration level.

Using those designs for Gram-positive (Table 1, assay 1) and Gram-negative species (Table 1, assay 2), significant quadratic models were obtained for all five species tested (Table S2 and S3 of the Supplemental material). The successful modelling of the three studied parameters (pH, DS and probe concentration) allowed the determination of the optimal conditions for the maximum fluorescence (Figure 2). Moreover, the confirmatory experiment showed an agreement between experimental and predicted values (Table 2). The average fluorescence for negative controls was equal or lower than 1 a.u. (data not shown), while for positive samples the values ranged from 7 to 150 a.u., depending on the microorganism and the conditions tested (Figure 2).

Analysing Table 2 we can also notice a difference in terms of fluorescence intensity, with Gram-negative *P. fluorescens* and *E. coli*, exhibiting a higher signal than Gram-positive species, *L. innocua*, *S. epidermidis* and *B. cereus*. This pattern was also reported in the previous optimization performed by Santos and colleagues (2014). FISH signal is influenced (not only) by accessibility and target content. While accessibility is dependent on the permeability of the cell envelope to the probes, target content is correlated with the growth rate of bacteria (DeLong *et al*., 1989; Roller *et al*., 1994; Wallner *et al*., 1993). With this in mind, the Gram-differences found can be attributed to varying ribosomal content as well as cell envelope permeability. Despite the observed Gram-differences, our results still show that positive results are achieved even using favourable Gram-negative hybridization protocols on Gram-positive and vice versa, since the outcome signal is still far greater than the respective negative control value.

From Table 2 we can observe as well that the optimal probe concentration for all species was the maximum tested and considered by the model, 300 nM (+1 factor). This was expected, since the probe concentration is a key factor for the nucleaction reaction

and the time needed for hybridization (Bruns *et al*., 2007). The nucleation reaction is the rate-limiting step in the hybridization of nucleic acids, being characterized by the formation of a small number of base pairs that initiate the hybridization, proceeding then as a rapid zippering of the remaining nucleotides (Bruns *et al*., 2007). If the concentration of hybrid strands in solution is similar, the hybridization follows a second order kinetics, meaning that the higher the concentration of hybrid strands in solution, the higher the annealing rate will be (Bruns *et al*., 2007). However, as FISH protocols usually use probe concentration in excess relatively to the number of target sequence(s) (Yilmaz and Noguera, 2004) a pseudo-first order kinetics is applied (Bruns *et al*., 2007), and in this case the hybridization depends only on the concentration of the target. However, the time required to hybridize the probe to the target remains inversely proportional to the probe concentration (Bruns *et al*., 2007). Other variables such as target accessibility, probe length and complexity have also an impact on the hybridization (Bruns *et al*., 2007), but these were not of concern since the same probe (PNA EUB338) was used throughout this work.

Interestingly, analyzing the results of the optimal pH and DS concentration (Table 2), it is possible to distinguish 2 different behaviors. A higher pH, approx. 10, combined with lower DS concentration, approx. 2% (w/v), were found to be favorable for Gram-negative species (*E. coli* and *P. fluorescens*), while near-neutral pH, approx. 8, together with higher DS concentrations, approx. 10% (w/v), favored Gram-positive species (*L. innocua*, *S. epidermidis* and *B. cereus*).

The application of DS in the hybridization solution has two main effects in FISH. On the one hand, higher concentrations of DS should be favorable to FISH as they cause an apparent increase in probe concentration (Azevedo, 2005; Cmarko and Koberna, 2007). On the other hand, it is well known that DS increases the viscosity of a

277 solution, hence decreasing molecular diffusion (Kosar and Phillips, 1995; Zustiak *et al*.,
278 2011). In order to understand why DS affected differently Gram-positive and Gram-
279 negative bacteria, we considered that the access of the probe to the target rRNA occurs
280 in three steps: 1) diffusion on the suspension, 2) diffusion through the cell envelop
281 (including the cell wall) and 3) diffusion in the cytoplasm. For the Gram-positive, the
282 limiting step is possibly 2), considering that they possess a peptidoglycan layer much
283 thicker than Gram-negative bacteria (Roller *et al*., 1994; Franks *et al*., 1998) and as
284 such, a higher probe concentration gradient is needed. For the Gram-negative the
285 limiting diffusion step is 1), so the increase in viscosity might be more relevant.
286 In order to explore this hypothesis of the interplay between viscosity and
287 optimum DS concentration needed for the probe to overcome the thick cell wall of
288 Gram-positive bacteria, we further tested different MW DS (besides the previously used
289 500 kDa in Table 1, assays 3 and 4), as the viscosity of DS molecules in solution
290 decreases with lower MW DS (Joosse *et al*., 2007). At 30ºC 10% (w/v) DS of 500 kDa
291 presents a viscosity of ≈35 mPa.s (Demetriades and McClements, 1998), whereas 10%
292 (w/v) DS of 10 kDa presents ≈2 mPa.s (Algotsson *et al*., 2013). So, using lower MW
293 DS we would expect to observe an increase in the optimum DS concentration values,
294 due to the lower viscosity of the hybridization solution obtained. The results presented
295 in Table 3 confirmed the anticipated outcome stated above.
296 Lastly, the pH of the hybridization solution may also impact FISH in 2 different
297 ways. On one side, it affects the ionization of nucleotides (Blackburn *et al*., 2006;
298 Vieregg, 2010). In fact, from pH 5 to 9 all bases are uncharged so hybridization occurs
299 without interference. At higher pH, guanine, uracil and thymine bases become
300 deprotonated (pK_a 9.2-9.7), while at lower pH, adenine and cytosine bases become
301 protonated (pK_a 3.5 and 4.2). This ultimately disfavors pairing, through an increase in

