# Standard operating procedure (SOP) for disk diffusion-based quorum sensing inhibition assays

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***Abstract***
***Introduction:*** *The emergence of multidrug-resistant bacterial strains is a severe global health issue, which is worsened by the inability of new antibiotics. Virulence inhibition is one of the novel strategies that have been proposed to combat bacterial pathogens more effectively, without the risk of exerting selection pressure on these microorganisms. Inhibition of bacterial cell-cell communication (quorum sensing; QS) is a promising approach however, rapid and cost-effective screening for compounds with QS-inhibitory activity is not yet well-established.* ***Aims:*** *The aim of the present study is to determine the ideal experimental conditions for the disk-diffusion based QS-inhibitory assay with the most frequently used QS-signal molecule-producing and reporter strains.*
***Methods:*** *In our study, the effects of growth characteristics, incubation time, temperature and the used culture media were studied on the used bacterial strains and results of the disk-diffusion based QS-inhibitory assay.*
***Results:*** *Based on our results, the ideal experimental setting includes a modified Luria-Bertani medium (LB*; complemented with nutrients and microelements), incubation at room temperature (25 °C) for 48 hours before the reading of results, where the density of the starting inocula has less influence of the results of the assay.*
***Conclusion:*** *Establishing standard operating procedures (SOPs) is a way to help carry out various operations, aiming to increase precision and efficiency. Adherence to the experimental settings defined based on our results may aid in improving the reproducibility, comparability and reliability of results obtained by this method.*

**Keywords:** *standard operating procedures, quorum sensing, QS, disk diffusion, Chromobacterium, violacein, Serratia, prodigiosin, Agrobacterium, pigment*

## 1. Introduction

Bacterial infections are still major factors of morbidity and mortality in both developing and developed countries worldwide therefore, antibiotics should be considered medicines of special importance [1]. In addition to being the causal therapy of often life-threatening infections (e.g., sepsis), antibiotics have paved the way for the development of many medical specialities (e.g., complex surgical procedures, organ transplantation, cancer chemotherapy, neonatology) [2, 3]. The continuous emergence of resistance bacterial strains (especially multidrug-resistant [MDR] pathogens) is becoming a severe global health issue [4,5]. One of the best ways to combat antimicrobial drug resistance is with the development of novel antibiotic drugs (which was the standard course of conduct during the 1960-1980's), nowadays however, the pharmaceutical companies are struggling to keep up with the continuous and detrimental developments in resistance trends [6,7]. The scarcity of new agents in the 'antibiotic pipeline' could be attributed to economical (antibiotics have high developmental costs and modest returns of investment, development of drugs for chronic illnesses and cancer is much more lucrative), clinical (the difficulties of arranging and tracking clinical trials) and microbiological (the emergence of resistant strains is inevitable) characteristics [8,9]. For this reason, no novel broad-spectrum agent has been developed since the discovery of the fluoroquinolones in the 1980's, while the dynamic increase in the prevalence of resistant isolates has been reported worldwide [10].

Due to the scarcity of available therapeutic options, novel strategies have been proposed to combat bacterial pathogens more effectively [11,12]. One of these strategies is combination therapy with the use of existing antibiotics, however, except for some well-defined clinical situations, the clinical utility of antibiotic combinations has been controversial, in addition to their costs for the healthcare infrastructure [13]. Another possible therapeutic alternative is to utilize adjuvant compounds (together with antibiotics) during therapy [12,14]. These antimicrobial adjuvants are classified to two distinct categories: Class I adjuvants affect the microorganism, while Class II adjuvants

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affect the cells of the host. Class I adjuvants include examples, such as β-lactamase inhibitors (which have been successfully used in therapy for many decades against various β-lactamase-producing pathogens), bacterial efflux pump inhibitors (e.g., phenylalanine-arginine β-naphthylamide (PAβN, although these compounds only have relevance in theoretical models and experimental settings for now, because most of them are toxic in the efflux pump inhibitory concentrations)) and modulators of bacterial membrane potential (e.g., loperamide) and compounds inhibiting bacterial toxin synthesis or neutralizing antibodies (e.g., bezlotoxumab against the toxins of *Clostridioides difficile*) [14,15]. Class I adjuvants may be useful, as they may in theory, make old antibiotics useful again, that have already been eliminated from clinical practice due to their widespread resistance. Class II adjuvants are usually compounds enhancing the immune response of the host organism against the foreign invaders (e.g., streptazolin, as a stimulant of macrophages and natural killer-cells) [14]. Another promising approach to fight bacterial infections is the use of virulence inhibitors: these compounds do not affect the viability of these cells, instead, they inhibit the synthesis or expression of bacterial virulence factors, which are key in their pathogenesis [16]. The potential advantage of these agents is that the selection pressure (and consequently, the chance of resistance development) is expected to be much lower [17].

