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Modulation of intracellular O_2 concentration in *Escherichia coli* strains using Oxygen Consuming Devices (OCDs)

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ABSTRACT

The use of cell factories for the production of bulk and value-added compounds is nowadays an advantageous alternative to the traditional petrochemical methods. Nevertheless, the efficiency and productivity of several of these processes can improve with the implementation of micro-oxic or anoxic conditions. In the industrial setting, laccases are appealing catalysts able to oxidize a wide range of substrates and reduce O_2 to H_2O. In this work, several laccase-based devices were designed and constructed foreseeing the modulation of intracellular oxygen concentration in bacterial chassis. These Oxygen Consuming Devices (OCDs) included *Escherichia coli*'s native laccase (CueO) and three variants of this protein obtained by directed evolution. The OCDs were initially characterized *in vitro* using *E. coli* DH5α protein extracts, and subsequently using extracts obtained from other *E. coli* strains and *in vivo*. Upon induction of the OCDs no major effect on growth was observed in four of the strains tested, and the analysis of the cell extracts protein profiles revealed increased levels of laccase. Moreover, oxygen consumption associated to the OCDs occurred in all conditions tested but the performance of the device was shown to be strain-dependent, highlighting the importance of the genetic background even in closely related strains. One of the laccase variants showed a 13- and 5-fold increase in oxidase activity and O_2 consumption rate, respectively. Furthermore, it was also possible to demonstrate O_2 consumption *in vivo* using L-DOPA as substrate, which represents the proof of concept that these OCDs generate an intracellular oxygen sink thereby manipulating the redox status of the cells. In addition, the modularity and orthogonality principles used for the development of these devices allow an easy reassembly and fine-tuning foreseeing their introduction into other chassis/systems.

Keywords: *Escherichia coli*; Bacterial chassis; Laccase; Oxygen Consuming Devices; Synthetic Biology.

INTRODUCTION

Micro-oxic and anoxic environments are fundamental to numerous processes with significance to clinical, environmental, and industrial fields. It is generally accepted that hypoxia is established during infectious processes[1-3] and that hypoxia inducible factors are central regulators of tumor phenotype contributing to malignancy.[4] Likewise, micro-oxic and anoxic processes are critical for maintenance of carbon, nitrogen and sulfur global cycles, the degradation of recalcitrant organic compounds or transformation of metals and metalloids to less toxic forms.[5,6] In industrial biotechnology, the applications in oxygen limited conditions have been largely overlooked compared to their counterparts in oxic conditions. Although ethanol, butanol, malate or polyhydroxybutyrate can be produced under micro-oxic or anoxic conditions,[7-10] the shift from fossil-based to biobased economy will benefit from an increased implementation of processes for biomass valorization or the synthesis of energy carriers, biofuels, bulk chemicals, probiotics, or bioactive compounds under O_2-limited conditions.[6] Currently, the production of many chemicals/compounds by aerobic processes has low productivity since cell metabolism favors biomass formation.[10,11] The presence of O_2 can also negatively affect processes by enzyme inhibition, for *e.g.*, the photobiological production of H_2 is severely compromised by the presence of oxygen due to the sensitivity of the H_2-evolving enzymes: hydrogenases and nitrogenases.[12,13] Therefore, the development of micro-oxic/anoxic processes is highly desirable to achieve high efficiencies and near theoretical yields.[11] In this context, several strategies were successfully implemented to increase the production of propanol, butanol, or ethanol in *Escherichia coli* [14-20] and synthetic biology has played a significant role in the development of these metabolic engineering approaches with the redesign of existing pathways or the *de novo* design.[21,22]

In an industrial setting, laccases are considered green catalysts due to the wide range of substrates catalyzed without the need for co-factors or production of toxic peroxide intermediates.[23,24] Laccases belong to the multicopper oxidase protein superfamily that is characterized by the highly conserved copper binding motifs: the T1 mononuclear copper center (where the one-electron substrate oxidation occurs), and the T2/T3 trinuclear copper center that catalyzes the four-electron reduction of oxygen to water.[25,26] The applications based on these enzymes are broad, ranging from paper, food and textile industries to bioremediation, biosensors, cosmetics, and organic synthesis.[23,27,28] Fungal laccases are often used for these purposes, but recently the interest in proteins from bacterial origin is increasing due to their higher thermal and pH stability. Moreover, the latter are more prone to activity and specificity improvements through protein engineering.[23] *E. coli*'s native multicopper oxidase (CueO) is a monomeric periplasmic protein involved in copper homeostasis.[29] Besides the characteristic copper binding motifs, CueO displays a methionine rich insert which provides extra copper binding sites that are essential for the cuprous oxidase activity related to copper detoxification, the physiological function recently attributed to the protein.[26,30] In addition, CueO also exhibits significant laccase (phenol oxidase) activity with broad substrate specificity. Taking advantage of this feature, several OCDs - Oxygen Consuming Devices based on the CueO laccase were assembled following the standardization and modularity principles and envisaging the modulation of the intracellular oxygen concentration in bacterial chassis. These OCDs were tested *in vitro* in different conditions and in different *E. coli* genetic backgrounds, and the proof of concept for the *in vivo* modulation of intracellular oxygen using these OCDs is also provided. Moreover, the approach followed here will allow extending the use of OCDs to other organisms/chassis, enabling the development of bioprocesses such as bioremediation

or production of biofuels and the elucidation of fundamental aspects of adaptation to oxygen limited/deprived conditions.

RESULTS AND DISCUSSION

Selection of Targets, Design and Assembly of the Lac OCD. Aiming at modulating the intracellular O_2 concentration in bacterial chassis commonly used in industrial processes, a bioinformatics tool (DESHARKY)[31] was used to identify metabolic routes involving the oxidation of natural compounds (metabolites) and consequent O_2 consumption. The search was based on a Monte Carlo heuristic algorithm, considering *E. coli* and *Synechocystis* sp. PCC 6803 as hosts, and O_2 as reactant source. From the tool outputs, the pathways comprising enzymes without information available in KEGG were eliminated and it was also imposed that the substrates for these reactions should be present in the hosts. Moreover, among pathways containing cycles of oxygen production-degradation, it was checked that the pathway effective stoichiometry involved the consumption of oxygen. Based on this search, several oxygen consuming devices were designed and tested (e.g. *A*-type flavoproteins, glucose oxidase) however, based on preliminary results and various technical difficulties that have arisen only the characterization of the OCDs based on *E. coli*'s K-12 native laccase (CueO) is presented here.

Envisaging the introduction of the OCDs in different bacteria, the 1551 bp sequence of *cueO* ORF was codon-optimized considering the codon usage of *E. coli* K-12, *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120 to obtain a single sequence that could be expressed in the three organisms – *cueO**. Comparison of the codon adaptiveness of the codon optimized and original *cueO* ORFs (Figure S1) revealed significant codon usage differences, with 27% of the codons showing codon usage differences above 50%.

For an approximate indication of the likely success of the codon optimized sequence expression in *E. coli*, the codon adaptation index (CAI) was calculated; since this index is a measurement of the relative adaptiveness of the codon usage of a gene towards the codon usage of highly expressed genes.[32] The original and codon-optimized *cueO* sequences showed similar CAI values, (0.816 and 0.782, respectively) indicating that the synthetic sequence would most likely be expressed in *E. coli*, despite the codon alterations made. Successful expression is also expected in *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120 since the CAI indexes obtained were 0.767 and 0.785, respectively. In addition to the codon-optimized *cueO* ORF (*cueO**), the synthetic DNA sequence includes the RBS BBa_B0034, double stop codons (TAATAA), and the prefix and suffix sequences of the BioBrick RFC[10] standard.[33] Subsequently, this sequence was cloned downstream the F2620 BioBrick originating the laccase-based OCD – Lac OCD (Figure 1). The F2620 BioBrick is a regulatory element that enables the device induction upon addition of signaling molecules of the acyl-homoserine lactone family (AHLs).

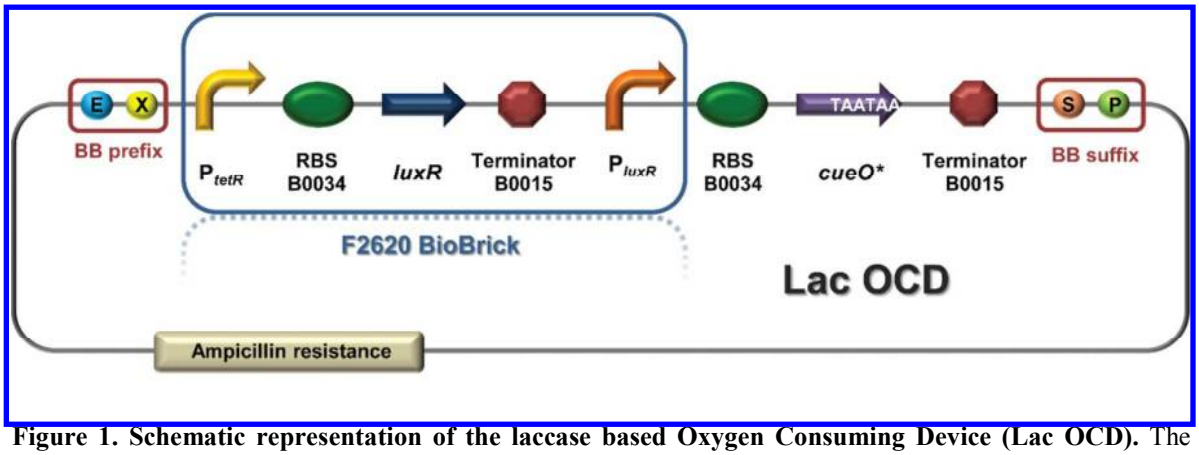

Figure 1. Schematic representation of the laccase based Oxygen Consuming Device (Lac OCD). The device includes the RBS B0034, the codon optimized *cueO* ORF (*cueO**) with double stop codons (TAATAA) followed by the double terminator B0015 and is regulated by the F2620 BioBrick. This regulatory element enables OCD regulation by the P_{luxR} promoter that is activated by the complex formed

between signaling molecules of the acyl-homoserine lactone class (AHLs) and the LuxR protein. The *luxR* ORF is preceded by the RBS B0034 and the constitutive promoter P_{tetR}. The Lac OCD is flanked by the BioBrick (BB) prefix (with the *Eco*RI (E) and the *Xba*I (X) restriction sites) and the BB suffix (with *Spe*I (S) and *Pst*I (P) restriction sites) and is in a BioBrick backbone, the pSB1A3 that harbors the gene conferring resistance to Ampicillin.