electrostatic repulsion at high pHs and destabilization of hydrogen bonding (Blackburn *et al*., 2006; Vieregg, 2010). On the other side, pH ionizes DS molecules, which affects its viscosity (Katchalsky, 1964). This was actually confirmed by viscosity measurements at 25 ± 1ºC of DS 500kDa 10% (w/v) solutions at pH 6, pH 9 and pH 12, having respectively 57.20 ± 0.01 mPa.s, 60.38 ± 0.02 mPa.s and 55.25 ± 0.03 mPa.s (Figure S1 of the Supplemental material).

Taking into account the viscosity measurements we could argue a limiting effect of increased viscosity with pH allied to a high content in DS. Nonetheless, viscosity readings show a rather small impact on this parameter when compared with the variance in viscosity of DS with different MW. Still, when using lower MW DS (Table 1, assay 3 and 4), that produces a far less viscous hybridization solution than the one using 500 kDa, we observe a higher optimum pH for Gram-positive (Table 3) close to the ones obtained for Gram-negative species with a DS of 500 kDa.

Taking into account the results obtained we were able to reach to an optimized PNA-FISH procedure for bacteria in terms of hybridization pH, DS and probe concentration. These results can be added to previous optimization disclosed by Santos *et al*. (2014) to greatly improve the efficiency of the hybridization protocols used. In fact, putting all this information together, a more optimized PNA-FISH hybridization procedure can be obtained in accordance to the properties of the target bacteria (Table 4).

It is possible that the optimized conditions of pH, dextran sulfate and probe concentration can be applicable to all protocols using PNA probes. Nonetheless, some minor adjustments to the optimum conditions described here cannot be excluded. We should point out that in order to access the effects of the conditions under study we worked with a simplified hybridization solution, so changes in composition and

viscosity or even probe length will impact PNA-FISH outcome. It is also important to notice that the optimization described here is not applicable to DNA, RNA and other nucleic acid mimics probes such as LNA or 2'OMe RNA, as their molecular structure differs markedly from PNA oligonucleotides (Cerqueira *et al*., 2008).

4. Conclusions

While optimum values/concentrations were obtained for the three parameters under study, an important observation of the present work was how pH and dextran sulfate interplay, affecting the probe gradient and consequently the hybridization efficiency. In Gram-positive bacteria, differently from Gram-negative species, a compromise between pH and DS concentration should be taken into consideration in order to maximize the hybridization efficiency (Figure 3).

Bacteria with thick peptidoglycan cell walls are harder to permeabilize (Roller *et al*., 1994), so a higher probe gradient between the extracellular environment and the cell cytoplasm is necessary to improve probe diffusion through the cell wall. This is accomplished using high concentrations of probe, 300 nM, and DS. The concentration of DS is, however, limited by the viscosity conferred by this molecule to the hybridization solution and in some extent by the pH. If the viscosity is too high, the diffusion of the probe in solution will be the limiting step, if it is too low, the probe gradient driving its diffusion across the cell envelope will be the limiting step for hybridization. So, a balance of DS and pH should always be considered for an efficient hybridization and this work might be used as a guideline according to the bacteria properties. Future work can expand the scope of this optimization to other steps of the FISH procedures, to a broader range of microorganisms, including species from the

other two Domains, *Archea* and *Eukarya* and eventually, to a set of different nucleic acid mimic probes.

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Table 1

Table 1 - Experimental levels for the variables used in the optimization of the PNA-FISH hybridization protocol for *E. coli*, *P. fluorescens*, *L. innocua*, *S. epidermidis* and *B. cereus* species.

Assay		Variables	Range and level				
			$-\alpha$	-1	0	$+1$	$+\alpha$
1[a]	x_1	pH	4.5	5.7	7.5	9.3	10.5
	x_2	[DS 500 kDa] % (w/v)	0.0	4.1	10.0	16.0	20.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
2[b]	x_1	pH	5.9	7.3	9.3	11.3	12.6
	x_2	[DS 500 kDa] % (w/v)	0.0	1.0	2.5	3.9	5.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
3[c]	x_1	pH	4.5	5.7	7.5	9.3	10.5
	x_2	[DS 10 kDa] % (w/v)	0.0	4.1	10.0	16.0	20.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
4[d]	x_1	pH	6.5	7.3	9.3	11.2	12.0
	x_2	[DS 0.5 kDa] % (w/v)	1.9	5.0	12.5	20.0	23.1

[a] Experimental levels set in the optimization protocol for *E. coli*, *P. fluorescens*, *L. innocua*, *S. epidermidis* and *B. cereus*.
[b] Experimental levels set in the optimization protocol for *E. coli* and *P. fluorescens*.
[c] Experimental levels set in the optimization protocol for *L. innocua*, *S. epidermidis* and *B. cereus*.
[d] Experimental levels set in the optimization protocol for *L. innocua*. Probe concentration at 200 nM.