Quorum sensing (QS; also called autoinduction) is a chemical-sociobiological mechanism of communication, during which bacteria can regulate the expression of specific genes (which are important for benefits in fitness and reproductive success in their niche), in response to the density of cells in the surrounding environment [18,19]. This includes the detection of signal molecules produced by surrounding cells and also self-produced signals (leading to positive feed-back; these autoinducers (or bacterial 'pheromones') diffuse into the specific niche, where their concentrations grow proportionally with the number of bacterial cells [20]. If the concentration of these signal molecules reaches a critical concentration (corresponding to a critical population density), these signal molecules initiate the transcription of various target genes [18-20]. Quorum sensing was first described in the marine bacterium *Vibrio fischeri,* a symbiont in the light organ of some marine animals: if bacteria reach a threshold population density, genes encoding bioluminescence are expressed [21]. QS mediates the expression of various features important in bacterial physiology and virulence, leading to phenotypic changes: expression of toxin genes (e.g., toxic shock syndrome toxin in *Staphylococcus aureus*, elastase in *Pseudomonas aeruginosa,* protease in *V. cholerae*), bacterial secretion systems (e.g., *Salmonella species*), efflux pumps (e.g., *P. aeruginosa*), biofilm-production (e.g., *P. aeruginosa*, *Acinetobacter baumannii*), induction of bacterial competence (*Streptococcus pneumoniae*), motility (e.g., *P. aeruginosa*) and production of pigments (e.g., *Chromobacterium violaceum, Serratia marcescens*) [22-27]. Quorum sensing has also been implicated in facilitating the spread of antibiotic-resistance genes [18,28-30].

QS signal molecules include a wide range of compounds with distinct structural characteristics [18-20]. In Gram-negative bacteria, derivatives of L-homoserine lactone (acyl-HSLs or AHLs) are the most prevalent, while in Gram-positive bacteria, peptide-based signal molecules (autoinducing peptides, AIPs, which are post-transcriptionally

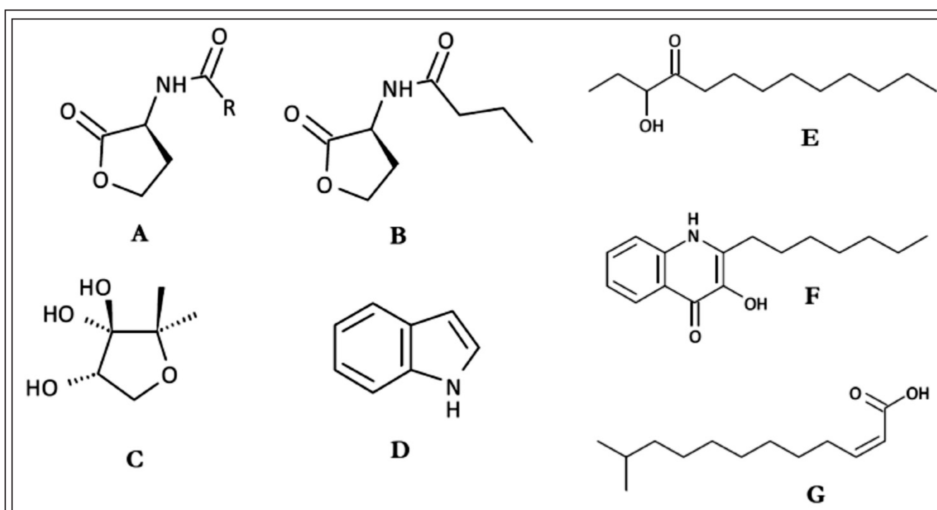


***Figure 1 Examples of quorum sensing signal molecules (autoinducers)*** *[18-27, 31]*
**A:** *Acyl-homoserine-lactones (AHL);* **B:** *Butanoyl-homoserine-lactones (BHL);* **C:** *Autoinducer-2 (AI);* **D:** *Indole;* **E:** *Cholera autoinducer (CAI-1/V. cholerae);* **F:** *Pseudomonas quinolone signal (PQS);* **G:** *Diffusible signal factor (DSF)*

