Amperometric detection of hormonal activity by a yeast cell biosensor

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Summary

The widespread use of natural and synthetic estrogens or chemicals with estrogenic activities is causing an increasing accumulation of estrogenic compounds in the environment. Already at very low concentrations these estrogenics can severely affect the wildlife, particularly in an aquatic environment. For these reasons measuring devices for detecting estrogen contaminations are in great demand. The majority of the analytical methods and bioassays on the market so far, lack *semi-online* adaptability, and usually cannot be used for automatic and continuous determination. Therefore, we have embarked on the development of new systems, which are able to fulfil those demands.

The EstraMonitor combines recombinant *A. adeninivorans* G1212/YRC102-hERα-phyK yeast cells as the microbial component with an amperometric detection method to analyze estrogenic contaminations. *A. adeninivorans* G1212/YRC102-hERα-phyK was constructed by Kaiser *et al.* (2010). These cells were engineered to co-express the human estrogen receptor (*hERα*) gene and the inducible phytase (*phyK*, derived from *Klebsiella sp*. ASR1) reporter gene under control of a promoter with estrogen response elements (EREs). In the presence of estrogenic substances, such as 17ß -estradiol (E2), the *phyK* gene is expressed and recombinant phytase is secreted into the media. The level of phytase is quantified by amperometric detection using substrate *p*-aminophenyl phosphate (*p*-APP). Phytase dephosphorylates *p*-aminophenyl phosphate (*p*-APP) into an intermediate product *p*-aminophenol (*p*-AP). *p*-AP is electroactive and oxidized at the electrode. This generates electrons and produces a current which is proportional to the level of phytase activity. Since phytase activity is directly correlated to the E2 concentration, the estrogenic activity can thus be calculated from the current measured.

The microbial component of the EstraMonitor, the non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK, works well with the amperometric method in a quantitative manner. The optimal applied potential determined for amperometric measurements was 150 mV and provided a low background signal for the amperometric detection. The half maximal effective concentration (EC_{50}) and limit of detection (LoD) values for E2 obtained from amperometric measurements with the EstraMonitor were 69.9 ng L^{-1} and 44.5 ng L^{-1}, respectively. These values are comparable to those of the widely used commercial Palmsens potentiostat (69.9 ng L^{-1} versus 78.1 ng L^{-1}, and 44.5 ng L^{-1} versus 43.9 ng L^{-1}).

The measuring procedure of the EstraMonitor system including incubation of *A. adeninivorans* G1212/YRC102-hERα-phyK cells with E2, subsequently incubation with electro-

chemical substrate (*p*-APP), and signal recordation is completed within only 4 h and 10 min. Out of this total time, amperometric detection including substrate incubation and signals recordation takes only 10 min out of total time.

The use of immobilized cells for a microbial biosensor is an essential advantage of the EstraMonitor system because it allows easy-handiness next to long-term stability and reusability. Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells revealed excellent properties which make them very suitable for *semi-online,* automatic and continuous monitoring. They were stable up to 30 days when stored at 4 °C. Furthermore, they could be reused up to 15 times. The EC_{50} and LoD values achieved for E2 using immobilized cells in combination with amperometric detection were 20.9 and 8.3 ng L^{-1}, respectively. Furthermore, this application also removes the need to separate cells by centrifugation, to sterilize the samples as well as to cultivate repeatly.

Additionally, both immobilized and non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells remain fully functional in a wide range of untreated wastewater samples and in environments containing up to 5% NaCl.

To enhance the sensitivity and reduce the time for estrogenic determination, an alternative *A. adeninivorans* G1214/YRC103-hERα-phyK strain was developed. This strain can produce a detectable amount of phytase within 2 h after induction with E2. It offers an improved microbial component in terms of sensitivity and time-effectiveness. In addition, to reduce the cost for estrogenic detection an alternative substrate, ascorbic acid 2-phosphate (AA2P), was tested. AA2P, which is both cheap and widely available, performed better than *p*-APP. The EC_{50} and LoD values for E2 obtained with AA2P were 15.69 and 0.92 ng L^{-1} versus 20.09 and 8.3 ng L^{-1} when examined with *p*-APP, respectively.

Taken together, the EstraMonitor is an automated system with respect to sample cycling, sample measuring and calibration supplemented with an alarm function. This system makes it possible to control estrogenic activity *semi-online*, automatically and continuously. These are advantages of the EstraMonitor compared to other estrogenic detection systems. It can thus be concluded that, the EstraMonitor is a powerful and feasible *semi-online* device for monitoring estrogenic activity especially adapted for the use in sewage treatment plants.

Zusammenfassung

Durch den ständig steigenden Einsatz von natürlichen und synthetischen Östrogenen bzw. Chemikalien mit östrogener Wirkung, kommt es heutzutage vermehrt zu Umweltbelastungen insbesondere für aquatisch lebende Organismen. Um auf derartige steigende Umweltbelastungen Einfluss nehmen zu können, war die Entwicklung eines geeigneten Nachweisverfahrens notwendig, welches die Nachteile bereits existierender Analyseverfahren und Bioassays zum Nachweis östrogener Verbindungen, beispielsweise bei der Automatisierung bzw. der kontinuierlichen Messung, verbessert.