Table 2

Table 2 - Optimum hybridization pH, DS and probe concentration predicted through the RSM models for the tested species. The negative control, predicted and obtained fluorescence values in those conditions are shown.

Bacteria	Optimum conditions			Predicted Fluorescence (a. u.)	Obtained Fluorescence (a. u.)	Negative Control (a.u.)
	pH	DS (% w/v)	Probe (nM)			
E. coli	9.87	1.93	300	37.1	37.7 ± 1.5	0.6 ± 0.1
P. fluorescens	10.83	2.32	300	98.2	171.7 ± 8.3	0.9 ± 0.1
L. innocua	8.36	7.94	300	24.9	21.6 ± 0.2	0.6 ± 0.1
B. cereus	6.92	11.70	300	37.9	30.6 ± 1.4	0.5 ± 0.1
S. epidermidis	8.56	12.84	300	18.0	17.4 ± 1.6	0.4 ± 0.1

Table 3

Table 3 - Optimum pH and DS concentration, for 500, 10 and 0.5 kDa MW molecules, in hybridization solution predicted through RSM models for Gram-positive species in study.

Dextran sulfate (MW)	Species					
	S. epidermidis		*L. innocua*		*B. cereus*	
	pH	[DS] (% w/v)	pH	[DS] (% w/v)	pH	[DS] (% w/v)
500 kDa	8.56	12.84	8.36	7.94	6.92	10.70
10 kDa	9.30	15.43	9.14	10.52	8.09	12.16
0.5 kDa	NE	NE	9.76	12.66	NE	NE

NE – Not Evaluated

Table 4

Table 4: Optimized hybridization variables for PNA-FISH in 5 Gram-positive and Gram-negative species, by RSM, obtained in this work and reported in Santos *et al*. (2014).

	Variable	Time (minutes)	Temperature (ºC)	Formamide (% v/v)	pH	DS (% w/v)	Probe (nM)
Bacteria	*E. coli* *P. fluorescens* *L. innocua*	55	60	5.5	10	2	≥ 300
	S. epidermidis				8	10	
	B. cereus	120		49.5			

Figure 1

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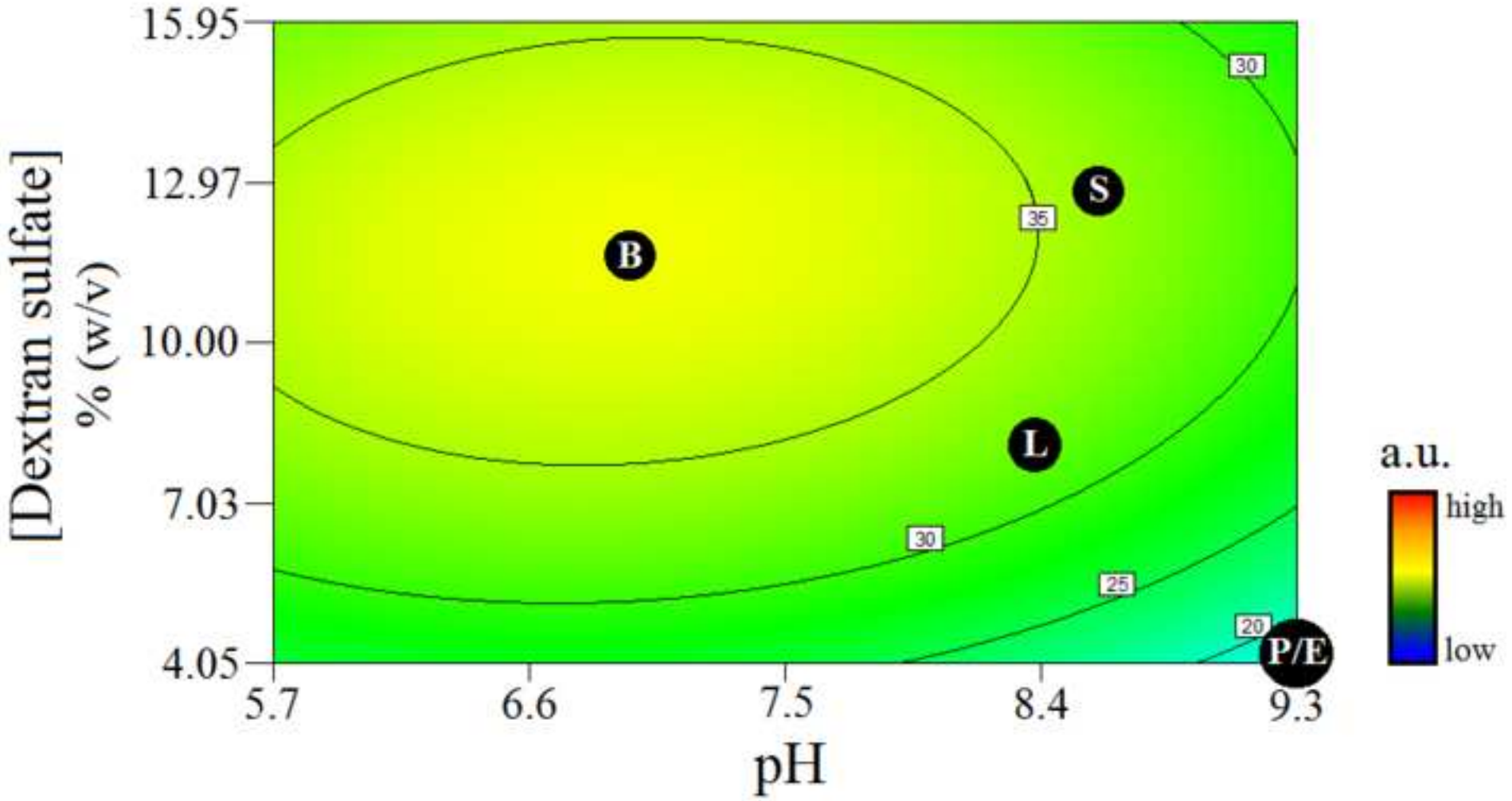