modified small peptides) are most frequently detected. Some signaling molecules are detected by both groups (e.g., AI-2, a derivative of dihydroxy-2,3-pentanedione) (for examples, see *Figure 1*) [18-23]. Although the signal molecules may differ, the consequent mechanism of activation caused by these molecules is very similar in all bacteria [18-23]. In Gram-negative bacteria AHLs may be characterized by the nature and length of the substitution at the 3-carbon position, and the presence of unsaturated chains within the acyl chain [18,19,31].

The elimination or inhibition of QS-signal transmission is termed quorum quenching (QQ) [32]. This may be a consequence of inhibition of autoinducer-synthesis, degradation of signal molecule or through the use of signal-antagonists, inhibiting the sensing of these signal molecules by the relevant bacteria [19,31,32]. It is no surprise that many organisms possess enzymes with activity against such signal molecules (e.g., the human paraoxonase (PON, a lactonase) can also degrade AHLs). Synthetic compounds (inhibition-based QQ) or enzymes (degradation-based QQ) do not kill pathogenic bacteria, instead they inhibit the signal transduction mechanisms important in the expression of their virulence determinants (thus, disarming them) [14,16,19,31,32]. QQ-compounds may be considered as potential therapeutic alternatives in the treatment of bacterial infections, as they are capable of eliminating the disease-causing capacity of bacteria, without the risk of rapid resistance development [18-20, 31-33]. Several *in vitro* and *in vivo* model systems have been developed for the qualitative and quantitative evaluation of a compounds QS-inhibitory activity: these methods may include the use of Petri-dish or microplate-based colorimetric methods, molecular biological techniques (e.g., polymerase chain reaction), animal models and transgenic constructs [34-36]. Disk diffusion is a simple method for screening the susceptibility of various microorganisms against drugs/candidate molecules: it is user-friendly, and there is a lot of experience accumulated due to its use in routine clinical microbiology. Disk-diffusion based QS-inhibitory (DDBQSI) assay utilizing QS-signal molecule producing strains and signal molecule-reporter strains (e.g., *Agrobacterium*, *Chromobacterium*, *Pseudomonas*, *Serratia* and *Vibrio* species) is the most frequently used method [36-40]. The advantage of this method is its simple execution, the high-throughput nature and its usability in resource-scarce settings [41]. Nevertheless, there are many different and conflicting experimental protocols described for DDBQSI-assays in the literature, which makes it difficult to evaluate and compare published results. Additionally, the reproducibility of positive results still represents an important challenge for laboratories, because growth characteristics and pigment production by these bacteria is also subject to some additional factors [37]. The aim of the present study is to determine the ideal experimental conditions (i.e., incubation time, temperature, culture media) for disk-diffusion based QS-inhibitory (DDBQSI) assays with the most frequently used QS-signal molecule producing and reporter strains, and to establish standard operating procedures (SOPs) to optimize reproducibility of these assays.

## 2. Materials and methods

### *2.1. Culture media*

- Mueller-Hinton broth (MH-B) and Mueller-Hinton agar (MH-A) (Bio-Rad Hungary Ltd., Budapest, Hungary)
- Nutrient broth (NB) and Nutrient agar (NA) (Bio-Rad Hungary Ltd., Budapest, Hungary)
- Luria-Bertani broth (LB-B) and Luria-Bertani agar (LB-A) Bio-Rad Hungary Ltd., Budapest, Hungary)
- Modified Luria-Bertani broth (LB*-B) and agar (LB*-A) (which were prepared in-house, containing 8.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, 2.0 g glucose, 1.0 g $K_2HPO_4$, 0.2 g $MgSO_4$ x $7H_2O$, 10 mL 3% $CaCl_2$ stock solution, 5 mL Fe-EDTA stock solution, 1 mL microelement stock solution and 12.0 g of bacteriological agar in case of the solid medium, per 1 L of media; pH was adjusted to 7.0-7.2)