Ziel dieser Arbeit war die Etablierung eines für die *semi-online* Messungen ausgelegten Messverfahrens zur Bestimmung von östrogen-wirksamen Substanzen. Dieses neue Messsystem, EstraMonitor, ist eine Kombination aus transgenen *A. adeninivorans* Hefezellen (G1212/YRC102-hERα-phyK) als mikrobieller Komponente und einer amperometrischen Messeinheit für östrogene Aktivität. Die Hefezellen (Kaiser *et al.,* 2010) co-exprimieren den humanen Östrogen-Rezeptor (*hERα*), sowie eine rekombinante Phytase (*phyK*, aus *Klebsiella sp.* ASR1) als Reporter, deren Gen unter Kontrolle eines induzierbaren Promotors mit Östrogen-Response-Elementen (ERE) steht. Damit sezernieren die transgenen Hefezellen in Gegenwart von östrogen-wirksamen Substanzen rekombinante Phytase in das Medium, wo sie als Substrat zugegebenes *p*-Aminophenylphosphat (*p*-APP) dephosphoryliert. Die Oxidation von *p*-Aminophenol (*p*-AP) wiederum setzt proportional zur Phytaseaktivität Elektronen frei, die sich amperometrisch messen lassen. Auf diese Weise kann über die Phytaseaktivität die Konzentration an östrogen-wirksamen Substanzen im Probenmaterial berechnet werden.

Für amperometrische Messungen mit nicht-immobilisierten *A. adeninivorans* G1212/YRC102-hERα-phyK Zellen wurde 150 mV als optimales Potential ermittelt. Die damit gemessenen EC_{50} und LoD Werte lagen für 17-Estradiol (E2) bei 69,9 ng L^{-1} bzw. 44,5 ng L^{-1}. Sie ließen sich mit einem kommerziellen Poteniostat (Palmsens) bestätigen (78,1 ng L^{-1} bzw. 43,9 ng L^{-1}). Das gesamte Prozedere des EstraMonitor Verfahrens kann innerhalb von nur ca. 4 h abgeschlossen werden.

Um die Handhabbarkeit, Langzeitstabilität und Wiederverwendbarkeit der mikrobiellen Sensorkomponente zu verbessern, wurden immobilisierte Hefezellen eingesetzt. Damit entfielen Arbeitsschritte wie Sterilisation, Kultivierung und Zentrifugation, was für eine *semi-online*, automatische und kontinuierliche Überwachung von z.B. Kläranlagen essentiell ist. Im Rahmen dieser Arbeit wurde ermittelt, dass immobilisierte *A. adeninivorans*

G1212/YRC102-hERα-phyK Zellen bei 4 °C bis zu 30 Tage stabil sind und bis zu 15 mal wieder verwendet werden können. Die amperometrisch detektierten EC_{50} und LoD Werte für E2 lagen bei 20,9 und 8,3 ng L^{-1}.

Erste Realprobenmessungen mit E2 belasteten, nicht-vorbehandelten Abwasserproben belegten, dass die *A. adeninivorans* G1212/YRC102-hERα-phyK Zellen aufgrund ihrer relativ hohen Salztoleranz bis zu einer Konzentration von 5% NaCl einsetzbar sind. Damit lässt sich der EstraMonitor zum Nachweis östrogener Aktivität im Abwasser ohne Vorbehandlung, Extraktion, Konzentration und Sterilisation der Proben einsetzen.

Um die Sensitivität der Nachweismethode, sowie vor allem deren Analysezeit weiter zu verkürzen, wurde im Rahmen dieser Arbeit mit *A. adeninivorans* G1214/YRC103-hERα-phyK eine neue mikrobielle Sensorkomponente konstruiert und damit erste Tests durchgeführt. Mit ihr ließ sich bereits nach 2-stündiger E2-Inkubation ein entsprechendes Messsignal detektieren. Damit konnte mit diesen verbesserten transgenen Hefezellen das Einsatzgebiet des EstraMonitors erweitert werden. Darüber hinaus war es ebenfalls möglich, die Kosten der Messmethode zu senken, indem das kostengünstigere Substrat Ascorbinsäure 2-phosphat (AA2P) eingesetzt wurde. Die damit erreichten EC_{50} und LoD Werte für E2 mit 15,69 und 0,92 ng L^{-1} waren im Vergleich zum bisher genutzten Substrat *p*-APP (20,09 und 8,3 ng L^{-1}) sensitiver.

Insgesamt kann festgestellt werden, dass der EstraMonitor ein komplett automatisiertes System zur Probenanalyse einschließlich Mess- und Kalibrier-Prozessen, sowie einer Alarmfunktion ist. Er bietet erstmals die Möglichkeit zur sensitiven, kostengünstigen, schnellen, automatischen und kontinuierlichen *semi-online* Kontrolle der östrogenen Aktivität, beispielsweise an Kläranlagen.

List of abbreviations

% (v/v)	volume percent
% (w/v)	mass percent
A. adeninivorans	*Arxula adeninivorans*
AA2P	ascorbic acid 2-phosphate
approx.	approximate(ly)
AF-1	activation function-1
AF-2	activation function-2
A-YES	*Arxula*-yeast-estrogen-screen
bp	base pair
BPA	bisphenol A
BLYES	Bioluminescent-based yeast-estrogen-screen
CPRG	chlorophenol red-β- D -galactopyranoside
°C	celsius degree
d25S	delta 25S
DBD	DNA-binding domain
DDT	dichlorodiphenyltrichloroethane
DEHP	di (2-ethylhexyl) phthalate
DES	diethylstilbestrol
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E2	17*ß*-estradiol
EC_{50}	half maximal effective concentration
EE2	17*α*-ethinylestradiol
EEQ	17*ß*-estradiol equivalent
ELISA	enzyme-linked immunosorbent assay
ELRA	enzyme-linked receptor assay
ER	estrogen receptor
ERE	estrogen responsive element
E-Screen	MCF-7 cell line
Fig.	figure
x g	times gravity speed
GAA	glucoamylase-promotor
GFP	green-fluorescent protein
$g\ L^{-1}$	grams per liter
hERα	human estrogen receptor *α*
Hsp-90	heat shock protein 90
YMM	yeast minimal media
lacZ	β-galactosidase-gene
LB	Luria-Bertani
LBD	ligand binding domain
LoD	limit of detection
luc	luciferase
LYES	lyticase-based yeast-estrogen-screen
min	minute
mM	millimolar