Figure 2a

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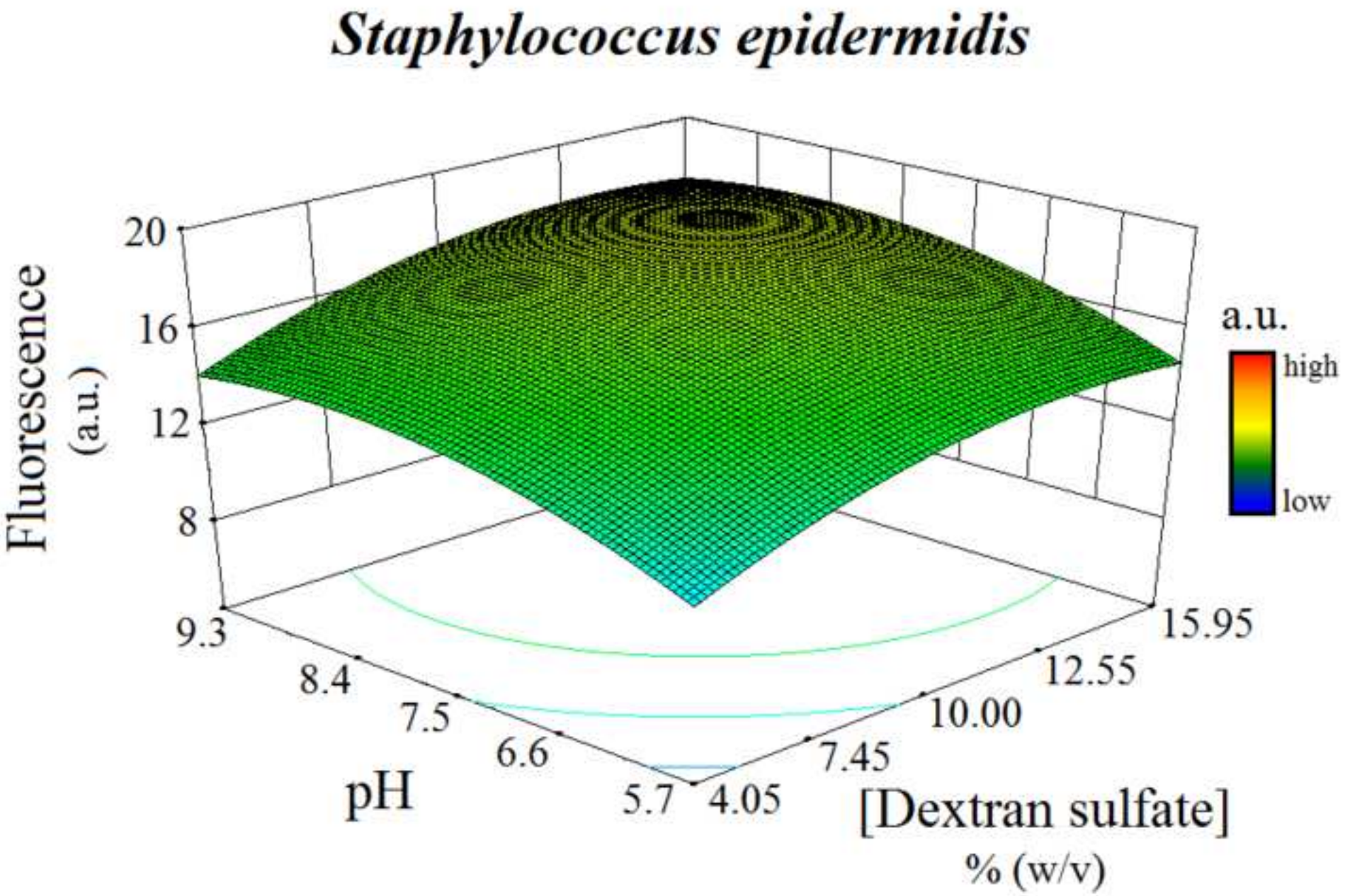