### *2.2. Bacterial strains*

The following bacterial strains were used during our experiments:

- *Chromobacterium violaceum* wt85 [36]
  *Taxomony:* Gram-negative, facultative anaerobic rod, member of the *Neisseriales* order
  *Function:* wild-type strain (control strain), characterized by the AHL signal molecule-mediated production of the purple violacein pigment, capable of endogenous QS-signal molecule-production (N-hexanoyl-L-HSL)
- *C. violaceum* CV026 [36]
  *axomony:* Gram-negative, facultative anaerobic rod, member of the *Neisseriales* order

*Function:* Tn5 transposase-mutant, AHL-signal molecule indicator strain (produces purple violacein pigment in the presence of AHLs), which is incapable of endogenous QS-signal molecule-production, but useful in the detection of external stimuli

- *Enterobacter cloacae* (clinical isolate no. 31298, isolated from a wound sample) [37]
  *Taxomony:* Gram-negative, facultative anaerobic rod, member of the *Enterobacterales* order
  *Function:* AHL-producing-strain (used with *C. violaceum* CV026)
- *Sphingomonas paucimobilis* Ezf 10-17 (isolated from a tumor of the "Ezertűfű" variety of the common grape vine [*Vitis vinifera*]) [36]
  *Taxomony:* Gram-negative, strict aerobic rod, member of the *Sphingomonadales* order
  *Function:* AHL-producing-strain (used with *C. violaceum* CV026)
- *Novosphingobium* spp. Rr 2-17 (isolated from a tumor of the "Rajnai rizling" variety of the common grape vine [*Vitis vinifera*]) [36]
  *Taxonomy:* Gram-negative, facultative anaerobic rod, member of the member of the *Sphingomonadales* order
  *Function:* AHL-producing-strain (used with *C. violaceum* CV026)
- *Serratia marcescens* AS-1 (Szeged Microbiological Culture Collection) [39]
  *Taxonomy:* Gram-negative, facultative anaerobic rod, member of the *Enterobacterales* order
  *Function:* characterized by the production AHL signal molecule-mediated production of the orange-red pigment prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin), capable of endogenous QS-signal molecule-production (N-hexanoyl-L-HSL)
- *A. tumefaciens* NTL4(pCF218)(pCF372) (isolated from a tumor of a wild cherry tree [*Prunus avium*]) [36,38]
  *Taxonomy:* Gram-negative, facultative anaerobic rod, member of the *Rhizobiales* order
  *Function:* characterized by the expression of β-galactosidase in the presence of a wide range of AHL signals, which may be detected in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the medium, resulting in a color change
- *A. tumefaciens C58* [36,38]
  *Taxonomy:* Gram-negative, facultative anaerobic rod, member of the *Rhizobiales* order
  *Function:* AHL-producing-strain (used with *Agrobacterium tumefaciens* NTL4(pCF218)(pCF372))

The bacterial strains for our experiments were kindly provided by Dr. Ernő Szegedi (Institute of Viticulture and Enology, National Agricultural Research Center). The bacterial strains were maintained on Luria-Bertani (LB) agar for shorter time periods (<1 month), while for longer periods, the strains were kept in a -80°C freezer, in a 1:4 mixture of 85% glycerol and liquid Luria-Bertani media. For the maintenance purposes of *C. violaceum* CV026 and *A. tumefaciens* NTL4(pCF218)(pCF372), media were also supplemented with kanamycin and carbenicillin, respectively [36,38].