mg mL^{-1}	milligrams per milliliter
mg L^{-1}	milligrams per liter
mV s^{-1}	millivolt per second
MCF-7	Michigan cancer foundation-7
nA	nanoampere
ng	nanogram
ng L^{-1}	nanograms per liter
nm	nanometer
nAES	new *Arxula* yeast-estrogen-screen
no.	number
NLS	nuclear localization signal
OD	optical density
p-	para-
p-AP	para-aminophenol
p-APP	para-aminophenyl phosphate
p-NPP	para-nitrophenyl phosphate
rDNA	ribosomal DNA
rYES	recombinant yeast-estrogen-screen
SPE	solid phase extraction
sec	second
URA	uracil
U μL^{-1}	unit per microliter
YES	yeast-estrogen-screen
YPD	yeast extract peptone dextrose
YRC	yeast rDNA integration cassette
YTH	yeast-two-hybrid
WT	wide-type
Δ	difference
µA	microampere
µg mL^{-1}	micrograms per milliter

1 Introduction

Nowadays, estrogenic compounds are accumulating in the environment. Next to natural estrogens, the main pollutants are synthetic forms and chemicals with estrogenic activities (section 1.1) which can mimic the behaviour of the natural estrogens like 17β-estradiol. They enter the cell directly, by passing through the membrane. Once inside the cell, they bind to the estrogen receptor (section 1.2). Through the mechanism of estrogen action (section 1.3), an estrogen response in the cells is triggered. However, a prevalence of estrogenic compounds in the environment can cause adverse effects in the wildlife, particularly in the aquatic organisms (section 1.4). Controlling estrogenic compounds is therefore in the focus of topical investigations. There are numerous methods for estrogenic detections (section 1.5), most of widely used ones are based on biosensors (section 1.6). Amongs these, microbial biosensors (section 1.7) have shown their benefits in monitoring environmental pollutants. Taking advantage of microbial biosensor, the EstraMonitor is designed for estrogenic monitoring in combining with amperometric detection method (section 1.11). This system is based on a microbial component, the immobilized *A. adeninivorans* (section 1.8 and 1.9) which was genetically modified using the Xplor 2 expression platform (section 1.10). In this study, the EstraMonitor is introduced to provide a novel *semi-online* and continuous system for estrogenic monitoring.

1.1. Estrogens and environmental estrogens

Estrogens are steroid hormones. Though present in both men and women, they usually occur in higher levels in women of reproductive age and are normally referred to as the female steroid hormones (Legler, 2002). Estrogens play an essential role in many reproductive and developmental processes. They influence growth, development and behaviour (puberty), regulate reproductive cycles (menstruation, pregnancy) and affect many body parts like bones, skin, arteries and brain in all vertebrates (Legler, 2002). While natural estrogens are steroid hormones, synthetic estrogens are non-steroidal. Natural estrogens in women contain three major types: estrone (E1), 17β-estradiol (E2) and estriol (E3) (Fig 1-1). Non-steroidal estrogens include synthetic products (xenoestrogen), plant products (phytoestrogens) and fungal products (mycoestrogens) with an estrogenic activity (Fang *et al.*, 2001; Watson *et al.*, 2007).