Figure 2b

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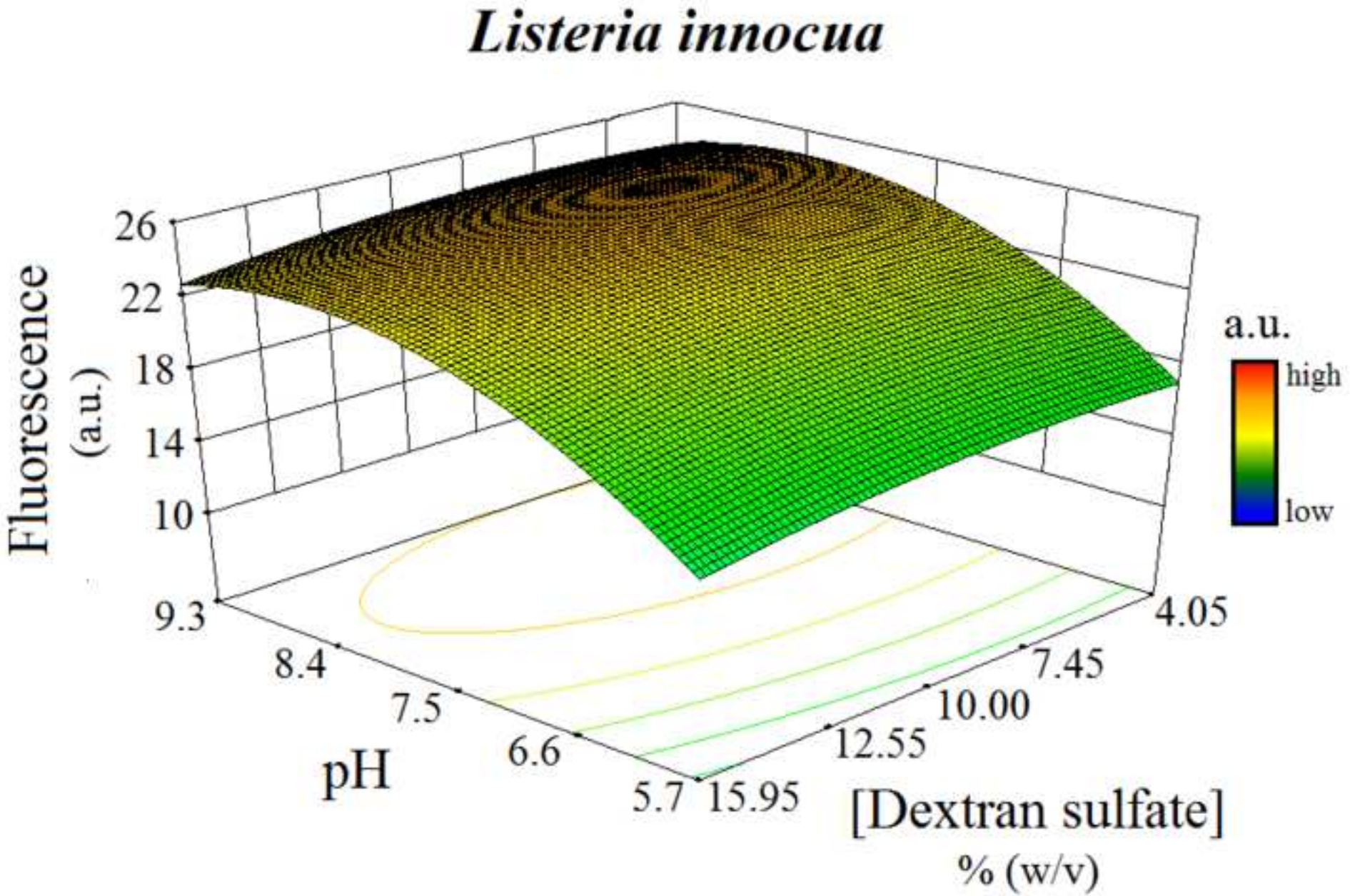