### *2.3. Chemicals*

Bacteriological agar (Bio-Rad Hungary Ltd.; Budapest, Hungary), tryptone (Thermo Fischer Scientific; Waltham, US), yeast extract (Thermo Fischer Scientific; Waltham, US), D-glucose (Sigma-Aldich; Budapest, Hungary), kanamycin (Sigma-Aldich; Budapest, Hungary), carbenicillin (Sigma-Aldich; Budapest, Hungary), NaCl (Sigma-Aldich; Budapest, Hungary), $K_2HPO_4$ (Sigma-Aldich; Budapest, Hungary), $KH_2PO_4$ (Sigma-Aldich; Budapest, Hungary), $MgSO_4x7H_20$ (Sigma-Aldich; Budapest, Hungary), $CaCl_2x2H_2O$ (Sigma-Aldich; Budapest, Hungary), $FeSO_4x7H_20$ (Sigma-Aldich; Budapest, Hungary), $Na_2EDTA$ (Sigma-Aldich; Budapest, Hungary), $MnSO_4x7H_2O$ (Sigma-Aldich; Budapest, Hungary), $ZnSO_4x7H_2O$ (Sigma-Aldich; Budapest, Hungary), $Na_2MoO_4x2H_2O$ (Sigma-Aldich; Budapest, Hungary), $CoCl_2x6H_2O$ (Sigma-Aldich; Budapest, Hungary), dimethyl-sulfoxide (DMSO; Sigma-Aldich; Budapest, Hungary), acridine orange (Sigma-Aldich; Budapest, Hungary) and phosphate buffered saline (PBS; Sigma-Aldich; Budapest, Hungary). During the preparation of the modified Luria-Bertani broth (LB*-B) and agar (LB*-A), the following stock solutions were used: 5% Fe-EDTA stock solution, 3% $CaCl_2$ stock solution and a microelement stock solution (containing 1.0 g $MnSO_4x7H_2O$, 0.5 g $ZnSO_4x7H_2O$, 25 mg $Na_2MoO_4x2H_2O$ and 2.5 mg $CoCl_2x6H_2O$ per 100 mL). The stock solutions were aliquoted in 50 mL centrifuge tubes and kept at -20°C.

### *2.4. Evaluation of growth characteristics and pigment production of relevant bacterial strains*

To identify the ideal experimental conditions, growth characteristics of the bacterial strains used were determined in Nutrient broth (NB), Mueller-Hinton (MH-B) and Luria-Bertani (LB-B) broths,

in addition to Nutrient agar (NA), Mueller-Hinton (MH-A) agar and Luria-Bertani agar (LB-A). In the assays, liquid and solid media were inoculated with the same primary culture for each bacterial strain using a calibrated loop (10 µl). The optical density of the liquid media ($OD_{580}$, using a photometer) and the number of colonies as well as the degree of pigment production were observed. Growth properties were studied at four different temperatures of incubation: 0°C (refrigerator), 10°C (cooled room with controlled temperature), 25°C (room temperature) and 37°C (incubator). The cultures were measured/read after 12, 24, 48 and 72 hours of incubation. The results of the experiments were from at least three independent experiments. Based on literature data, our study was later complemented with a modified Luria-Bertani (LB*) medium, which was compared to the classical LB medium [42] (see *2.1. Culture media*).

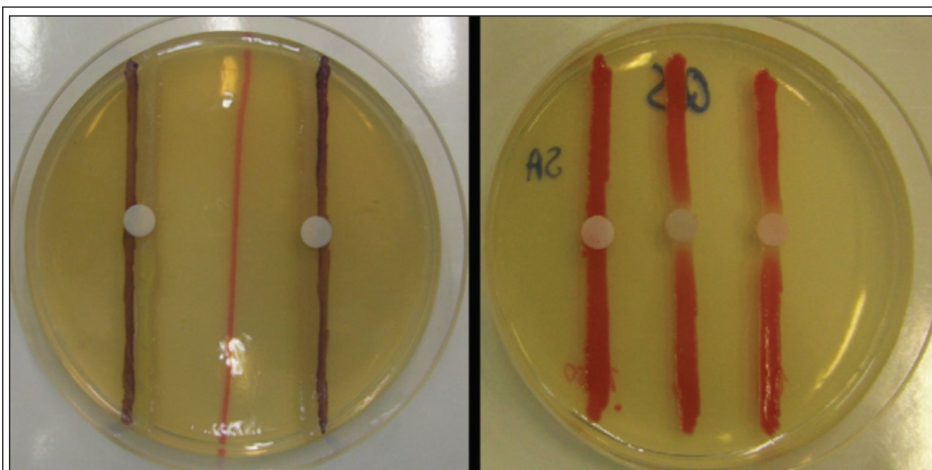


***Figure 2*** *Disk diffusion quorum-sensing inhibitory assay using C. violaceum CV026 and E. cloacae 31298 (left) and S. marcescens AS-1 (right)*