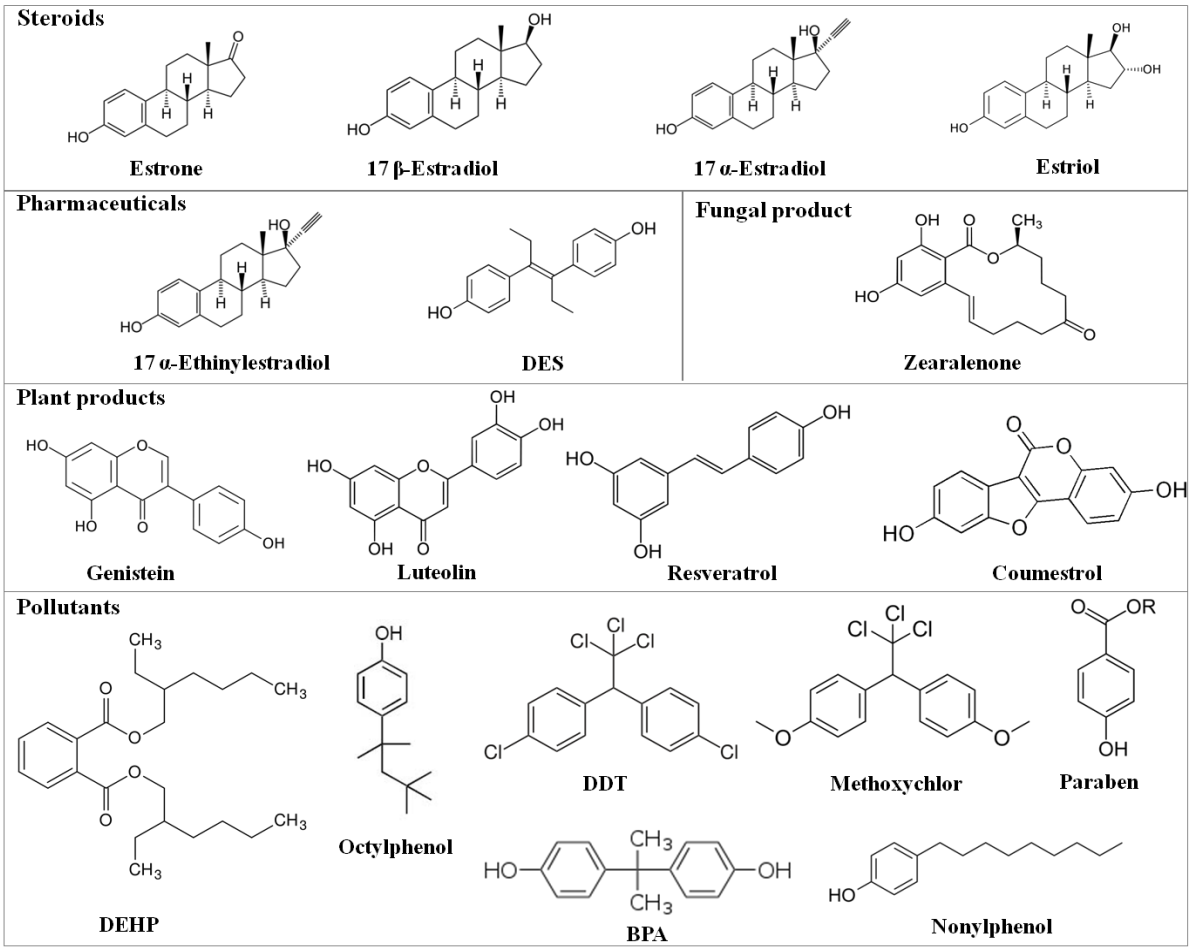

Fig. 1-1. Structural diversity of presentative estrogens and xenoestrogens.

Environmental estrogens are a group of compounds that occur not only as residues of natural sexual hormones of humans and animals such as 17β-estradiol, estrone and estriol, but also as metabolites of plants (phytoestrogens) and fungi (mycoestrogens) (McLachlan, 2001; Seifert *et al.*, 2003). Phytoestrogens include genistein, luteolin, resveratrol and coumestrol. Mycoestrogens contain zearalenone. To the natural estrogens come the synthetic estrogens which are specifically synthesized pharmaceuticals such as diethystilbestrol and the anti-conception pill (17α-ethinylestradiol) or estrogenic cosmetics preservatives like paraben (Legler, 2002). The third group of environmental estrogens is industrialized by-products with estrogenic effects, the so-called xenoestrogens (Legler, 2002; McLachlan, 2001; Seifert *et al.*, 2003). Xenoestrogens comprise among others organochlorine pesticides like DDT and methoxychlor, which due their stability and widespread usage in the past are still present in the environment contaminating many agriculture and run-off sites (Watson *et al.*, 2007). Also detergents like *p*-nonylphenol and plastic monomers like bisphenol A act as xenoestrogens. Both are by-products of plastic manufacturing or substances used in producing plastic materials (DEHP) and are widespread pollutants in food and water via packaging, and as manufac-

turing by-products in the environment (Sonnenschein and Soto, 1998). Structures of some natural, synthetic and xenobiotic estrogenic chemicals are shown in the Fig. 1-1.

1.2. Estrogen receptor

Estrogen receptors (ER) are found in a broad range of species (e.g. human, mice and rat) (Jensen and Jacobson, 1962; Green *et al.*, 1986a; Sluyser *et al.*, 1998). They are nuclear transcription factors belonging to the family of nuclear hormone receptors. The human ER (*hER*) was cloned and sequenced from MCF-7 human breast cancer cells (Green *et al.*, 1986a). hER is a large protein containing 595 amino acids with a molecular weight of 66 kDa. This protein harbours several distinct regions that are responsible for different functions (Green *et al.*, 1986a; McLachlan and Arnold, 1996). The hER protein contains six functional domains (Fig 1-2) (Kumar *et al.*, 1986, 1987; Macgregor and Jordan, 1998). Two of these, the DNA binding domain (DBD) and the ligand binding domain (LBD) are highly conserved among the members of the nuclear hormone receptors (Macgregor and Jordan, 1998). They contain two activation function regions (AFs). AF-1 is located in the amino-terminal region (NH_2-terminal region) in the DBD and AF-2 in the carboxyl-terminal region (C-terminal region) in LBD.

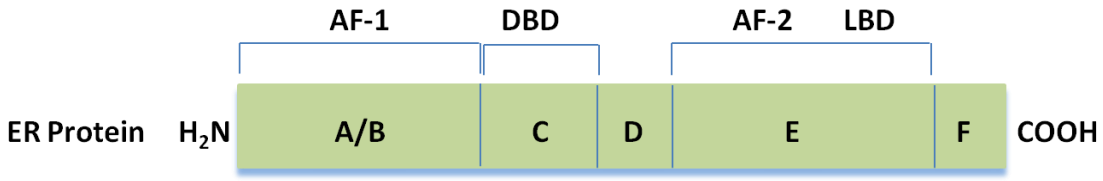

Fig. 1-2. Schematic representation of the domain structure of an ER (Macgregor and Jordan, 1998)**.** The receptor consists of six domains. The A/B region at the NH2-terminus contains the AF-1 domain. The C region comprises the DNA binding domain (DBD). The D region acts as a hinge between the DBD and the LBD. The E region composes of the ligand binding domain (LBD) and AF-2 domain. The F region contains C-terminal domain.

The A/B region of the ER contains an activation function 1 (AF-1) and is able to act in a ligand-independent way when placed outside of the receptor (Germain *et al.*, 2006). Length and sequence of the A/B region are highly variable in the different nuclear receptors, demonstrating a very weak evolutionary conservation (Germain *et al.*, 2006). In contrast to AF-2, which is induced upon hormone binding to the receptor (Kumar *et al.*, 1987; Lees *et al.*, 1989; Tora *et al.*, 1989; Webster *et al.*, 1988, 1989), AF-1 is constitutively active (Macgregor and Jordan, 1998). The C region contains the DBD and a dimerization domain (Germain *et al.*,

2006; Macgregor and Jordan, 1998). This is the most highly conserved region in the nuclear hormone receptors (Germain *et al.*, 2006; Macgregor and Jordan, 1998). Through this domain, ER binds to a specific DNA sequence called estrogen response element (EREs) (Kumar *et al.*, 1986). The C region can bind to heat shock protein 90 (Hsp 90) (Chambraud *et al.*, 1990) and is responsible for the nuclear localization of the receptor (Macgregor and Jordan, 1998). The D region, a poorly conserved domain, acts as a hinge between the DBD and the LBD, allowing rotation of the DBD (Germain *et al.*, 2006). This domain also harbours a nuclear localization signal or at least some elements of a functional nuclear localization signal (Germain *et al.*, 2006). The E region, LBD, consists of an activation function 2 (AF-2), Hsp 90 binding function, a NLS (nuclear localization signal) and a dimerization domain (Germain *et al.*, 2006; Macgregor and Jordan, 1998). This domain, which is less conserved than the DBD, is responsible for the ligand binding and subsequent dimerization and contains a ligand-dependent trans-activation function (Germain *et al.*, 2006; Macgregor and Jordan, 1998). The C-terminal F domain has a function in distinguishing estrogen agonists versus antagonists, possibly through interaction with cell-specific factors (Montano *et al.*, 1995).