Effect of Laccase OCD Activation on Growth and Protein Profiles of Five *E. coli* Strains. It is widely recognized that the performance of synthetic parts is dependent on the genomic and metabolic context.[34,35] Therefore the Lac OCD was introduced and characterized in different *E. coli* strains routinely used in laboratory: DH5α, BL21(DE3), MG1655, SURE and Top10. In addition to the strains containing the Lac OCD, control strains harboring the plasmid with the F2620 BioBrick (F2620) only were also generated.

The growth of the *E. coli* strains (DH5α, BL21(DE3), MG1655, SURE and Top10) containing the F2620 only or the F2620 plus the Lac OCD (Lac OCD) was compared to the respective wild-type, in absence/presence of the inducer AHL (*N*-(β-ketocaproyl)-L-homoserine lactone, 3OC6HSL). No significant differences in growth were observed, except for the SURE and DH5α strains (Figure 2a and S2). In the *E. coli* SURE strain, a premature onset of the stationary phase was observed in the wild-type and cells harboring the Lac OCD while for cells harboring the F2620 the growth was unstable (Figure S2). This reduced fitness may be due to the strain's impaired DNA repair, recombination and restriction systems. For *E. coli* DH5α, the cells containing the Lac OCD showed hindered growth in the 6 hours that followed the addition of AHL (Figure 2a), with a decrease in growth rate to 36% (0.12 h^{-1}) of the wild-type rate (0.35 h^{-1}). However, after this period, the growth behavior was similar to the wild-type. Previous studies report that

metabolic burden leads to growth alterations that can be related to the reallocation of cell's resources to maintain the plasmid used to transform cells,[36] which is not the case here since no differences in growth were observed between the wild-type and the cells harboring the F2620.

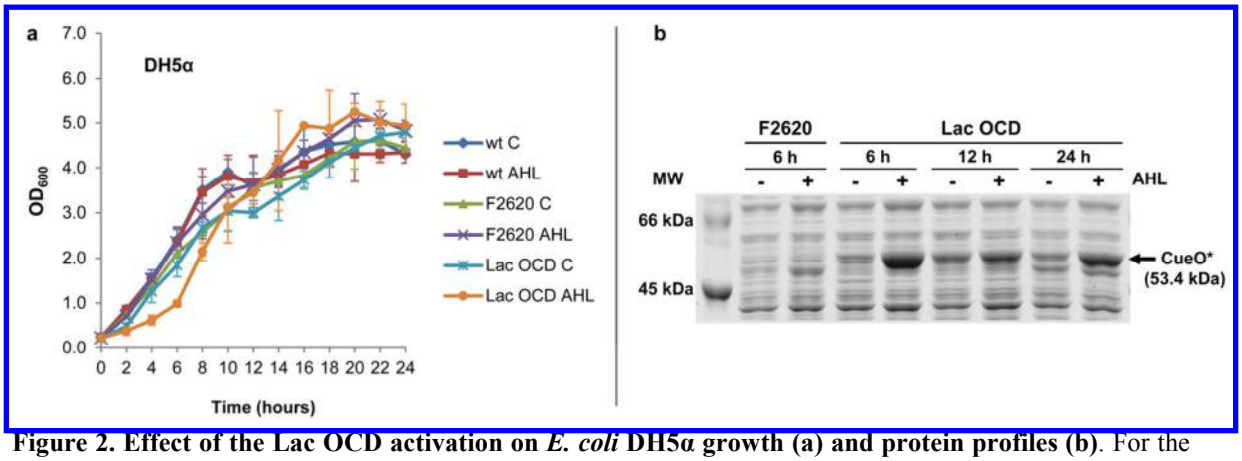

Figure 2. Effect of the Lac OCD activation on *E. coli* DH5α growth (a) and protein profiles (b). For the growth experiment (**a**) the wild-type (wt), the cells containing the F2620 only or the F2620 plus the Lac OCD (Lac OCD) were grown in absence (C) or presence of the inducer (AHL). Growth was monitored measuring the OD_{600} every 2 hours for a 24 hour period. Error bars represent the standard deviation of biological replicates (n = 3). Protein extracts obtained from *E. coli* DH5α cultures harboring the F2620 and the F2620 plus the Lac OCD (Lac OCD), in presence (+) or absence (-) of AHL and collected at different time-points after OCD induction (6, 12 and 24 h) were separated by electrophoresis on SDS-PAGE and stained with Coomassie Blue (**b**). The protein band corresponding to the laccase (CueO*) is highlighted by the black arrow. MW – low molecular weight protein marker (GE Healthcare).

Increased protein expression can also lead to metabolic burden due to the depletion of aminoacyl-tRNAs or amino acid pools that are known to be dependent on the growth rate.[36,37] Therefore, DH5α cells containing the Lac OCD and grown in presence or absence of AHL were collected 6, 12, and 24 hours after induction and the protein profiles were

visualized on Coomassie blue stained SDS polyacrylamide gels (Figure 2b). In contrast with the F2620 transformants, a substantial amount of laccase was readily observed in extracts obtained from cultures harboring the device, particularly in the presence of the AHL inducer. The highest levels of laccase were observed 6 hours after induction. In the subsequent points, although the protein levels decrease, laccase was still easily observed. Therefore, our results suggest that the overexpression of laccase leads to a growth delay in DH5α cells. The subsequent reduction in protein levels may alleviate the metabolic burden allowing the recovery of the growth rate after 6 hours, being the culture final OD similar to the wild-type.

Additionally, the protein profiles of the other *E. coli* strains (BL21, MG1655, SURE and Top10) were also analyzed (Figure 3). Similarly to DH5α, a marked difference between the protein profiles of the cells harboring F2620 or the F2620 plus the Lac OCD was observed, with increased laccase levels in extracts obtained from cells harboring the device and grown in presence of the inducer (Figure 3, arrowheads). In the protein profiles of cells containing the OCD but in absence of AHL, increased levels of laccase could also be observed compared to the extracts of cells with F2620, suggesting read through from P_{tetR} or leakiness of the P_{luxR} promoter. This leaky behavior was previously reported to be dependent both on abiotic and genetic factors such as temperature, media, chassis' genetic background or the embedded sequence context.[38] For the *E. coli* strain MG1655 an inconsistent behavior was registered, as frequently no induction of laccase expression was observed.

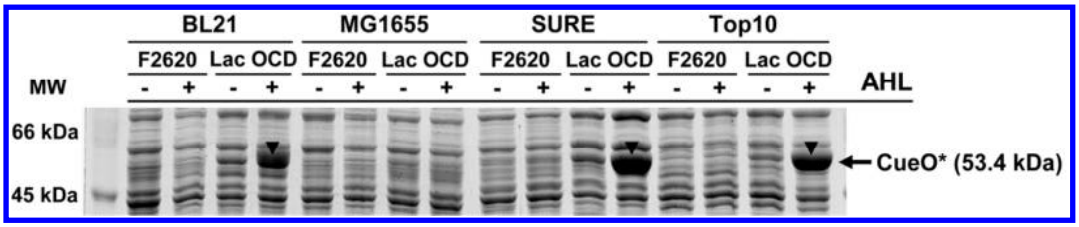

Figure 3. Coomassie stained SDS-PAGE of the protein profiles of different *E. coli* strains. Protein extracts were obtained from *E. coli* BL21, MG1655, SURE and Top10 cultures harboring the F2620 or the F2620 plus the Lac OCD (Lac OCD), in presence (+) or absence (-) of AHL, collected 6 hours after induction. The protein band corresponding to the laccase (CueO*) is highlighted by the black arrow, and for each strain the induced protein expression is highlighted by the black arrowheads. MW – low molecular weight protein marker (GE Healthcare).