*2.5. Disk diffusion quorum-sensing inhibitory assay*

Quorum sensing inhibitory activity was monitored by the disk diffusion method. During the assay, cultures of $OD_{580}$~0.5 overnight bacteria grown in LB*-B broth were inoculated directly onto LB*-A agar surface. Filter paper disks (7.0 mm in diameter, Whatmann 3MM), were impregnated with 10 µL of acridine orange (AO; used as a positive control; 25.0 mg/mL in phosphate buffered saline) or DMSO (used as a negative control, 2 V/V%) [37]. The disks were placed on the surface of LB*-A agar surface between the parallel inoculations of sensor (*C. violaceum* CV026) and AHL-producer (*S. paucimobilis* Ezf 10-17, *Novosphingobium* spp. Rr 2-17 and *E. cloacae* 31298) strains; the exception was *S. marcescens* AS-1 (capable of producing prodigiosin from endogenous AHL-signals), where disks were placed on the center of the inoculated line (*Figure 2*) [36-39]. To quantify the QS inhibitory effect, the diameter of the QS-inhibition zones (i.e., the culture of discolored but intact bacteria) around the disks was measured using a ruler, after 12, 24, 48 and 72 hours of incubation [36-39]. The results of the studies are derived from the average of at least three independent experiments. The *A. tumefaciens* NTL4(pCF218)(pCF372) and *A. tumefaciens* C58 indicator-AHL-producer pair was not included in this experiment, as the presence of X-gal is required in the media for the colour change to occur.

## 3. Results and discussion

*3.1. Growth characteristics of bacterial strains*

There were no relevant differences detected in the growth rate of bacterial strains between the different liquid broths (NB, MH, LB). The growth of bacterial strains was inhibited at low temperatures (0 and 10 ° C) resulting in $OD_{580}$ values of 0-0.05, 0.05-0.1 and 0.1-0.2 for 12, 24, 48 and 72 hours of incubation, respectively, which was inadequate to perform further experiments. There was no difference in bacterial growth between 25°C and 37°C incubations (resulting in $OD_{580}$ values of 0.4-0.5 after 12 hours (i.e. overnight), 0.8-1 after 24 hours, and >1 after 48 hours), except in the case of *C. violaceum* wt85 and *C. violaceum* CV026, where higher reads were observed at 37°C, but in both cases, the OD of the bacterial cultures was appropriate for performing further experiments. The use of 48 hour- and 72 hour-cultures is not recommended, due to the accumulation of dead bacterial cells and autolysis, a consequence of the depletion of nutrients in the culture media (in fact, the $OD_{580}$ values after 72 hours showed decreasing tendencies), which may lead to distorted results in the experiments later on. Similarly, there were no relevant differences detected in the growth rate of

bacterial strains between the solid agar media (NB, MH-B and LB-B). It should be highlighted, that in case of *S. marcescens*, the temperature had a pronounced effect on pigment production in both liquid and solid media (pigment production ceased at 37°C, this effect was not observed for *C. violaceum* wt85). For this reason, 25°C was set as the reference temperature for the additional experiments.

Based on previous reports, it was found that the concentration of several metal ions in the environment has a pronounced effect of quorum sensing in bacteria [37]. After a thorough literature survey, an additional medium was included in our optimization studies, namely the modified LB (or LB*) broth and solid media, which is supplemented by additional nutrients and a microelement solution (containing various metal ions) [42]. The tested strains showed no relevant differences in the growth characteristics in LB-B and LB*-B broths in the same experimental setup previously described. However, during the comparison of LB-A and LB*-A solid media, it was evident that colony formation (number and size of bacterial colonies) and pigmentation of the colonies occurred more rapidly, therefore, the growth properties of the relevant strains were further characterized on this media (*Table I*). During the bacterial growth experiments on the LB*-A solid media, it was observed that bacterial colonies' growth and pigment production on LB * agar were stable after 48 hours when incubated at 25°C. In addition, if the read-