The three subtypes of ERs, ERα, ERβ and ERγ (Greene *et al.*, 1986; Green *et al.*, 1986; Hawkins *et al.*, 2000; Kuiper *et al.*, 1996; Mosselman *et al.*, 1996) differ in amino acid sequence, tissue distribution, ligand binding affinities and transactivation function (Legler, 2002). ERα, ERβ are found in humans and in several animals (Green *et al.*, 1986a, b; Kuiper *et al.*, 1996; Mosselman *et al.*, 1996), ERγ has so far only been found in fish (Hawkins *et al.*, 2000). ERs are expressed in many tissues such as reproductive organs and accessory sex organs, brain, bone and liver.

In the absence of hormone, hERα is available within the nuclei of target cells in an inactive form (Wijayaratne and McDonnell, 2001). The presence of estrogenic hormones on the other hand leads to ligand binding to hERα. This results in a conformation change allowing the interaction with particular cofactors and DNA response elements within gene promoters which activates target gene expression (Beekman *et al.*, 1993; Horwitz *et al.*, 1996; Wijayaratne and McDonnell, 2001).

1.3. Proposed modes of action of estrogens

Like all lipophilic steroid hormones, estrogens readily diffuse across the plasma membrane and bind to the ER (Heffner and Schust, 2010; Macgregor and Jordan, 1998). Because ER is a nuclear transcription factor, binding between ER and estrogens initially takes place in

the nucleus (King and Greene, 1984; Welshons *et al.*, 1984). In response to the binding Hsp 90 dissociates and the ER undergoes a conformational change leading to dimerization (Klinge *et al.*, 1997; Macgregor and Jordan, 1998). As a consequence, estrogen fits into the hydrophobic region of the LBD (Mueller, 2002). Helix 12 of the LBD acts as a "lid" sealing hydrophobic crack and stabilizing the binding of estrogen (Brzozowski *et al.*, 1997; Pike *et al.*, 1999; Shiau *et al.*, 1998). Once ER has bound to estrogen and dimerized, it binds through its DBD to EREs with high affinity and transactivates gene expression (Klinge, 2001). ERE is a specific DNA sequence (about 13 base pairs) located upstream of the transcriptional start site in the promoter region of the target gene (Macgregor and Jordan, 1998; Mueller, 2002). Illustrated scheme of these events is presented in the Fig 1-3.

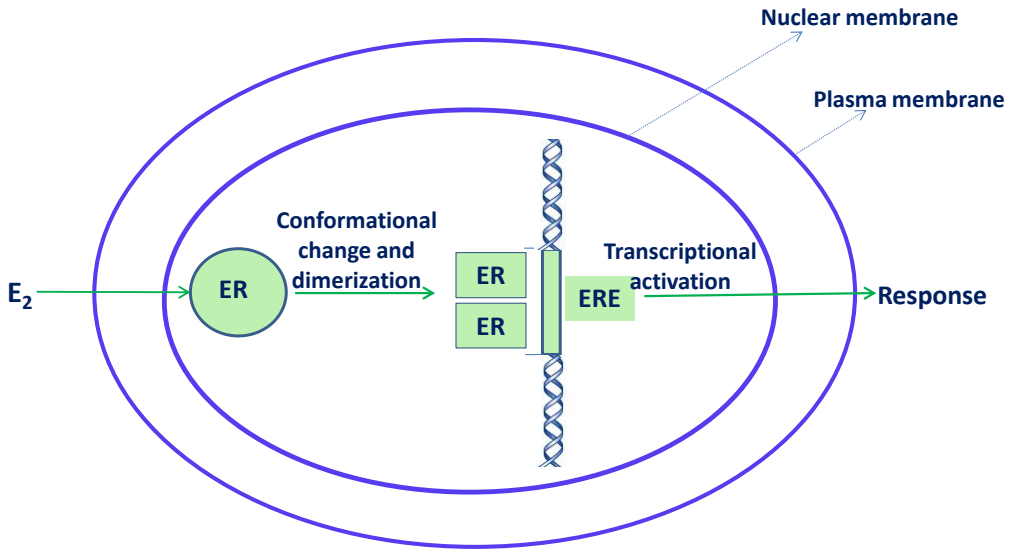

Fig. 1-3. Mechanism of steroid hormone receptor action in the cell (Macgregor and Jordan, 1998). Estrogens (E), like 17β-estradiol, easily pass through the plasma membrane through diffusion because of their non-polarity. Inside the cell, they bind to the estrogen receptor which changes its conformation and dimerization occurs. The estrogen-estrogen receptor dimer complex then binds to the EREs located in the target gene promoter region and activates transcription.

1.4. Effects of estrogenic substances in the environment

Numerous estrogenic compounds are accumulating in the environment due to the broad applications of natural and synthetic estrogens (e.g. pharmaceuticals, pesticides, cosmetic additives and industrial by-products). These estrogenic substances are emitted into the environment either directly or after they have passed through municipal wastewater treatment plants and effluent sources (Ingrand *et al.*, 2003; Koerner *et al.*, 1999; Lopezde-Alda and Barcelo 2001). The low concentration of estrogenic compounds coupled with their metabolic "novelty", leads to incomplete removal from wastewater (Daughton and Ternes, 1999). Be-

cause of incomplete removal or conversion into an active form during the process of sewage treatment, estrogens and xenoestrogens are released into surface water like rivers, lakes and seas or adsorbed to sewage sludge or sediment (Liney *et al.*, 2006). These chemicals are found in low parts per trillion in the aquatic environment (Ternes *et al.*, 1999).