In sum, the Lac OCD was consistently expressed in four of the five *E. coli* strains tested and its activation had only a transient impact on the growth of one of the strains. Moreover, the device was shown to be robust since increased levels of laccase could still be detected 24 hours after the induction, in agreement with previous reports on CueO's high stability.[39]

***In Vitro* Performance of the Laccase OCD in *E. coli* DH5α.** An extensive characterization of the Lac OCD was carried out in *E.coli* DH5α to determine the dynamic range of the device performance. For this purpose, phenol oxidase activity and O_2 consumption rates were determined using protein extracts obtained from cells induced in exponential or stationary phase, and grown in M9 minimal medium or M9 supplemented with copper. In addition, cells were collected at different time-points: 6, 12 or 24 hours after AHL induction. The influence of the different parameters on the OCD performance was statistically determined performing a four-way ANOVA, where a model with all the

effects and interactions up to the third order was tested. The statistical analysis revealed that the medium used for cell growth does not influence the device performance in terms of laccase activity or O_2 consumption (p-value = $2.20x10^{-02}$ and $6.78x10^{-02}$, respectively). For this reason, the results obtained using M9 medium supplemented with copper (Figure 4) were shown separately from those obtained with M9 medium (Figure S3). Although the time of induction was shown to have an influence in the Lac OCD, with a significant p-value of $2.36x10^{-05}$, the impact of this parameter is modest (with a partial Eta squared value of 0.062). The growth phase in which the device was induced showed to have an impact on the performance (p-value = $1.97x10^{-19}$), with cells grown in M9 medium and induced in stationary phase showing higher O_2 consumption rates than cells induced in exponential phase (Figure S3b and d). Even though the Lac OCD was partly active in absence of AHL, as expected the statistical analysis revealed that the presence of the inducer has the most influence in performance, with significant p-values of $2.13x10^{-30}$ for laccase activity and $2.29x10^{-23}$ for O_2 consumption. In fact, compared to the other factors (growth phase or time of induction), a clear on-off switching effect of the inducer can be easily observed for most conditions tested (Figure 4 and Figure S3).

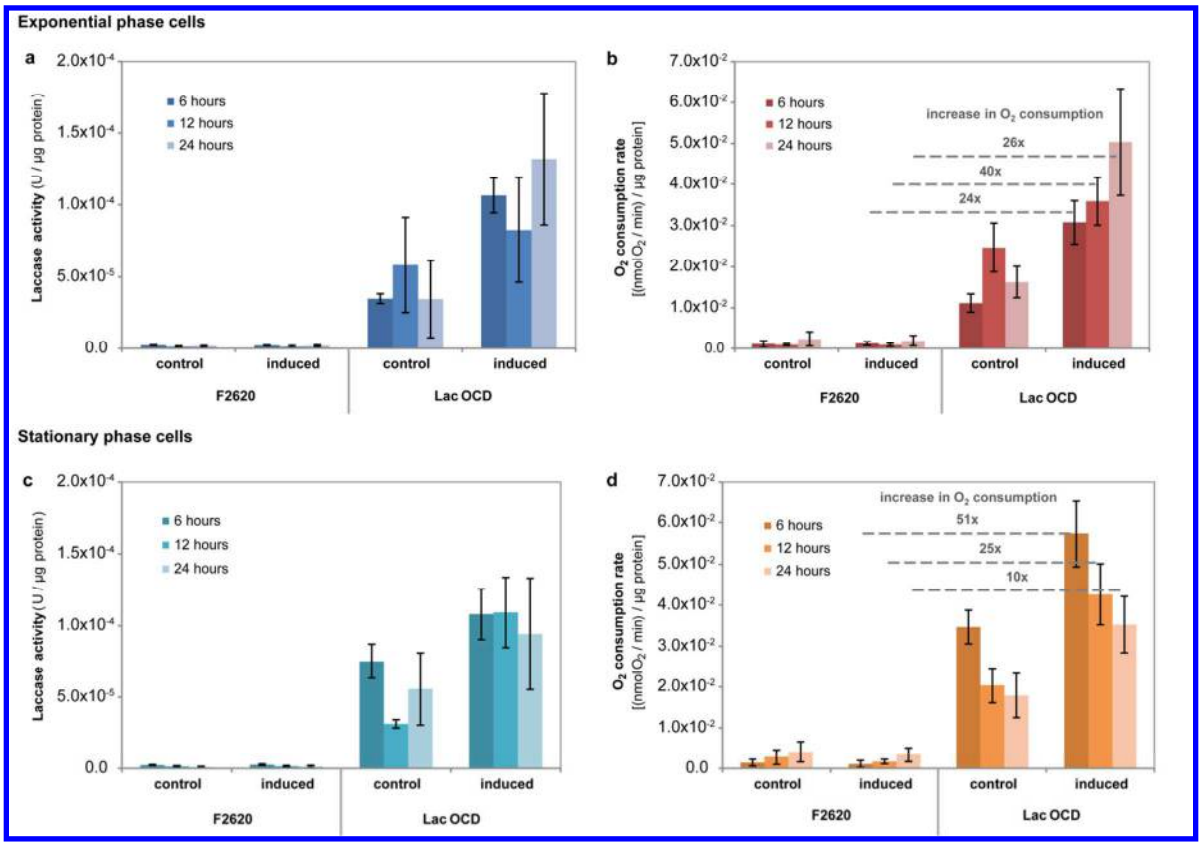

Figure 4. Characterization of the Lac OCD in *E. coli* DH5α cells grown in M9 medium supplemented with Cu^{2+}. Specific laccase activity (**a** and **c**) and O_2 consumption rate measurements (**b** and **d**) were performed using protein extracts obtained from cultures harboring the F2620 or the F2620 plus the Lac OCD (Lac OCD). Cultures were induced in exponential (top panel) or stationary phase (lower panel) and collected 6, 12 and 24 hours after induction. Cells were grown in the absence of inducer (control) or with 10 µM AHL (induced). The fold increase in O_2 consumption (induced Lac OCD *vs.* induced F2620) at 6, 12 or 24 hours after induction is shown in **b** and **d**. Results were normalized per µg of protein. Error bars represent the standard deviation of biological replicates (n = 3).

This *in vitro* characterization of the Lac OCD revealed that this is a robust device able to consume O_2 in all the conditions tested. Furthermore, O_2 consumption rates were found to increase 10- to 58-fold in cells harboring the Lac OCD compared to cells harboring the F2620 only (Figure 4 and Figure S3).

***In Vitro* Performance of the Laccase OCD in Other Genetic Backgrounds.** The *in vitro* characterization of the Lac OCD was also carried out in other *E. coli* strains to evaluate the performance of the device in different genetic backgrounds. For this purpose, protein extracts obtained from cells grown in M9 Cu^{2+}, induced in exponential phase and harvested 6 hours after induction were used. For *E. coli* DH5α, BL21, SURE, and Top10, the addition of AHL led to the consistent activation of the OCD; while for the MG1655 strain, the device behavior was erratic, with most protein extracts showing no laccase activity. This is in agreement with our previous experiments, and yet unexpected since the F2620 BioBrick was reported to be functional in *E. coli* MG1655 using AHL as inducer.[33] The functionality of the F2620 BioBrick was assessed, replacing the *cueO** ORF in the OCD by the *gfp* ORF (BBa_E0040). This device was introduced into MG1655 and, for all the biological replicates tested, the expression of Gfp was detected after the addition of the AHL inducer (Figure S4), confirming the functionality of the regulatory element in this *E. coli* strain. In addition, the OCD was reassembled with the P_{trc1O} promoter[34], reintroduced and tested in MG1655, showing consistent laccase activity (Figure S5). However, we were not able to obtain consistent laccase activity when *cueO** is under the regulation of the F2620. This may be due to the device secondary structure that may interfere with transcription or, since CueO is a native protein from *E. coli*, the high expression levels achieved with the F2620 Biobrick in MG1655 may trigger a regulatory mechanism (e.g. degradation). Therefore, the behavior of the Lac OCD regulated by the F2620 BioBrick was compared only in the other four strains (Figure 5).

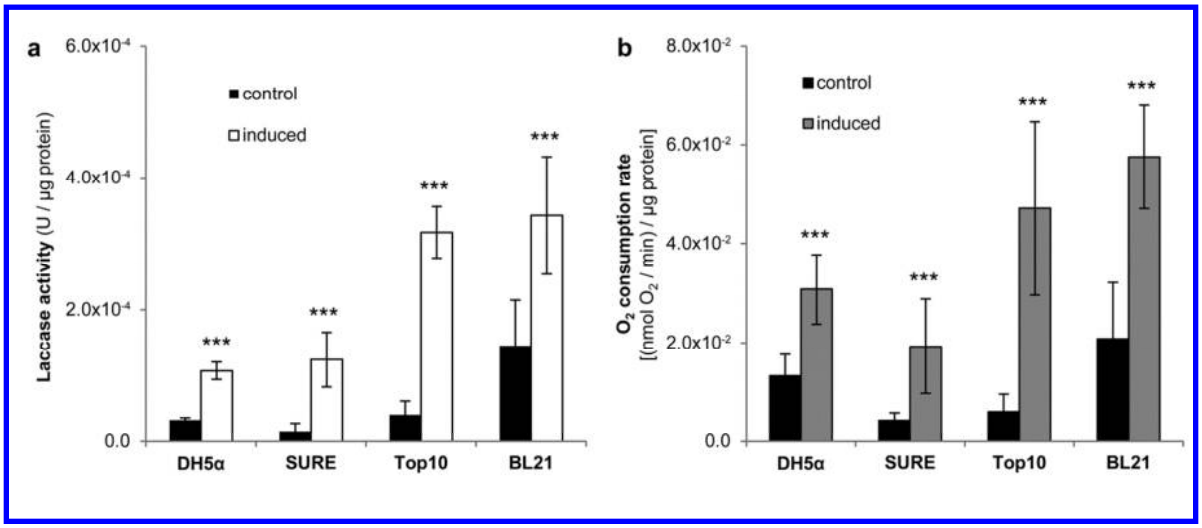

Figure 5. Characterization of the Lac OCD in different *E. coli* strains. (**a**) Specific laccase activity measurements. (**b**) O_2 consumption rate measurements using a Clark-type O_2 electrode. Protein extracts were obtained from cells harboring the F2620 plus the Lac OCD (Lac OCD) and grown in M9 minimal medium supplemented with Cu^{2+}. Cultures were induced in exponential phase and harvested 6 hours after induction with 10 µM AHL. Results were normalized per µg of protein. Error bars represent the standard deviation of biological replicates (n = 3), *** represents p-value <0.001.