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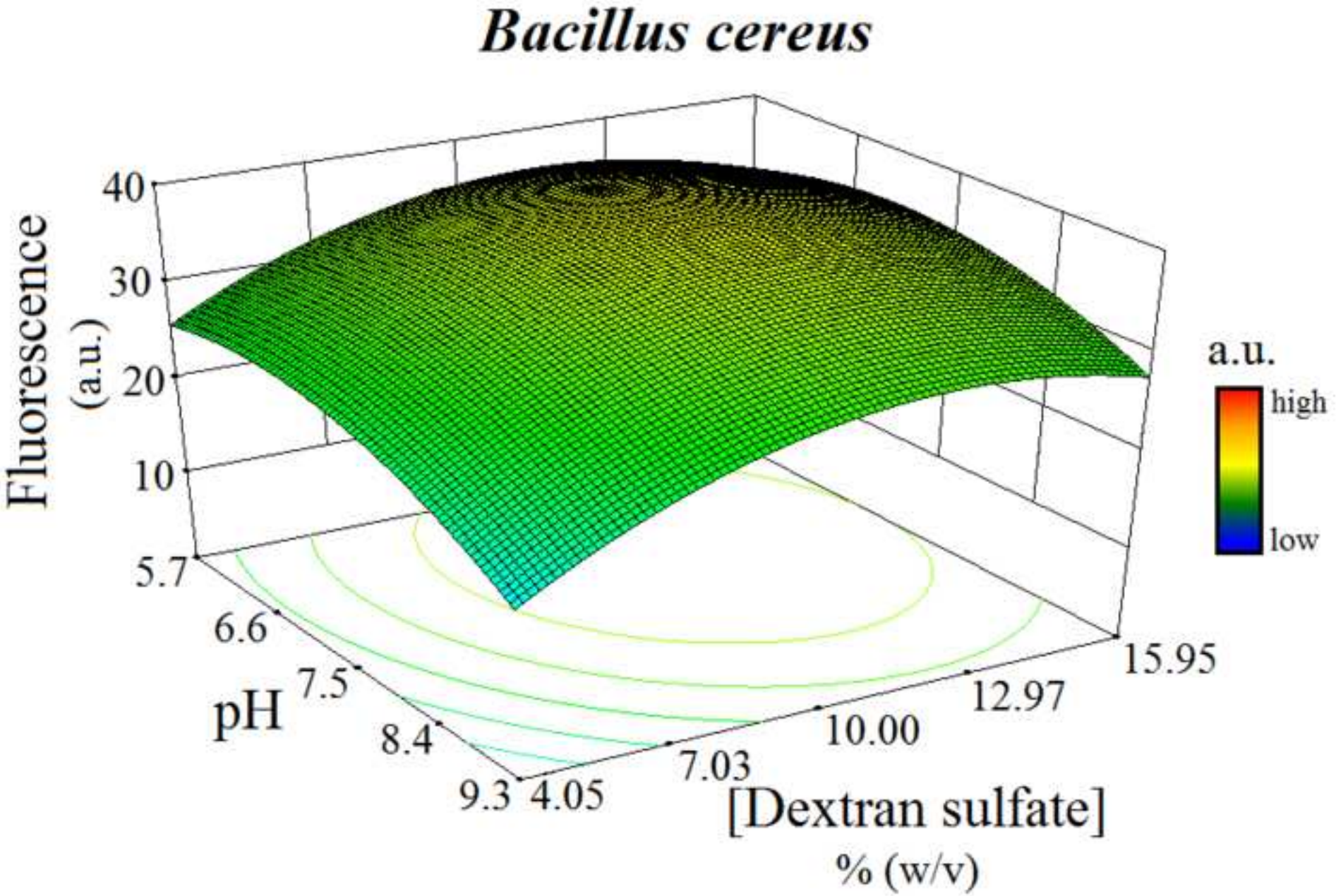

Figure 2d

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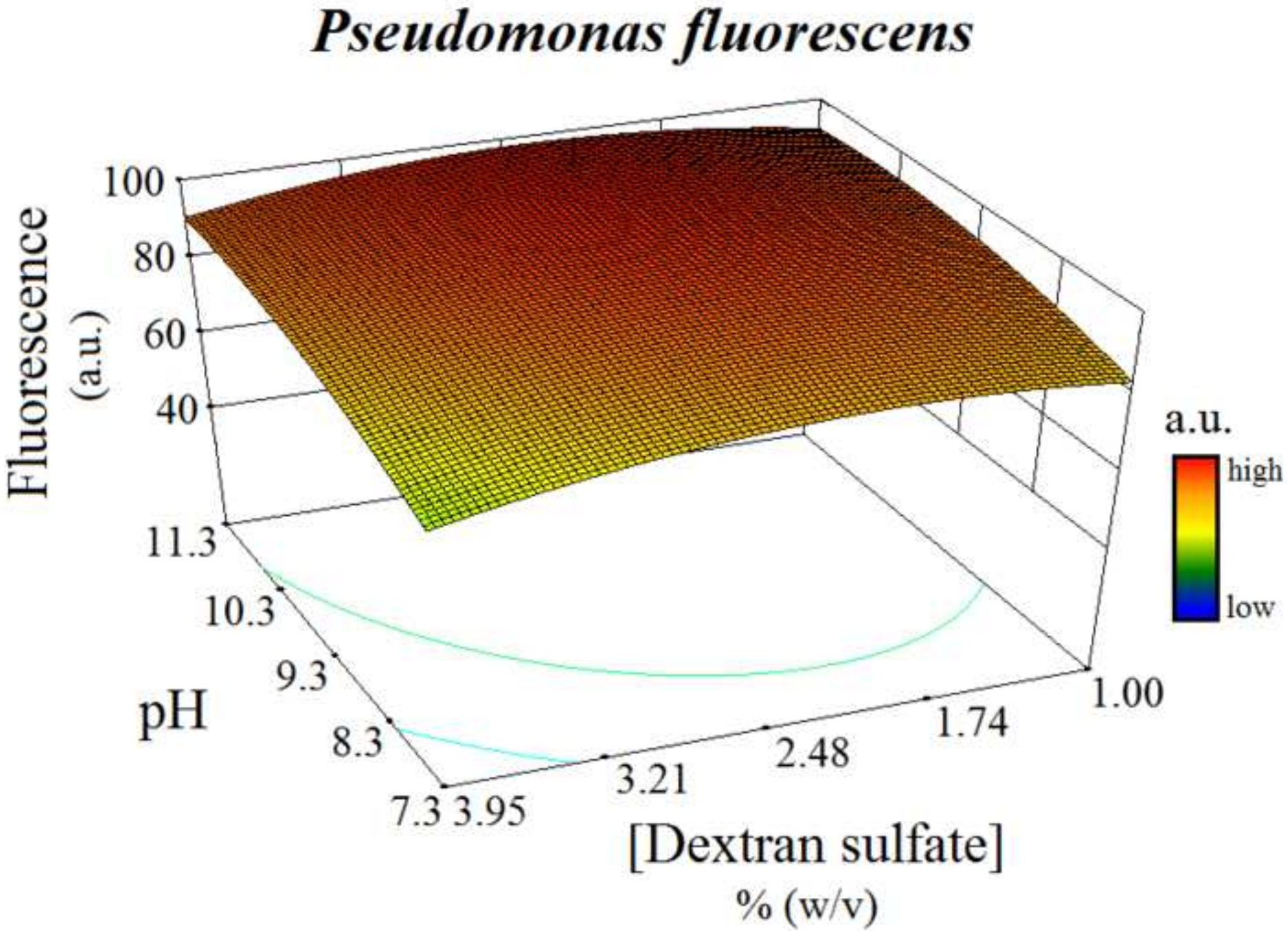