It has been demonstrated that estrogenic compounds can mimic the natural estrogen (E2) by binding to estrogen receptors and interfering with endocrine signalling pathways (Vos *et al.*, 2000). The estrogenic contaminations residing in the environment can disrupt the normal endocrine functioning of human and wildlife species. Consequently, they cause permanent alterations in the structure and function of the endocrine system (De Boever *et al.*, 2001; Tyler *et al.*, 1998). Furthermore, estrogenic compounds are thought to be responsible for disturbances in reproduction, development and other biological pathways especially in the aquatic environment (Legler *et al.*, 2002; Montagnani *et al.*, 1996; Routledge and Sumpter, 1996; Sonnenschein and Soto, 1998). This view is supported by Matthienssen and Sumpter (1998) who found that a number of natural and synthetic estrogenic hormones, natural plant sterols, synthetic alkylphenols, and certain organochlorine substances affect vertebrate wildlife.

There is considerable evidence that the estrogenic effects can be mostly described as feminization in fish species (Gross-Sorokin *et al.*, 2006; Jobling *et al.*, 2006; Johnson *et al.*, 2008; Matthienssen and Sumpter, 1998; Sumpter, 1995). Vitellogenin is normally produced by sexually mature females only. Male fish do not produce vitellogenin, however, they can be stimulated to do so when exposed to estrogenic substances. Therefore, the concentration of vitellogenin in blood plasma of male fish is an ideal biomarker for exposure to estrogenic compounds (Hylland and Haux, 1997; Sumpter and Jobling, 1995; Tyler *et al.*, 1996). Vitellogenin production has been found to be induced by 17β-estradiol concentrations as low as 5 ng L^{-1} (Tabata *et al.*, 2001), estrone concentrations as low as 3.2 ng L^{-1} (Thorpe *et al.*, 2001) and 17α-ethinylestradiol around 1 ng L^{-1} (Fenske *et al.*, 2001; Thorpe *et al.*, 2001).

It has been observed that the reproductive impacts on fish also include the induction of intersex (Jobling *et al.*, 2002a), lowered hormone levels (Folmar *et al.*, 1996) and reduced gamete production and fertilization capacity (Jobling *et al.*, 2002a, b). The intersex condition has been recorded in different fish species due to exposures to both 17β-estradiol and estrone with concentrations as low as 10 ng L^{-1} (Metcalfe *et al.*, 2001; Tabata *et al.*, 2001) and 17α-ethinylestradiol as low as 4 ng L^{-1} (Lange *et al.*, 2001). Other estrogene induced effects include altered mating behaviour (Wenzel *et al.*, 2001), reduced testicular growth (Jobling *et*

al., 1996) and reduced reproductive output (van den Belt *et al.*, 2001; Wenzel *et al.*, 2001). In addition, environmental estrogens may be responsible for the increasing rate of human cancer (Bhatt, 2000; Davis, 1997) and abnormalities in the human reproductive system (Andersen *et al.*, 2000; Massaad *et al.*, 2002).

1.5. Estrogenic detection methods

In the light of the adverse effects of the estrogenic compounds mentioned above, there is an urgent need to detect and control estrogenic activity especially in the aquatic environment. To date, there is a wide variety of methods used for estrogenic detection. Most of these detections are analytical and biological methods.

In earlier times, estrogenic contaminations were identified and quantified by classical analytical techniques such as gas chromatography–mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), LC-tandem mass spectrometry (LC-MS/MS), high performance liquid chromatography (HPLC/MS) and enzyme-linked immunosorbent assays (ELISA) (Diaz-Cruz *et al.*, 2003; Farre *et al.*, 2006; Lopezde-Alda and Barcelo, 2001; Ternes *et al.*, 2002). The sensitivity, accuracy and high reproducibility of these analytical methods make them excellent tools for estrogens screening. However, these techniques are time-consuming because of sample preparation and concentration, expensiveness and they cannot be performed easily outside the laboratory. In addition, they are restricted to a limited set of substances like environmental chemicals with estrogenic activities (Petrovic *et al.*, 2004).

At present, there are numerous assays available both *in vivo* and *in vitro* (Fang *et al.*, 2000; Parrott, 2001) for the detection of estrogenic activity in environmental samples. To determine total estrogenic activities, *in vivo* exposure studies such as cell differentiation, protein expression, and enzyme activities are the most relevant biological methods (Branham *et al.*, 1993; Heppell *et al.*, 1995; Rutishauser *et al.*, 2004; Sumpter and Jobling, 1995). However, they are expensive and time-consuming. Additionally, *in vivo* assays employ the complex responses such as induction of uterine wet weight that normally acts as the biomarker of the estrogenic activity. This response may be modulated through modes of action which do not directly involve the ER (Clark *et al.*, 1980; Korach and McLachlan, 1995; Nelson *et al.*, 1978; Zacharweski, 1997). Therefore, *in vivo* assays may not detect the compounds which act through the ER and are thus not a good choice for screening.

In order to improve detection methods, monitoring of various estrogenic pollutants in environmental matrices has entered a new phase during the last decade. The requirements of

improvements in instrumentation, sampling and sample preparation techniques become essential to fulfil these demands: detection at low-level range; achieving economic, less time-consuming and *semi-online* methods. To this end, the following categories of *in vitro* assays have become more popular (Baker *et al.*, 1999; Beresford *et al.*, 2000; Bolger *et al.*, 1998; Gaido *et al.*, 1997; Payne *et al.*, 2000; Schultis and Metzger, 2004; Soto, 1995).

