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The determination of pharmaceuticals in wastewater using a recombinant *Arxula adeninivorans* whole cell biosensor

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ABSTRACT

An *Arxula adeninivorans* based microbial biosensor has been developed for the determination of pharmaceuticals and chemicals such as omeprazole, lansoprazole, β-naphtoflavone and methylcholanthrene within 5 h using biochemical detection and 4 h and 10 min using amperometric detection. The biosensor consists of genetically modified *A. adeninivorans* G1212/YRC102-hAhR-hARNT-phyK (*hAhR* – human arylhydrocarbon receptor; *hARNT* – human arylhydrocarbon receptor nuclear translocator; inducible *phyK* – derived from *Klebsiella* sp. ASR1) as the biological component and coupled with either a biochemical or an amperometric detection method. The combination between *hAh* receptor gene, *hARNT* and the *A. adeninivorans*-derived *glucoseamylase* promoter of the reporter gene (*GAA*) containing specific *cyp1A1*-derived core sequence created a construct which enabled specific induction by pharmaceuticals. This offers a new cell-based biosensor for the pharmaceutical determination. The half maximum effective concentration (EC_{50}) and the limit of detection (LoD) were found to be 236.13, 95.01 and 174.72, 83.65 μg/l for omeprazole and lansoprazole, respectively. These two pharmaceuticals are among the most widely used internationally. Additionally *A. adeninivorans* G1212/YRC102-hAhR-hARNT-phyK cells allow the measurement in raw wastewater, i.e. not concentrated, unpurified and untreated which will allow on-site operation in sewage treatment plants.

1. Introduction

Pharmaceuticals are a structurally diverse class of emerging contaminants. The continuously increasing pharmaceutical consumption worldwide is leading to increasing occurrence of pharmaceuticals in the environment. Pharmaceuticals are released into the environment in untreated wastewater. Additionally some pharmaceuticals are not easily removed during conventional wastewater treatment and are discharged in treated wastewater [1–5]. They may also be discharged to the environment via other routes, such as urban or agricultural runoff. Some pharmaceuticals also persist in drinking water supplies and ultimately contaminate consumer tap water [5–7]. It is known that the concentrations of pharmaceuticals in these environments range from ng/l to μg/l [8–12]. This raises concerns regarding possible adverse ecological effects in aquatic environment and also for human health. For example, pharmaceuticals such as estrogenic substances can cause abnormalities in the development and reproduction of living organisms or feminization in fish [13,14]. In addition, antibiotics can promote bacterial resistance in the environment due to continuous exposure [12,15–17].

Although there are a number of methods for the determination of pharmaceuticals, the low (ng/l) concentration of pharmaceuticals in surface water and wastewater requires more simple analytical methods for their determination. Most commonly used methods are gas chromatography–mass spectrometry (GC–MS)

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[18–20], liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS) [5,21,22], liquid chromatography with ultraviolet detection (LC-UV) [23] and high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (LC/MS/MS) [24–26]. The sensitivities of these analytical methods are general high, however, they are time-consuming, lab intensive, complicated and usually with complex sample separation and concentration steps.

Other widely used methods are based on biosensors which focus most on the determination of hormones (estrogenic, androgenic, gestagenic molecules) for example the YES-assay [27], E-Screen assay [28], A-YES assay [29], nAES assay [30], EstraMonitor apparatus [31,32], A-YAS [33] and recombinant cell bioassays [34]. There are several bacterial and yeast-based assays for pharmaceutical screening [35,36] which are used for detecting genotoxicity. There are also a few publications on cell-based biosensors for the determination of other pharmaceuticals in contaminated environments. Among cell-based biosensors, yeast genetic systems have emerged as powerful tools for measuring the activities of proteins, detecting hormonal activities and characterizing molecular interactions [37–39].

Here our aim is to demonstrate the determination of pharmaceuticals (especially omeprazole and lansoprazole) using an *Arxula adeninivorans* based microbial biosensor. This biosensor employs recombinant *A. adeninivorans* G1212/YRC102-hAhR-hARNT-phyK yeast cells as the microbial component combined with an amperometric or biochemical transducer. *A. adeninivorans* G1212/YRC102-hAhR-hARNT-phyK was engineered to co-express the human arylhydrocarbon receptor (*hAhR*), human arylhydrocarbon receptor nuclear translocator genes and the inducible phytase (*phyK*, derived from *Klebsiella* sp. ASR1) reporter gene under control of a promoter containing xenobiotic response elements. In the presence of pharmaceuticals such as omeprazole, lansoprazole, β-naphtoflavone, hAhR is expressed, activated, translocated into the nucleus and finally dimerized with ARNT. This activated complex binds to the xenobiotic response elements of the reporter gene promoter and initiates transcription of the *phyK* gene resulting in secretion recombinant phytase into the media. The level of phytase is quantified by amperometric detection using the substrate, ascorbic acid 2-phosphate (AA2P) or by biochemical detection using the substrate *p*-nitrophenyl phosphate disodium salt hexahydrate (pNPP). In the amperometric detection method, phytase dephosphorylates AA2P into an intermediate product ascorbic acid (AA), which is electro-active and can be oxidized at an electrode. This produces a current, which is proportional to the level of phytase activity. In the biochemical detection method, hydrolysis of *p*-NPP by phytase results in the formation of a coloured product under alkaline conditions that absorbs light at 405 nm. Since phytase activity is directly correlated to the pharmaceutical concentration, the total pharmaceutical activity can be calculated from the current or from the absorbance.