Basal levels of O_2 consumption were detected for all strains even in the absence of inducer. The highest leakage was registered for the BL21 strain. This behavior was previously reported in the characterization of genetic logic gates[38]. The absence of the Lon protease, in the BL21 strain, results in reduced proteolysis and protein accumulation that may lead to crosstalk activation of the P_{lux} promoter. Nevertheless, the on-off switching characteristics of the Lac OCD were maintained and, a significant increase in laccase activity and O_2 consumption was observed for all strains. The highest fold increase (induced/control) was observed for Top10 and SURE, with 7-fold increase in laccase activity and 8- and 4-fold increase in O_2 consumption, respectively. Since abiotic factors (such as medium and temperature) and also the component embedded sequence were

maintained, differences registered in the device performance are attributed to the strain specific genetic context. The DH5α, SURE, and Top10 strains display mutations that were introduced for molecular biology purposes and resulted in similar phenotypes. However, *E. coli* Top10 does not harbor the *glnV44* and *relA1* mutations, present in the SURE and DH5α strains, that enable RNA synthesis in absence of translation. Interestingly, this characterization demonstrates that the device performance is dependent on the genetic background, even in closely related strains.

***In vitro* Performance of OCDs Based on Laccase-Variants.** Additional OCDs, based on three laccase variants with improved activity and obtained through directed evolution C7 (Leu170→Ser, Gly363→Glu, Glu476→Lys), E11 (Asp356→Glu, Asp380→Tyr), and H7 (Met385→Ile, Gly436→Ser) (provided by Prof. Dan Tawfik, Weizmann Institute of Science, Israel), were assembled maintaining the context embedded sequence of the Lac OCD, using the F2620 BioBrick as regulatory element and the RBS BBa_B0034. These OCDs were characterized *in vitro* using protein extracts obtained from cells collected 6 hours after the addition of the inducer AHL (Figure 6). Moreover, to gain insights on the location of the mutations in a 3D context, the protein structure of the variants was predicted by homology modelling with the CueO structure, using the online tool suite Phyre2.[40] As previously mentioned, the CueO protein displays the MCOs typical T1 and T2/T3 copper centers, associated with substrate oxidation and oxygen reduction. This protein also has the unusual methionine-rich sequence with a helix that blocks the access of organic substrates to the T1 copper center and three additional copper binding sites essential for cuprous oxidase activity.[30,39,41]

The characterization showed that the E11 variant does not have a significant improvement in performance (Figure 6). The two residues mutated in this variant,

Asp356→Glu, Asp380→Tyr, are part of CueO's methionine- histidine-rich helical domain (Pro357-His406). The analysis of the mutational sensitivity, using the SuSPect tool,[42] revealed that mutations on residues 356 and 380 are unlikely to lead to alterations in protein performance as it was observed the *in vitro* assays performed in this work. The 3 mutations in the C7 variant (Leu170→Ser, Gly363→Glu, Glu476→Lys) resulted in a 2-fold increase in laccase activity but no significant improvement in terms of O_2 consumption (Figure 6). The SuSPect analysis revealed that mutations of the 170 and 476 residues are likely to have a phenotypic effect and, from the protein structure it is possible to discern that these two residues are in the vicinity of the CueO domain interfaces, where the T1 Cu and the T2/T3 trinuclear Cu centers are located. The third mutation in the 363 residue, located in the helical domain that covers the T1 Cu site, similarly to the mutations detected in the E11 variant, has a low mutational sensitivity. Notably, the H7 variant exhibited a significant increase in performance with a 13-fold increase in laccase activity and 5-fold increase in O_2 consumption (Figure 6). Two mutations were detected in this protein, the 385 residue that is one of the methionines located in the methionine-histidine-rich helical domain, and the 436 residue that it is in the vicinity of the Cu5, the T1 Cu ligands, and the hydrogen bond that connects them.[26] Therefore, the increased performance displayed by this variant implies that the mutations are interfering with electron transfer route and/or with substrate access to the oxidation sites.

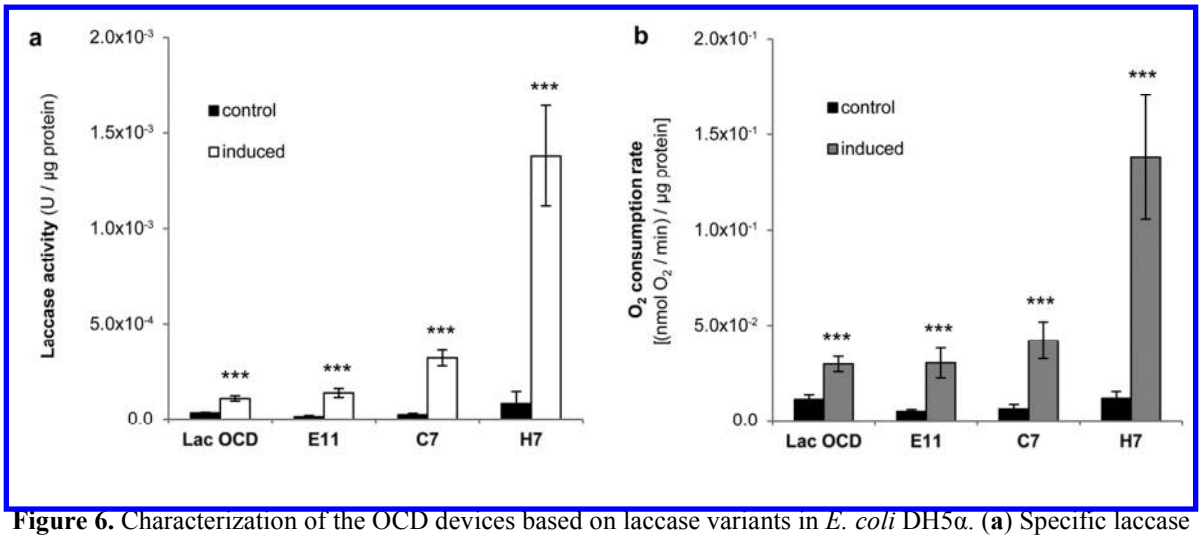

Figure 6. Characterization of the OCD devices based on laccase variants in *E. coli* DH5α. (**a**) Specific laccase activity measurements. (**b**) O_2 consumption rate measurements using a Clark-type O_2 electrode. Protein extracts were obtained from cells harboring F2620 plus the Lac OCD (Lac OCD) and grown in M9 minimal medium supplemented with Cu^{2+}. Cultures were induced with 10 µM AHL in exponential phase, and harvested 6 hours after induction. Results were normalized per µg of protein. Error bars represent the standard deviation of biological replicates (n = 3), *** represents p-value <0.001.

***In Vivo* Performance of OCDs.** To demonstrate the functionality of the OCDs *in vivo*, we started by performing a substrate screening since the substrate used for the *in vitro* assays – ABTS – can only be oxidized in acidic conditions. Different substrates known to be oxidized by laccase-like MCOs in neutral pH conditions were tested: syringaldazine, 2,6-dimethoxyphenol (DMP), L-dihydroxyphenylalanine (L-DOPA), and tyrosine. *In vitro*, oxygen consumption was detected using syringaldazine, DMP, and L-DOPA. However, *in vivo* O_2 consumption was observed only in presence of L-DOPA, probably because syringaldazine and DMP do not enter the cells. Therefore, L-DOPA was used as substrate to test the performance of the Lac and the H7 OCDs *in vivo*, using *E. coli* DH5α and Top10 cells (Figure 7). A significant increase in O_2 consumption associated to L-DOPA oxidation was detected in cells harboring the Lac OCD compared to the control (F2620), with a 100-

fold increase registered for DH5α and 300-fold for Top10. However, this characterization revealed that the H7 variant does not show improved performance *in vivo* with L-DOPA (Figure 7). A similar result was obtained *in vitro* using the same substrate (Figure S6). These results are not surprising, since the improvement of the laccase by directed evolution was performed using ABTS as substrate. If indeed the H7 variant mutations facilitate substrate accessibility to the Type 1 Cu when higher molecular weight molecules (such as ABTS) are used, they have no effect when smaller molecules (like L-DOPA) are provided. The on-off switching characteristics of the device were not verified *in vivo* (Figure 7). This issue can be overcome by changing the regulatory element or abiotic factors such as temperature. Previous reports demonstrated that P_{lux} promoter is leakier at 30 ºC than at 37 ºC, suggesting that temperature can have an impact in the binding affinity between the operator sequence and the LuxR protein.[38]