- Competitive ligand binding assays: One of these is referred to as the receptor binding assay with hERα and hERβ based on fluorescence polarization. Exogenous estrogenic compounds can replace the fluorescent ligand from a prepared "slowly tumbling estrogen receptor-ligand complex". Thus, the concentration of a competing estrogenic compounds is inversely related to the intensity of the fluorescent signal. Therefore, by measuring fluorescent intensity, the concentration of the estrogenic compounds is defined (Schultis *et al.*, 2002; Schultis and Metzger, 2004). These assays are normally fast (about 4 h) and inexpensive, however, the sensitivity is low since it cannot distinguish between receptor agonists and antagonists (Zacharewski, 1997).

- Cell proliferation assays: In these assays estrogenic activities are determined by a proliferation test with human estrogen receptor-positive MCF-7 breast cancer cells (E-Screen assay) (Körner *et al.*, 1999). The used medium contains charcoal-dextran stripped human serum which inhibits the proliferation of MCF-7 cells. Estrogenic substances induce the cell proliferation by negating the inhibitory effect of a cell proliferation repressing factor (Soto *et al.*, 1995; Zacharewski, 1997). The E-Screen assay is simple and highly sensitive but time-consuming (approx. 7 days) and costly.

- Recombinant receptor/reporter gene assays: Widely used are yeast-based assays, such as the YES-assay (Routledge and Sumpter, 1996), E-Screen assay (Körner *et al.*, 1999), rYES assay (De Boever *et al.*, 2001), BLYES assay (Sanseverino *et al.*, 2005), LYES assay (Schultis and Metzger, 2004), A-YES assay (Hahn *et al.*, 2006) and nAES assay (Kaiser *et al.*, 2010). Most of these assays require recombinant yeast cells (*S. cerevisiae* or *A. adeninivorans*) to identify estrogenic activities via interaction between estrogenic substances with the hERα. The recombinant yeast cells used in these assays harbour an estrogen receptor expression cassette accompanied with a reporter construct. In the presence of estrogenic compounds, the ER form dimerizes and binds to the EREs in the reporter gene cassette. This results in the expression of the reporter gene encoding for the designed enzyme. *lacZ* and *phyK* genes encoding for β-galactosidase and phytase enzymes, respectively, are mostly used in these assays. Depending on the substrates employed for the enzyme-substrate reactions, the

detection methods are bioluminescent, chemiluminescent, colorimetric or photometric. For example, the current A-YES and nAES assays are based on robust recombinant *A. aden-inivorans* cells harbouring a gene for human estrogen receptor α (hERα) and a reporter gene *phyK* encoding the phytase enzyme (Hahn *et al.*, 2006; Kaiser *et al.*, 2010). In the presence of estrogenic compounds, the phytase enzyme is secreted into the sample and detected photometrically (Hahn *et al.*, 2006; Kaiser *et al.*, 2010).

The recombinant receptor/reporter gene assays are designed on the principle of biosensors (Liang *et al.*, 2011) and are usually characterized by high responsiveness, sensitivity, low cost and easy handling. Nevertheless, some time-consuming and most lack automation and *online* properties.

1.6. Biosensors

Biosensors contain a biological component (bioreceptor) which is either closely connected to or integrated within a transducer device (e.g., a kind of electrode) to produce a signal proportional to the analyte concentration (Chan, 1999; Lei *et al.*, 2006; Mulchandani and Rogers, 1998; Riedel *et al.*, 2002; Shantilatha *et al.*, 2003; Tran, 1993; Turner *et al.*, 1992). The transducer eventually converts the biochemical reaction into a quantifiable and measurable response signal which can be further amplified, processed and stored for later analysis (Mulchandani and Rogers., 1998; Riedel *et al.*, 2002; Tran, 1993; Turner *et al.*, 1992) (Fig. 1-4).

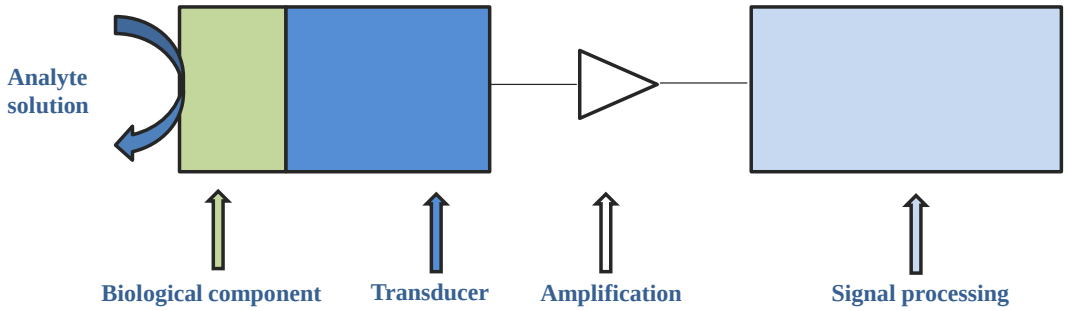

Fig. 1-4. Schematic diagram of a biosensor. A biosensor composes of a bioreceptor and a transducer. A bioreceptor can recognize selectively the particular compound based on their specific interaction. The transducer transforms a biological signal into a measurable signal which can be amplified and processed.

]

The biological component (bioreceptor) is the first essential component of a biosensor and consists of either microorganisms, enzymes, antibodies, receptors, organelles, tissues or cells from animals or plants (Table 1-1). These can recognize selectively particular compounds on the basis of specific interactions. A signal transducer, the second important part of a biosensor, converts the biological signal from the bio-receptor into a measurable signal. The particular class of transducer employed, is based on the parameter which are measured, for example, electrochemical (amperometric, potentiometric and conductometric), optical (colormetric, luminescence and fluorescence) or calorimetric (thermistor) signals. The most commonly used biological component and transducers are listed in Table 1-1.