2. Materials and methods

2.1. Chemicals, electrodes, yeast strains and cultivation conditions

p-Nitrophenyl phosphate disodium salt hexahydrate (*p*NPP), ascorbic acid 2-phosphate (AA2P) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (Germany).

27 reference standard substances (listed in Table 3) which belong to different therapeutic classes (e.g. steroids, antibiotics, antisecretory compounds, etc.), used in this paper were purchased from Sigma–Aldrich, Roth, Aldrich (Germany). All solutions were prepared in 1% DMSO.

Cryovial tubes were purchased from Cryo TubeTM Vials, Denmark. Nunc MaxiSorp® flat-bottom 96 well plates were purchased from Thermo scientific, Germany.

Thick film electrodes (Pt–Ag/AgCl–Pt) were obtained from BST Bio Sensor Technology GmbH (Germany).

Escherichia coli TOP 10 [F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ80lacZΔM15ΔlacX74 recA1 araD139* Δ(*ara leu*) *7697 galU galK rspL* (*Strr*) *endA1nupG*], obtained from Invitrogen (Carlsbad/USA), served as the host strain for bacterial transformation and plasmid isolation. The strain was grown in LB medium supplemented with 50 μg/ml ampicillin or 30 mg/ml kanamycin (AppliChem, Darmstadt/Germany) when required for selection.

The auxotrophic tryptophan mutant, *A. adeninivorans* G1212 [*aleu2 atrp1::ALEU2*], was used as a transformation host for the integration of YRC102-hAhR-hARNT-phyK into the genome [40]. Yeast strains were grown at 30 °C in a complex medium (YEPD) or under selective conditions in a yeast minimal media supplemented with 2% (w/v) glucose, sorbitol or maltose (YMM-glucose, YMM-sorbitol, and YMM-maltose). Solid media plates were prepared by adding 1.6% (w/v) agar to the liquid YMM [41,42]. *hAh* receptor and *hARNT* sequences were adapted to the codon usage of *A. adeninivorans.* They were optimized and synthesized under demission of undesired restriction recognition sequences by Geneart AG, Heidelberg, Germany.

2.2. Construction of the resistance marker-free microbial component of the biosensor

All constructs are based on the Xplor®2 transformation/expression platform which has been successfully established as a common transformation system in *A. adeninivorans* [43]. The plasmid Xplor2 provides the d25S rDNA (Δ25S rDNA) segment which is disrupted by a multi-cloning site (MCS) for the insertion of the expression modules. The *Sal*I–*Afe*I flanked modules for selection (*ALEU2* promoter – *ATRP1m*, del*ALEU2* promoter – *ATRP1m*) can directly incorporated into this MCS [40].

The shuttle vectors containing *hAhR* and *hARNT* were digested by *Eco*RI and *Bam*HI respectively to release the *hAhR* and *hARNT* fragments. They were isolated and then cloned into the previously constructed vectors pBS-TEF-PHO5-SA and pBS-TEF-PHO5-SS, respectively [30,43]. These resulted in the expression modules (*TEF1* promoter – *hAhR* gene – *PHO5* terminator) flanked by *Sal*I-*Apa*I and (*TEF1* promoter – *hARNT* gene – *PHO5* terminator) flanked by *Spe*I-*Sac*II. Both modules were subsequently inserted stepwise into MCS of the Xplor2 vector.

The inducible reporter gene module (*GAA* promoter – *phyK* gene – *PHO5* terminator) variants were constructed by ligation of the amplification products (*phyK* gene including *PHO5* terminator (PhyK-BsaI-5′ and PHO5-SalI-3′ primers were used – Table 1) and the modified versions of *GAA* promoter (GAA-BsaI-5′ and GAA-SalI-3′ – Table 1) into the *Sal*I site of the Xplor2-hAhR-hARNT expression vector. *Sal*I and *Bsa*I restriction sites were inserted by PCR at the 5′ prime and 3′ end of *GAA* promoter fragment and *Bsa*I and *Sal*I restriction sites were inserted at the 5′ prime and 3′ end of the *phyK* coding region, including *PHO*5 terminator. PCR fragments were digested with *Sal*I and *Bsa*I and simultaneously ligated into *Sal*I site of Xplor2-hAhR-ARNT. A mixture of 20 plasmids, containing 8 variants was used to transform *A. adeninivorans* G1212.

2.3. Transformation procedures, recovery of stable recombinant A. adeninivorans

E. coli and *A. adeninivorans* cells were transformed according to Böer et al. [43]. After transformation, the stable recombinant *A. adeninivorans* G1212/YRC102-hAhR-hARNT-phyK yeast cells were