Figure 2e

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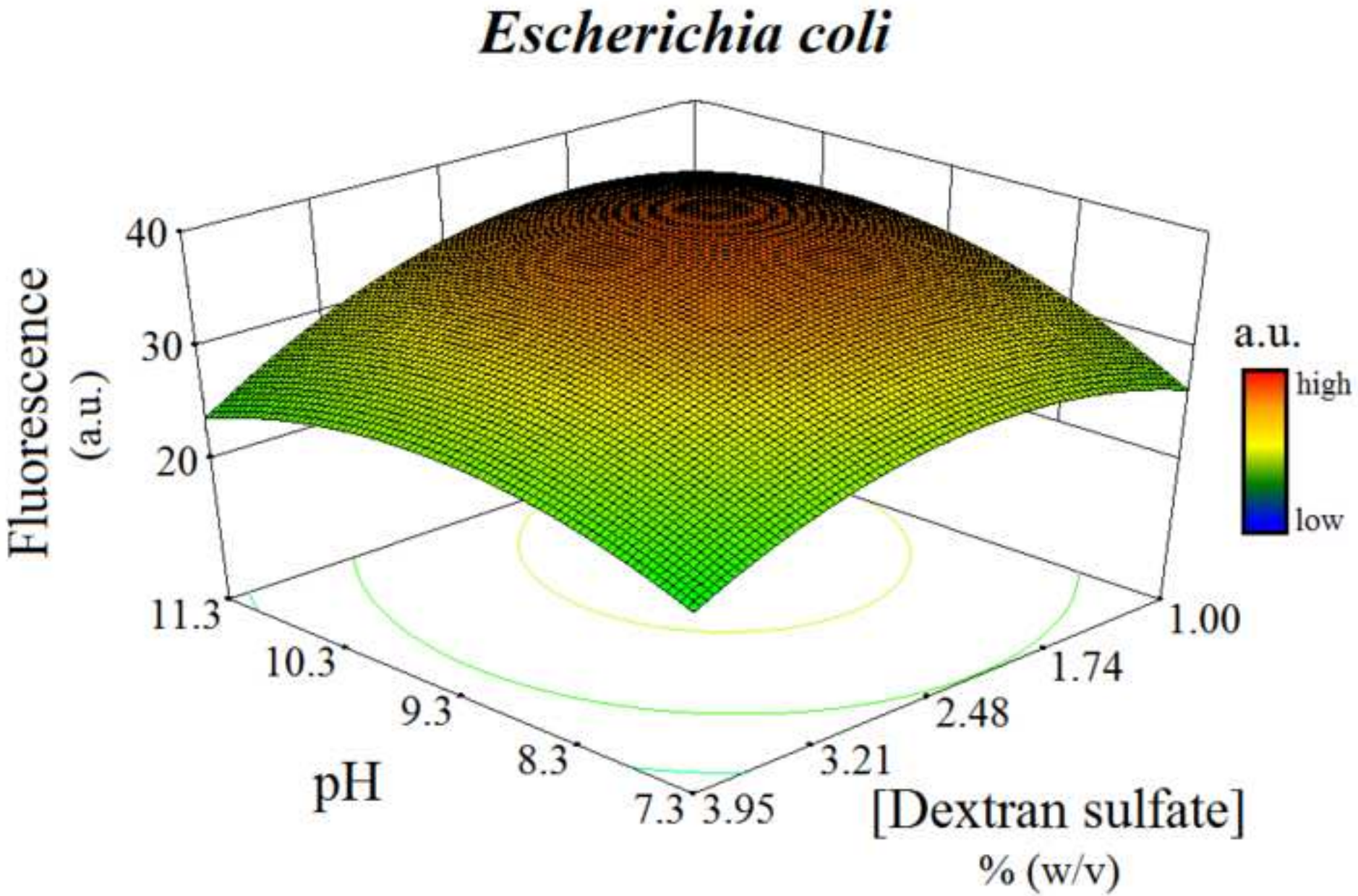