***Table I*** *Growth characteristics of tested QS-strains on LB*-A media at room temperature (25 °C)*

| | **Optical density ($OD_{580}$) of bacterial inoculum used** | | | | |
|---|---|---|---|---|---|
| **After 24 hours** | 0.1 | 0.3 | 0.5 | 0.7 | 1.0 |
| ***Chromobacterium violaceum* CV026** | ø | ø /+ | + | + | ++ |
| ***Chromobacterium violaceum* wt85** | ø | ø/+ | + | + | ++ (!) |
| ***Sphingomonas paucimobilis* Ezf 10-17** | ø/+ | ø/+ | + | ++ | ++ |
| ***Novosphingobium spp.* Rr 2-17** | ø | ø | ø | + | + |
| ***Serratia marcescens* AS-1** | ++ | ++ | +++ | +++ | +++ |
| ***Enterobacter cloacae* 31298** | ++ | ++ | ++ | +++ | +++ |
| ***Agrobacterium tumefaciens* NTL4** | -/+ | -/+ | + | ++ | ++ |
| ***Agrobacterium tumefaciens* C58** | -/+ | -/+ | + | ++ | ++ |
| | | | | | |
| **After 48 hours** | 0.1 | 0.3 | 0.5 | 0.7 | 1 |
| ***Chromobacterium violaceum* CV026** | ++ | +++ | +++ | +++ | +++ |
| ***Chromobacterium violaceum* wt85** | +++ (!) | +++ (!) | +++ (!) | +++ (!) | +++ (!) |
| ***Sphingomonas paucimobilis* Ezf 10-17** | ++ | ++ | +++ | +++ | +++ |
| ***Novosphingobium spp.* Rr 2-17** | ++ | ++ | ++ | ++ | +++ |
| ***Serratia marcescens* AS-1** | +++ (!) | +++ (!) | ++++ (!) | ++++ (!) | ++++ (!) |
| ***Enterobacter cloacae* 31298** | +++ | +++ | +++ | +++ | +++ |
| ***Agrobacterium tumefaciens* NTL4** | +++ | +++ | +++ | +++ | +++ |
| ***Agrobacterium tumefaciens* C58** | +++ | +++ | +++ | +++ | +++ |
| | | | | | |
| **After 72 hours** | 0.1 | 0.3 | 0.5 | 0.7 | 1 |
| ***Chromobacterium violaceum* CV026** | +++ | +++ | +++ | +++ | +++ |
| ***Chromobacterium violaceum* wt85** | +++ (!) | +++ (!) | +++ (!) | +++ (!) | +++ (!) |
| ***Sphingomonas paucimobilis* Ezf 10-17** | +++ | +++ | +++ | +++ | +++ |
| ***Novosphingobium spp.* Rr 2-17** | ++ | +++ | +++ | ++++ | ++++ |
| ***Serratia marcescens* AS-1** | +++ (!) | +++ (!) | ++++ (!) | ++++ (!) | ++++ (!) |
| ***Enterobacter cloacae* 31298** | +++ | +++ | ++++ | ++++ | ++++ |
| ***Agrobacterium tumefaciens* NTL4** | +++ | +++ | ++++ | ++++ | ++++ |
| ***Agrobacterium tumefaciens* C58** | +++ | +++ | ++++ | ++++ | ++++ |

*Legend: ø: no growth, +: weak bacterial growth, ++: moderate bacterial growth, +++: adequate bacterial growth, ++++: strong bacterial growth (!): pigment production*

ing of the plates occurred after 48 hours, the colony growth and pigment production has shown to be independent from the optical density of the initial inoculum in the range $OD_{580} \geq 0.5$, while this number was $OD_{580} \geq 0.1$ if the reading occurred after 72 hours (*Table I*).

*3.2. Disk-diffusion quorum-sensing inhibitory assay*

The results of the optimization experiments with the positive control acridine orange (AO) are presented in *Table II*, where the quorum-sensing inhibition zones are shown for the parallel inoculations between QS-sensor strain *C. violaceum* and the AHL-producer strains, and for *S. marcescens* AS-1, respectively (*Figure 3*). No quantifiable QS-inhibition zone (i.e. loss of purple violacein pigmentation) was detected in case of the CV026-AHL-producers after 12 hours, at least 24 hours were needed for the discoloration to develop, except for the *S. marcescens* AS-1, where minor inhibition was present. Based on our results, the inhibition zone was still subject to change at the 24 hour-reading of plates, however, the results after 48 hours may be considered to be final, additional incubation and observation did not change the results. According to the data presented, the *Serratia* model system was the most sensitive for the QS-inhibitory activity of AO. DMSO was used as a negative control, no measureable QS-inhibition zone was detected.