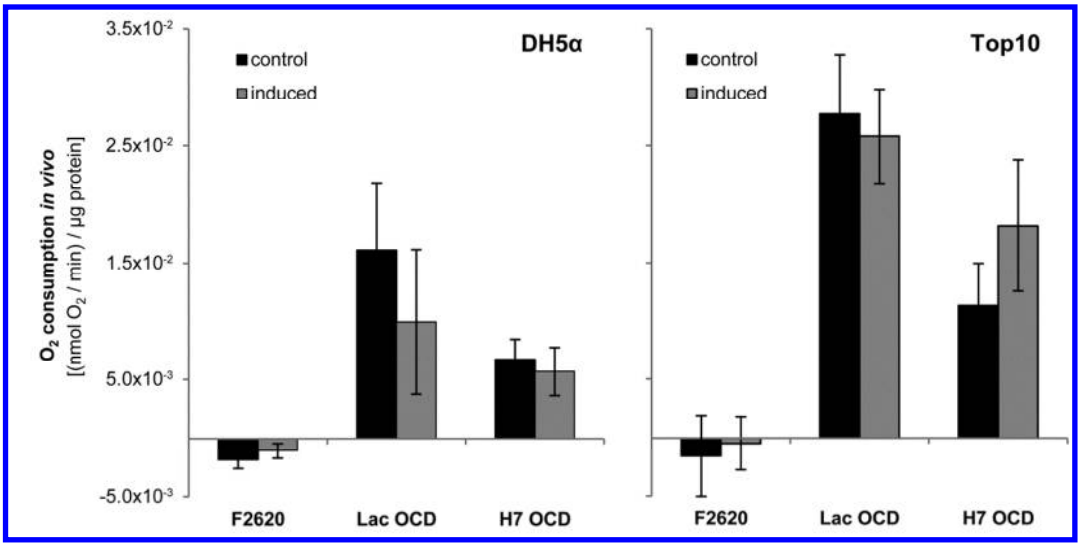

Figure 7. *In vivo* characterization of the Lac and H7 OCDs in *E. coli* DH5α and Top10. O_2 consumption rates were measured using DH5α (left) or Top10 (right) cell suspensions grown in M9 medium supplemented with Cu^{2+} in control or induced conditions (presence or absence of AHL, respectively), and using L-DOPA as

substrate. Results were normalized per μg of protein. Error bars represent the standard deviation of biological replicates (n = 3).

For further validation of the OCD functionality *in vivo*, *E. coli* Top10 cell suspensions were incubated in presence or absence of L-DOPA using sealed flasks and the dissolved O_2 concentration was measured at different time-points (Figure S7). In cell suspensions incubated in the presence of L-DOPA the dissolved O_2 is significantly reduced, representing 20-30% of the dissolved O_2 in absence of the substrate (O_2-saturated conditions). After 90 minutes of incubation, a similar concentration of dissolved oxygen in presence of L-DOPA or glucose (O_2 consumed by respiration) was observed. These results demonstrate that O_2 consumption by the synthetic OCD device is effectively able to modulate the dissolved oxygen concentration in cell suspensions.

Conclusions. In this work, we were able to successfully demonstrate that OCDs based on *E. coli*'s native laccase CueO can be used to modulate intracellular O_2 concentration. The assessment of devices based on CueO's protein variants confirms the advantages of using a bacterial laccase, namely the possibility to improve its activity through protein engineering. In addition, and since laccases are broad range catalysts, the choice of substrate can be adapted to the chassis (genetic background) and the specific application envisaged. Furthermore, the OCDs robustness was attested by the hosts O_2 consumption registered in all conditions tested. Interestingly, the devices were consistently functional in four of the five *E. coli* strains investigated but the performances were different, even in closely related strains. This work represents the proof of concept/stepping stone for the use of OCDs to elucidate or improve processes in micro-oxic/anoxic conditions and can be extended to

other bacterial chassis and specific applications. For example, coupling the OCD to a hydrogen producing device and an oxygen sensor, the latter already developed for the model cyanobacterium *Synechocystis* sp. PCC 6803,[43] could establish a circuit for an efficient hydrogen production by this photoautotrophic chassis. The use of OCDs can also be applied to improve the production of alcohols (butanol, ethanol or propanol), glutamate, itaconate or 3-Hydroxybutyric acid by heterotrophic chassis, namely *E. coli*. The use of scaffolds may also be considered in applications that involve enzymes requiring a tight control of oxygen concentration.

MATERIALS AND METHODS

Chemicals. The M9 medium components, casamino acids and thiamine hydrochloride were obtained from BD Biosciences (SanJose, CA, USA) and VWR (Radnor, PA, USA), respectively. *N*-(β-ketocaproyl)-L-Homoserine lactone (3OC6HSL, AHL) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and all other reagents from Sigma Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany). All DNA-modifying enzymes and polymerases were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Strains and Media. For cloning purposes, *Escherichia coli* strain DH5α (Agilent, Santa Clara, CA, USA) was used and the transformants were cultivated at 37 ºC in lysogeny broth (LB)[44], supplemented with 100 µg/mL ampicillin.

The *E. coli* strains BL21(DE3) (Agilent), DH5α, MG1655 (*E. coli* genetic stock center, New Haven, CT, USA), SURE and Top10 (Invitrogen, Waltham, MA, USA) were used for the characterization of the laccase-based OCDs. Cell cultures were grown in M9 minimal

medium supplemented with 1 mM thiamine hydrochloride, 0.4% (vol/vol) glycerol, 0.2% (wt/vol) casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ (M9),[44] or in M9 supplemented with 20 μM $CuSO_4$ (M9 Cu^{2+}). Single colonies were inoculated into 5 mL M9 ampicillin (100 μg/mL) and grown overnight at 37 ºC with rotary shaking (120 rpm). Cultures were renewed and after overnight growth were used as inocula for the experiments. The cell cultures were grown in Erlenmeyer flasks maintaining a medium to headspace proportion of 1:4, the inocula were diluted to an initial OD_{600} of 0.05 and grown to the desired optical density.

Selection of Protein Targets, Device Design and DNA Synthesis. The DESHARKY tool was used for the identification of pathways involving O_2 consumption in *Escherichia coli* and *Synechocystis* sp. PCC 6803.[31] The devices were designed based on the bioinformatics search outputs, in the BioBrick (BB) format (with Bb prefix and suffix), including the ribosome binding site (RBS) BBa_B0034 and the double terminator BBa_B0015 (http://parts.igem.org).[33] The Open Reading Frame (ORF) sequence from *E. coli* laccase (*cueO*) was codon optimized for *E. coli* K-12, *Synechocystis* sp. PCC 6803, and *Nostoc* sp. PCC 7120 using the software Gene Designer 2.0 (DNA 2.0, Menlo Park, CA, USA) to obtain a single sequence that could be potentially expressed in the three organisms. The DNA sequence comprising the BioBrick prefix, RBS, ORF, double terminator, and BioBrick suffix was synthesized by Epoch Life Science (Sugar Land, TX, USA).

OCDs Assembly. For the generation of the Lac OCD, the composite Biobrick BBa_F2620 (P_{tetR} – *luxR* – P_{luxR}) was assembled with the previously synthesized DNA sequence using the standard assembly protocol.[33] Briefly, the vectors containing the codon optimized *cueO* ORF and the BBa_F2620 were digested with *Xba*I/*Pst*I and *Spe*I/*Pst*I, respectively. The DNA fragments were isolated from agarose gel or purified directly using the NZYGelpure

kit (NZYTech, Lisbon, Portugal), according to manufacturer's instructions. Subsequently, the purified DNA fragments were ligated using the T4 DNA ligase. The Lac OCD construct was confirmed by restriction analysis and DNA sequencing (STAB VIDA, Lisbon, Portugal) using primers VF2 and VR (Table S1).[45]

To obtain laccase protein variants a direct evolution approach was used (work carried out at Professor Dan Tawfik's laboratory, Department of Biomolecular Sciences, Weizmann Institute of Science, Israel). For this purpose, a library of proteins was generated using the Megawhop method[46] and subsequently screened using the phenoloxidase activity assay and ABTS as substrate (see In Vitro *Assays* below). For the assembly of the OCDs based on these laccase variants, the ORFs of the protein variants C7, E11, and H7 were amplified from the pGem®-T Easy (Promega, Madison, WI, USA), using the primers Bblac_F2 and Bblac_R2 that include the BioBrick™ prefix and suffix, respectively (Table S1). Each PCR mixture (20 µL) contained: 0.5 U of GoTaq Flexi DNA Polymerase (Promega), 1x GoTaq Flexi buffer, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 1 µM of each primer, and 5 ng of template DNA. The PCR reaction profile was: 2 min at 95 ºC followed by 25 cycles of 30 s at 95 ºC, 30 s at 50 ºC and 3 min at 72 ºC, and a final extension at 72 ºC for 7 min. The amplicons were purified, digested with *Xba*I/*Pst*I and stepwise assembled with the BioBrick RBS part BBa_B0034 and then with the BBa_F2620 BioBrick, both digested with *Spe*I/*Pst*I. The sequence of the three OCDs based on laccase protein variants were confirmed by DNA sequencing (STAB VIDA) using primers: VF2, VR, Ec_cueO_F and Ec_cueO_R (Table S1).

Effect of the Lac OCD Induction on *E. coli* Growth. Cell cultures were grown until the early exponential phase (OD_{600} = 0.2-0.3) and, at this point, split into "control" and

"induced". The latter received AHL ($3OC_6HSL$) to a final concentration of 10 μM. The growth was monitored by measuring the OD_{600} every 2 hours for a 24 hours period.