Figure 3

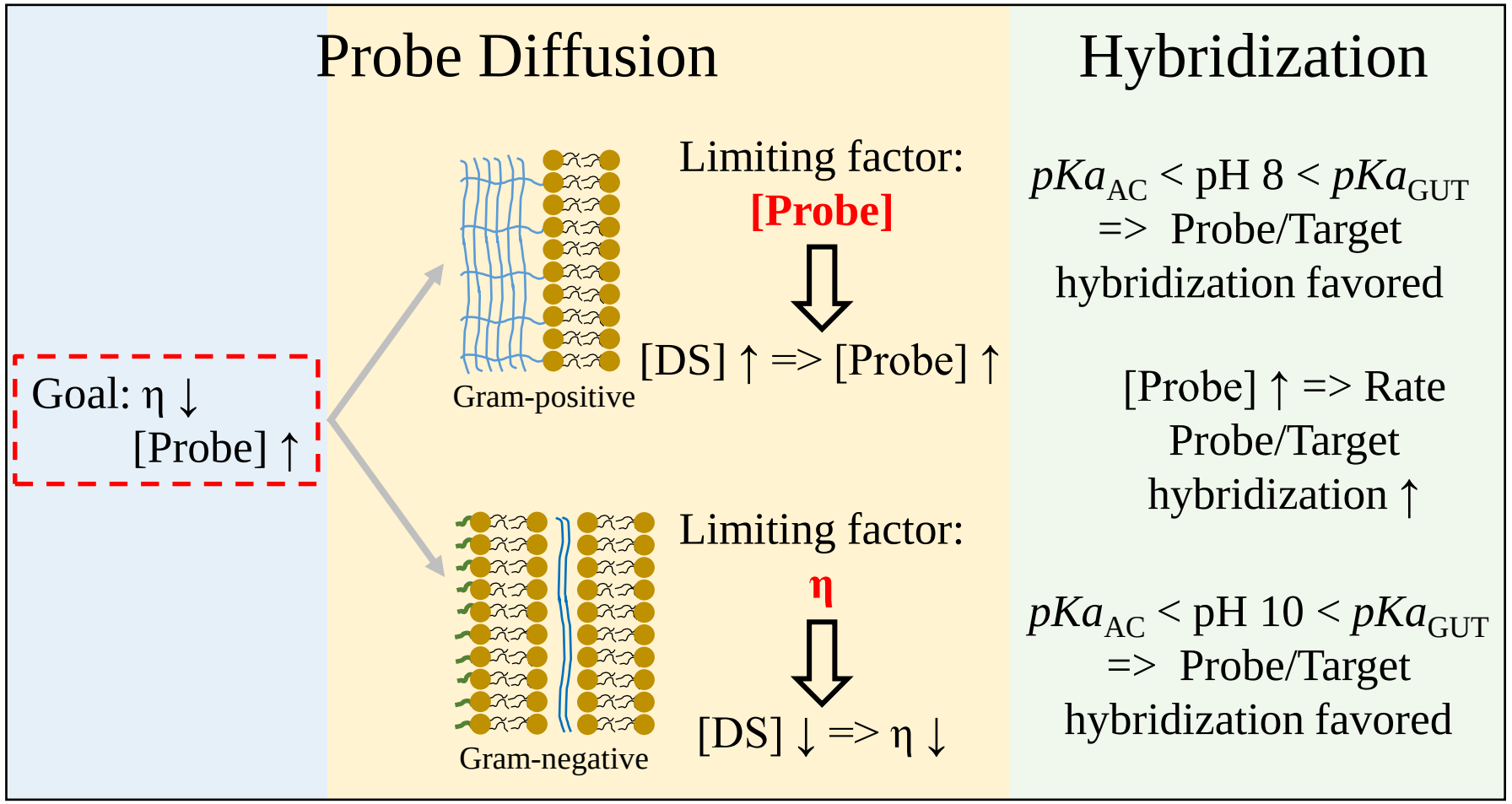
Probe Diffusion
Hybridization
Goal: η ↓
[Probe] ↑
Gram-positive
Limiting factor:
[Probe]
[DS] ↑ => [Probe] ↑
Gram-negative
Limiting factor:
η
[DS] ↓ => η ↓
pKa_AC < pH 8 < pKa_GUT
=> Probe/Target
hybridization favored
[Probe] ↑ => Rate
Probe/Target
hybridization ↑
pKa_AC < pH 10 < pKa_GUT
=> Probe/Target
hybridization favored

Figure legends

Figure 1 - Contour plot of *B. cereus* showing the effect of pH and DS (500 kDa) concentration on the fluorescence intensity (with probe concentration at the optimum of 300 nM). The fluorescence values (in arbitrary units) of the contour lines are the ones obtained for *B. cereus*. The optimum points predicted by the software for *E. coli*, *P. fluorescens*, *L. innocua*, *B.cereus* and *S. epidermidis* are represented in black circles with its respective initial letter. For *E. coli* and *P. fluorescens* no optimum value was obtained, but the overall behaviour observed indicates that lower DS concentrations and higher pH values should be preferred to redefine the testing concentrations.

Figure 2 - Surface response plots representing the interaction effect of pH and DS on the fluorescence response of *S. epidermidis*, *L. innocua*, *B. cereus*, *E. coli* and *P. fluorescens*. The optimal PNA EUB338 probe concentration was 300 nM for all 5 strains. Fluorescence values are presented in arbitrary units (a.u.).

Figure 3 - Schematic illustration showing the influence of pH, DS and probe concentration in PNA-FISH for Gram-positive and Gram-negative species. The identification of the limiting factor for Gram-positive and Gram-negative bacteria regarding probe diffusion inside the cell and the adjustment in terms of [DS] needed in order to maximize it. The η stand for viscosity and the A; C; G; U and T in front of *pKa* stand for the Watson-Crick nucleotide bases.

Supplementary File figure

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