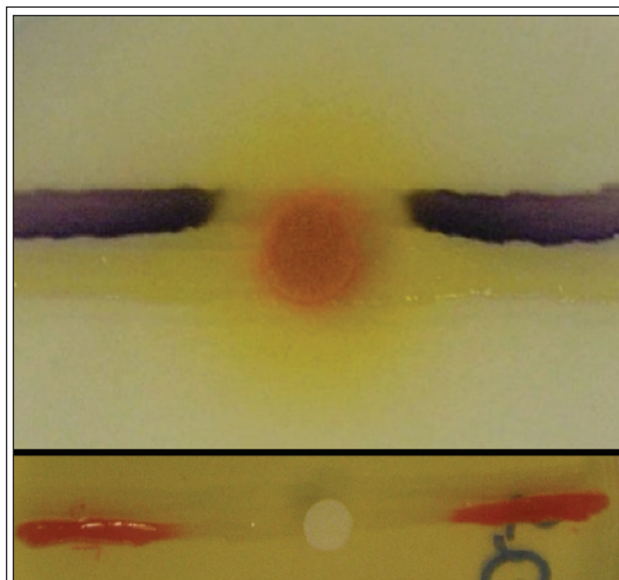

***Figure 3*** *Quorum sensing-inhibition zones using C. violaceum CV026 and E. cloacae 31298 (top) and S. marcescens AS-1 (bottom) after 48 hours of incubation*

## 4. Conclusions

The emergence of multidrug resistance in bacterial infections significantly hinders the appropriate therapy of patients, and with the current disinterest of pharmaceutical companies to develop new antibiotics, alternative approaches should be considered for the therapy of these infections. Quorum sensing is a form of bacterial cell-cell communication, whereby these microorganisms use diffusible signal molecules as proxy to detect the surrounding cell density and produce metabolically costly products when the sufficient biomass has been reached. Inhibitors of quorum sensing may be potent modulators of bacterial virulence, eliminating their pathogenic potential, without killing them (therefore the selection pressure would be lower), however, the development and screening for the QS-activity of these compounds is not well-established. A standard operating procedure (SOP) is a designated set of step-by-step instructions compiled by relevant (qualified) individuals or an organization to help carry out various operations, aiming to increase precision and efficiency. The aim of our study was to characterize the appropriate conditions for the disk diffusion-based QS-inhibition assay, consisting of QS-signal sensor and AHL-producer strains. Based on our results, the ideal experimental setting includes a modified Luria-Bertani medium (complemented with nutrients and microelements), incubation at room temperature (25°C) for 48 hours before reading of the results, where the density of the starting

***Table II*** *Quorum-sensing inhibitory activity of acridine orange (OA) in various model systems, corresponding to different plate-reading times*

| | **Quorum-sensing inhibition zone diameter (mm±SD)** | | | |
|---|---|---|---|---|
| **Bacterial model system** | 12 hours | 24 hours | **48 hours** | 72 hours |
| ***C. violaceum* CV026 + *E. cloacae* 31298** | ø | 13 ± 2.2 | **14 ± 1.2** | 14 ± 1.2 |
| ***C. violaceum* CV026 + *S. paucimobilis* Ezf 10-17** | ø | 14 ± 1.6 | **16 ± 0.9** | 16 ± 0.9 |
| ***Novosphingobium* spp. Rr 2-17** | ø | 11 ± 2.0 | **13 ± 1.0** | 13 ± 1.0 |
| | | | | |
| ***Serratia marcescens* AS-1** | 6 ± 2.3 | 17 ± 1.5 | **19 ± 0.8** | 19 ± 0.8 |

inocula has less influence of the results of the assay. Adherence to the abovementioned criteria may aid in improving the reproducibility, comparability and reliability of results obtained by this method.

## 5. Acknowledgements

The authors would like to thank Dr. Ernő Szegedi (Institute of Viticulture and Enology, National Agricultural Research Center) for providing the bacterial strains used in our experiments. Part of this study was presented at the 18th International Congress of the Hungarian Society for Microbiology (MMT; Budapest, Hungary).

## 6. Competing interests

The authors declare no conflict of interest, monetary or otherwise.

## 7. References

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