Sample Collection and Cell Extracts Preparation. For the collection of samples, cell cultures were grown in M9 or M9 Cu^{2+} media until the early exponential (OD_{600} = 0.2-0.3) or stationary ($OD_{600} \approx 2$) phase. At these stages, the cultures were divided in two: one was induced by the addition of AHL (final concentration 10 μM) while the other served as control. Samples were collected at different time-points after induction (6, 12 or 24 hours) by centrifuging 20 mL of culture for 8 min at 4 500*g*, 4 ºC. The cell pellets were washed once using cold 50 mM phosphate buffer, pH 7.0 (KPi) and stored at -80 ºC until further use.

For the preparation of cell extracts, the pellets were resuspended in 250 μL KPi containing protease inhibitors (Complete™ Mini EDTA-free Protease Inhibitor, Roche, Basel, Switzerland), that were added according to manufacturer's instructions. Cells were then disrupted by sonication on ice with a Branson Sonifier 250 using 2 cycles of 15 s (50% dutty cycle, output 3) intercalated with 1 min off duty and then centrifuged for 8 min at 16 000*g*, 4 ºC. The protein concentration of the extracts was measured using the BCA Protein Assay Kit (Thermo Scientific) according to the instructions.

Confirmation of Protein Expression. Protein samples were separated by electrophoresis on SDS (sodium dodecyl sulfate)-polyacrylamide gels: in brief, electrophoresis was performed on a vertical mighty tall system (Hoefer, Holliston, MA, USA) according to the method of Laemmli.[47] A 10% gel was prepared and samples, that were mixed with Laemmli sample buffer and heated at 95 ºC for 5 min, were loaded on the gel. Protein separation was carried out at 12 mA. After protein separation, proteins were visualized using Coomassie Brilliant Blue G250 (BIO-RAD, Hercules, CA, USA).

OCDs Characterization.

In Vitro *Assays.* The phenol oxidase activity of the CueO multicopper oxidase (MCO) was measured using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) as electron donor.[48] Prior to the assay, the protein extracts were incubated with 1 mM $CuSO_4$ for 1 min and the appropriate volume of sample was added to the assay mixture in order to obtain linear oxidation kinetics. The assay mixture (500 µL) contained 50 mM sodium acetate buffer pH 5.0, protein extract (1-20 µL) and the reaction was started by the addition of ABTS to a final concentration of 3 mM. Substrate oxidation was monitored measuring the increase in A_{420} for 1 min at 30 ºC (ε_{420} = 36 000 L/mol·cm). The specific laccase activity was expressed as units of activity per µg of protein, where one activity unit represents one µmol of ABTS oxidized per min.

The oxygen consumption rates were determined polarographically using a Clark-type O_2-electrode (Hansatech Instruments, Norfolk, UK). The assay previously described for phenol oxidase activity was performed using 1 mL working volume and a final concentration of 30 mM ABTS; oxygen uptake was expressed as nmol O_2 consumed per min and normalized per µg of protein.

For the screening of additional CueO substrates, the oxidation activities of syringaldazine, 2,6-dimethoxyphenol (DMP); L-dihydroxyphenylalanine (L-DOPA) and tyrosine were spectrophotometrically determined as described previously.[49-51] The oxygen consumption measurements were performed using the same setup described for ABTS and 10 mM syringaldazine, 20 mM L-DOPA and 20 mM DMP were used in the assays.

In Vivo *Assays.* Cultures grown in absence or presence of AHL (inducer) and 12-24 h after induction were used. Cells were centrifuged for 8 min at 4 500*g*, 4 ºC and washed twice using cold 40 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer pH 7.0. Finally,

cells were resuspended in MOPS buffer to a final $OD_{600} \approx 10$ and kept on ice until 30 min before the assays, when cells were transferred to a water bath at 30 ºC. The assays were performed in 1 mL working volume containing 50 mM sodium phosphate buffer pH 6.0, 20 mM L-DOPA and cells to a final $OD_{600} = 1.0$, 2.0 or 4.0. For the control of cell fitness an assay was performed using 20 mM glucose as substrate. The oxygen consumption rates were determined polarographically as described above, and expressed as nmol O_2 consumed per min and normalized per μg of protein.

Online Tools and Statistical Analyses. To determine the likely success of sequence expression, the codon adaptation index (CAI) of the original and codon-optimized *cueO* sequences was calculated using the online tool CAICal.[32] The *E. coli* K-12, *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120 codon usage tables used in the CAI calculations were retrieved from the Codon Usage Database (www.kazusa.or.jp/codon/).

The relative codon adaptiveness values for the original and the codon optimized *cueO* sequences were obtained by the "each triplet position *vs*. usage table" method using the online graphical codon user analyzer and the *E. coli* K-12 codon usage table provided therein.[52]

The online tool suite Phyre2 was used to predict the structure of the laccase protein variants by homology modelling with the CueO structure.[40] The mutational sensitivity of protein residues was determined using the SuSPect tool[42] also available in the Phyre2 online tool suite.

Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student's *t*-test and the 0.05 probability level was chosen as the point of statistical significance throughout. For the analysis of the factors affecting the

OCDs performance *in vitro*, a multi-way ANOVA was performed using the IBM SPSS software (Armonk, NY, USA).

ASSOCIATED CONTENT

Supporting Information

Oligonucleotides used in this work (Table S1), comparison of the codon adaptiveness of the codon optimized and the original *cueO* ORFs (Figure S1), effect of the Lac OCD activation on *E. coli* BL21, MG1655, SURE and Top10 growth (Figure S2), characterization of the Lac OCD in *E. coli* DH5α cells grown in M9 medium (Figure S3), normalized GFP fluorescence of *E. coli* DH5α and MG1655 cultures harboring the F2620 BioBrick + *gfp* (Figure S4), characterization of the Lac OCD reassembled with the P_{trc1O} promoter in *E. coli* DH5α and MG1655 (Figure S5), *in vitro* characterization of the Lac OCD in *E. coli* DH5α cells using L-DOPA as substrate (Figure S6), dissolved O_2 measurements using *E. coli* Top10 suspensions incubated in sealed flasks (Figure S7) and Supporting methods, GFP fluorescence analysis and dissolved O_2 concentration measurements using sealed cultures.

Abbreviations

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt;

AHL - *N*-(β-ketocaproyl)-L-homoserine lactone;

CAI - codon adaptation index;

L-DOPA - L-dihydroxyphenylalanine;

MCO – Multicopper Oxidase;

OCDs – Oxygen Consuming Devices

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Authors' contributions

PT, AJ and CCP conceived and designed the study; AJ, JC, GR performed the analysis with Desharky; CCP, ZB and FP performed the experiments; PT, CCP, ZB and FP analyzed the data; PT, CCP and FP drafted the manuscript and all authors revised the manuscript.

Notes

The authors declare no competing financial interests.

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Modulation of intracellular O_2 concentration in *Escherichia coli* strains using Oxygen Consuming Devices (OCDs)

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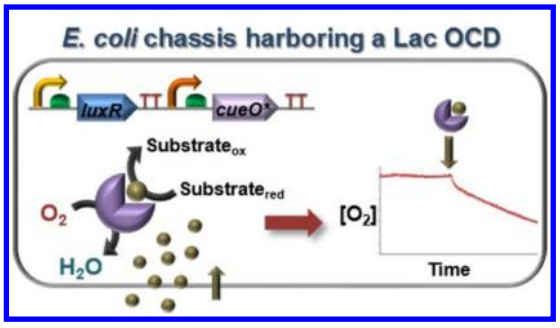

Supporting Information

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Supporting Materials and Methods

Table S1. Oligonucleotide used in this work.

Primer name	Sequence 5' → 3'	Reference
VF2	TGCCACCTGACGTCTAAGAA	1
VR	ATTACCGCCTTTGAGTGAGC	
Bblac_F2*	ACCATC**GAATTC**GCGGCCGCT**TCTAGA**GGATGCAACGTCGTG	This work
Bblac_R2*	TCTTT**ACTAGT**AGCGGCCG**CTGCAG**GGTTATACCGTAAACC	
Ec_cueO_F	ATCAACCTGCCGCTACCTGC	
Ec_cueO_R	TGTTGGCATGGTGGAAATCG	

*BioBrick prefix and suffix sequences are underlined and restriction sites highlighted in blue.

(1) Shetty, R. P., Endy, D., and Knight Jr., T. F. (2008) Engineering BioBrick vectors from BioBrick parts. *J Biol Eng 2*, 5.

Supporting Figures

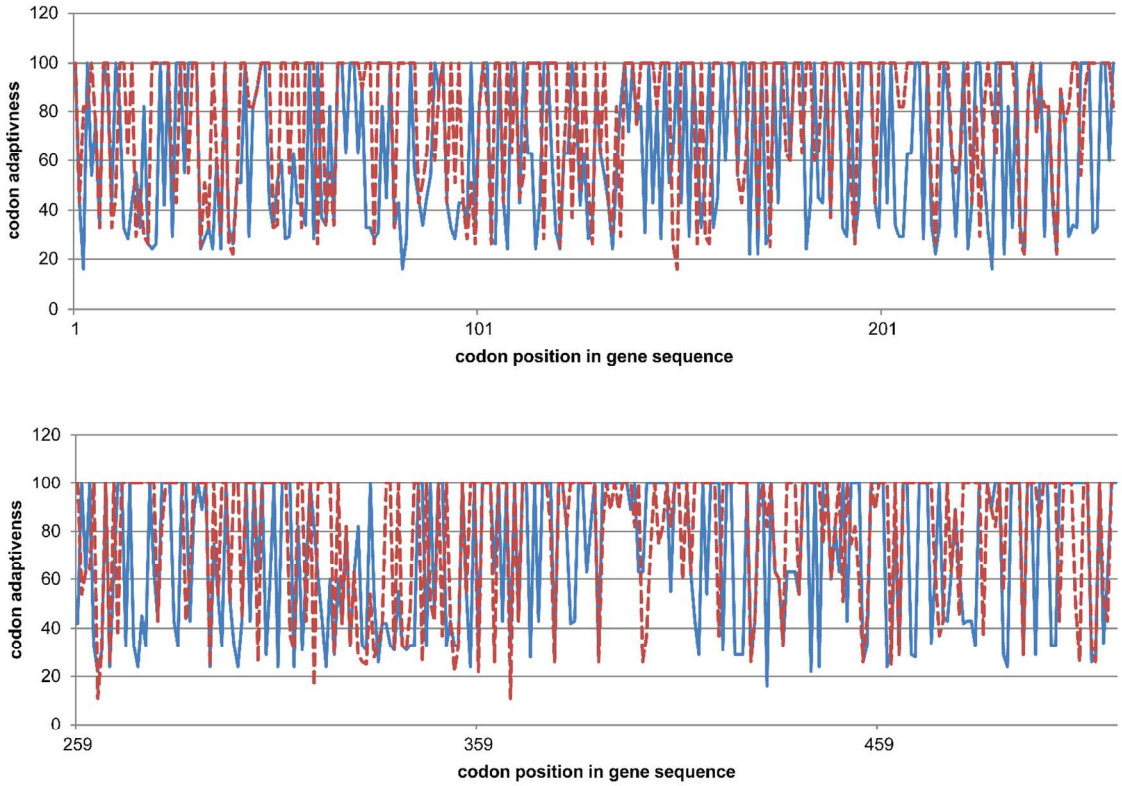

Figure S1. Comparison of the codon adaptiveness of the codon optimized (blue line) and the original (red dashed line) *cueO* ORFs. Codon adaptiveness along the codon position in gene sequence is plotted.

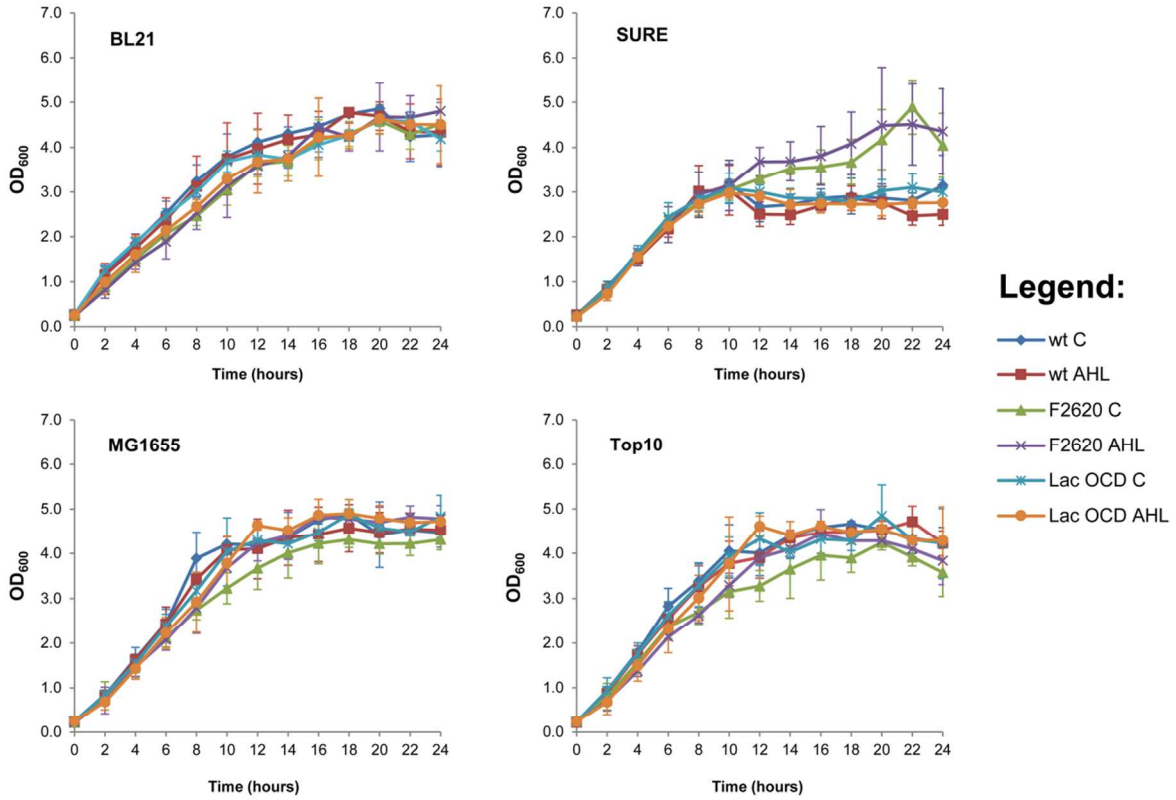

Figure S2. Effect of the Lac OCD activation on *E. coli* BL21, MG1655, SURE and Top10 growth. The wild-type (wt), the cells containing only the F2620 or F2620 plus the Lac OCD (Lac OCD) were grown in absence (C) or presence of the inducer (AHL). Growth was monitored measuring the OD_{600} every 2 hours for a 24 hours period. Error bars represent the standard deviation of biological replicates (n = 3).

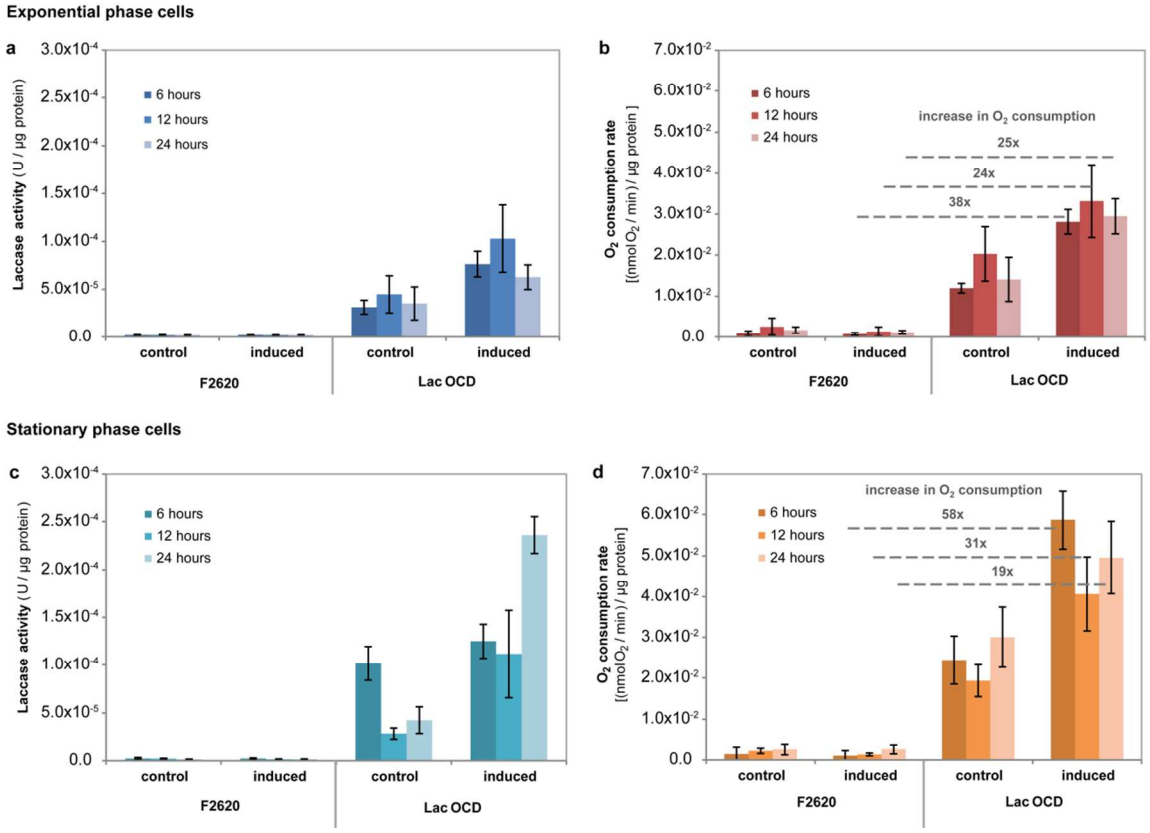

Figure S3. Characterization of the Lac OCD in *E. coli* DH5α cells grown in M9 medium. Specific laccase activity (**a** and **c**) and O_2 consumption rate measurements (**b** and **d**) were performed using protein extracts obtained from cultures harboring the F2620 or the F2620 plus the Lac OCD (Lac OCD). Cultures were induced in exponential (top panel) or stationary phase (lower panel) and collected 6, 12 and 24 hours after induction. Cells were grown in the absence of inducer (control) or with 10 µM AHL (induced). The fold increase in O_2 consumption (induced Lac OCD *vs.* induced F2620) at 6, 12 or 24 hours after induction is shown in **b** and **d**. Results were normalized per µg of protein. Error bars represent the standard deviation of biological replicates (n = 3).

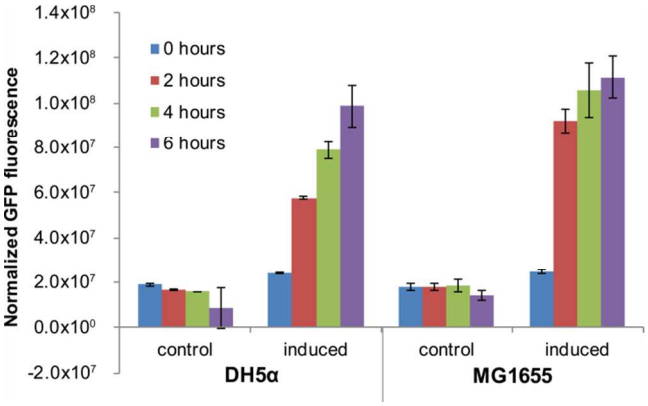

Figure S4. Normalized GFP fluorescence of *E. coli* DH5α and MG1655 cultures harboring the F2620 BioBrick + *gfp*. 96-well plates were setup using DH5α and MG1655 cultures (OD_{600} = 0.1) that were grown in the absence of inducer (control) or presence of 10 µM AHL (induced). Measurements were performed 0, 2, 4, and 6 hours after plate setup and the fluorescence was normalized to Abs_{620}. The results are representative of three biological replicates (with exception of MG1655 induced cultures, n = 10), with technical duplicates (measured in duplicate), error bars show ±S.D.

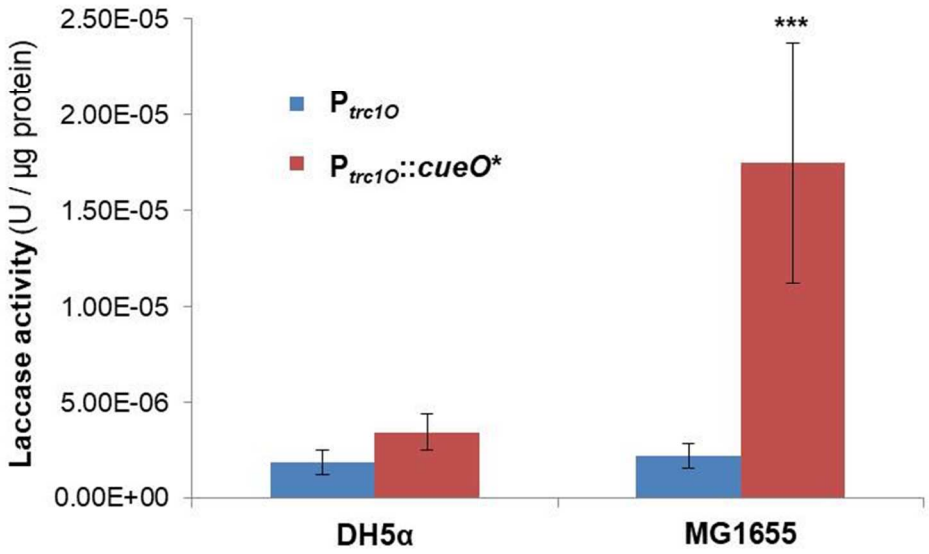

Figure S5. Characterization of the Lac OCD reassembled with the P_{trc1O} promoter in *E. coli* DH5α and MG1655. Specific laccase activity was measured using protein extracts obtained from cells harboring the P_{trc1O} or P_{trc1O} plus the Lac OCD (P_{trc1O}::*cueO**) and grown in M9 minimal medium supplemented with Cu^{2+}. Cultures were harvested 18 - 24 hours after inoculation. Results were normalized per µg of protein. Error bars represent the standard deviation of biological replicates (n = 4), *** represents p-value <0.001.

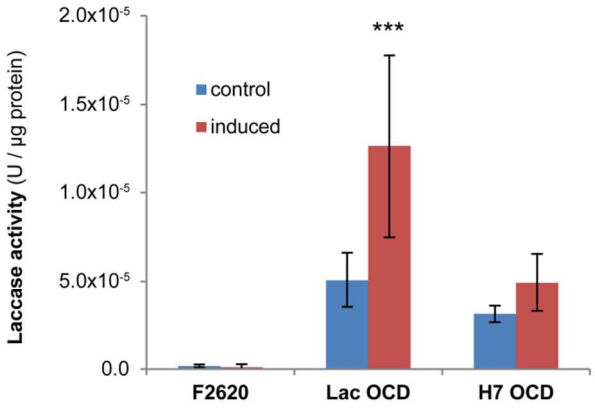

Figure S6. *In vitro* characterization of the Lac OCD in *E. coli* DH5α cells using L-DOPA as substrate. Specific laccase activity was measured using protein extracts obtained from the cultures used in the *in vivo* characterization. Cells harboring the F2620, the F2620 plus the Lac OCD (Lac OCD) or the F2620 plus the Lac H7 variant (H7 OCD) were grown in M9 medium supplemented with Cu^{2+} in control and induced conditions (presence or absence of AHL, respectively). Results were normalized per µg of protein. Error bars represent the standard deviation of biological replicates (n = 3), *** represents p-value <0.001.

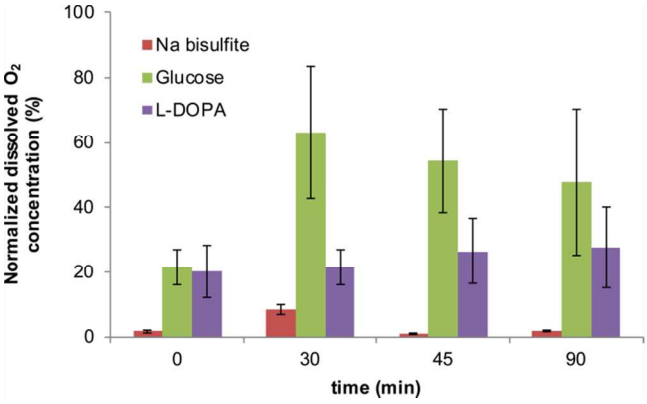

Figure S7. Dissolved O_2 measurements using *E. coli* Top10 suspensions incubated in sealed flasks. The cell suspensions were incubated with 30 mM L-DOPA (laccase substrate), 20 mM glucose (control for O_2 consumption) and 100 mM sodium bisulfite (Na bisulfite, control for O_2-deprived conditions) at 37 °C with shaking. One mL samples were removed at different time points (0, 30, 45 and 90 min) using sterile syringes and needles; and the dissolved O_2 concentration was measured using a Clark-type O_2 electrode. Results were normalized to the dissolved O_2 in saturated conditions (corresponding to 100% dissolved O_2). Error bars represent the standard deviation of biological replicates (n = 2).

Supporting Methods

GFP Fluorescence Analysis. For the evaluation of GFP expression, cultures of *E. coli* DH5α and MG1655 harboring the F2620 BioBrick or the F2620::*gfp* were grown overnight (final $OD_{600} \approx 3$) in 50 mL flasks at 37 °C with shaking (120 rpm). The cultures were diluted to a final $OD_{600} \approx 0.2$ and 100 µL aliquots were distributed in Nunc™ MicroWell™ 96-Well Optical-Bottom Plates (Thermo Fisher Scientific) containing 100 µL of M9 medium or M9 with 20 µM of AHL (to obtain a final concentration of 10 µM AHL in 200 µL). The 96-wells plates were incubated at 37 ºC with shaking (120 rpm), and measurements were carried out in duplicate 0, 2, 4 and 6 hours after plate setup. GFP fluorescence and Abs_{620} were detected using the Synergy 2 Multi-Mode Microplate Reader and the Gen5™ software (BioTek Instruments, Winooski, VT, USA). For fluorescence detection, an excitation filter of 485/20 nm and an emission filter of 528/20 nm were used (sensibility set for 110). The experiments included 3 biological replicates, with the exception of MG1655 harboring the F2620::*gfp* for which 10 biological replicates were analyzed and technical duplicates were included for all samples. For data analysis, the background fluorescence and absorbance of the M9 medium was subtracted from the values obtained for the samples and, the fluorescence values were normalized by optical density.

Dissolved O_2 Concentration Measurements Using Sealed Cultures. Cultures of *E. coli* Top10 harboring the OCD (grown overnight) were washed and resuspended in 50 mM phosphate buffer, pH 7.0 (KPi) to a final $OD_{600} \approx 1.0$. This cell suspension was distributed by different 4 Erlenmeyers (100 mL): the oxygen-saturated medium control (containing only cells), the oxygen-deprived medium control (cells + 100 mM sodium bisulfite) and the other two contained 20 mM L-DOPA (laccase substrate) or 20 mM glucose (used as O_2 consumption control). The Erlenmeyer's were sealed using Suba Seal rubber stoppers (Sigma), and were incubated at 37 °C with shaking. At different time points (0, 30, 45 and 90 min), 1 mL samples were removed through the stopper septa using a sterile syringes and needles and, the dissolved O_2 concentration was measured using a

Clark-type O_2-electrode (Hansatech Instruments). Results were normalized to the dissolved O_2 in saturated conditions (corresponding to 100% dissolved O_2).