Table 1-1. Common components of a biosensor.

Biological component	**Transducer**	**Out put**
Organisms	Electrochemical	
Tissues	Amperometric	Applied current
Cells	Potentiometric	Voltage
Organelles	Conductometric	Impedance
Nucleic acids	Optical	
Enzymes	Colormetric	Color
Receptors	Luminescence	Light intensity
Antibodies	Fluorescence	Light intensity
	Calorimetric	
	Thermistor	Temperature

Biological components used for biosensors

Enzymes are proteins with high catalytic activity and selectivity towards substrates. They have been used to examine the concentration of the diverse analytes (Guibault, 1976). They are the most common components of biosensors (Lei *et al.*, 2006; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Tran, 1993; Turner *et al.*, 1992). Although their commercial availability at high purity levels makes them very specific for their substrates or inhibitors, several limitations still remain such as time-consumption, costs, requirement of multiple enzymes to produce the measurable product, the need of cofactor/coenzyme or reduced activities at temperatures above 60 °C. As a part of biosensors, enzymes have been immobilized at the surface of the transducer by adsorption, covalent attachment and entrapment in a gel or an electrochemically generated polymer, in bilipid membranes or in solution

behind a selective membrane (Bartlett and Cooper, 1993; Bartlett and Whitake, 1987; Bidan, 1992; Cosnier, 1999; Guibault, 1983; Reyes-De-Corcuera and Cavalieri, 2003; Scouten, 1987; Tien *et al.*, 1997).

Antibodies are proteins with great selectivity. They are produced by β-lymphocytes in response to antigenic structures which (usually) are foreign to the organism. Molecules larger than about 10 kDa are able to stimulate an immune response. Smaller molecules like vitamins or steroids can be considered as antigens (also called haptens). However, they are not able to cause an immune response unless they are coupled to larger molecules (bovine serum albumin) (Reyes-De-Corcuera and Cavalieri, 2003). Antibodies are often immobilized on the surface of the transducer by covalent attachment via amino, carboxyl, aldehyde or sulfhydryl groups (Hermanson, 1996; Reyes-De-Corcuera and Cavalieri, 2003). Like enzymes antibodies have similar limitations, binding may not be reversible and regeneration of the surface may require drastic changes in conditions like low pH, high ionic strength or detergents (Reyes-De-Corcuera and Cavalieri, 2003). Therefore, efforts are made to produce low cost, single use sensors. Probably the main potential advantage of immunosensors over traditional immunoassays is that they theoretically allow faster and in-field measurements (Reyes-De-Corcuera and Cavalieri, 2003). Immunosensors usually employ optical or acoustic transducers.

Microorganisms used as biological components include *Torulopsis candida* (Sangeetha *et al.*, 1996), *Bacillus subtilis* (Riedel *et al.*, 1988; Tan and Qian, 1997), yeast cells such as *A. adeninivorans* and *S. cerevisiae* (Baronian, 2004; Chan *et al.*, 1999; Parascandola *et al.*, 2006; Riedel *et al.*, 1998; Tag *et al.*, 2000a, b), *Pseudomonas* sp. (Li and Chu, 1991) and thermophilic bacteria (Karube *et al.*, 1989). The particular choice of organism is mostly based on the electrochemical measurement of their metabolism (Karube, 1987). Microorganisms offer the ability to detect a wide range of chemical substances. Furthermore, they are easily accessible for genetic modifications and usually have a broad operating pH and temperature range (Blum and Coulet, 1991; Lei *et al.*, 2006; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Riedel *et al.*, 1998; Tran, 1993; Turner *et al.*, 1992). Additionally, microorganisms are cheaper than enzymes or antibodies and already have shown their potential with a variety of transducers (Blum and Coulet, 1991; D'Souza, 2001; Lei *et al.*, 2006; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Nikolelis *et al.*, 1998; Ramsay, 1998; Tran, 1993; Turner *et al.*, 1992).

Transducer

Electrochemical

Electrochemical transducers can be potentiometric, amperometric or conductometric.

Potentiometric transducers consist of an ion-selective electrode (pH, ammonium, chloride and so on) or a gas-sensing electrode (p_{CO2} and p_{NH3}). They measure the difference in potential resulting from ion accumulation or depletion between a working and a reference electrode. The measured signal is correlated to the analyte concentration (Mulchandani and Rogers, 1998; Simonian *et al.*, 1998; Tran, 1993). Because of a logarithmic relationship between the potential generated and analyte concentration, a wide detection range is possible (Mulchandani, 1998).

Amperometric transducers comprise electrodes made of metals such as platinum, gold, silver, stainless steel or carbon-based materials. These materials are inert at potential at which the electrochemical reaction takes place. Amperometric electrodes measure the current generated by the oxidation or reduction of the electrochemical active product which is proportional to the concentration of the analyte of interest. In contrast to the potentiometric transducers where the potential is changed, the potential in the amperometric transducer is fixed with respect to a reference electrode (Mulchandani, 1998). At present, the amperometric transducers are the main commercial devices on the market.

Conductometric transducers compose of thin-film intedigitated electrodes (Dzyadevych *et al.*, 2001; Dzydevich *et al.*, 1994; Korpan *et al.*, 1994; Shul'ga *et al.*, 1994; Watson *et al.*, 1987, 1988). They measure the electrical conductivity of a solution in the course of a chemical reaction. Many enzyme-catalyzed reactions involve either consumption or production of charged species, and thus lead to a change in the ionic composition of the sample tested. However, because of the measurement of solution conductance is nonspecific, analytical use of conductometric transducer is limited (Mulchandani, 1998).

Optical

Optical transducers monitor the change in optical properties such as UV absoption, bio- and chemiluminescence, reflectance and fluorescence which result from the interaction of the biocatalyst with the analyte of interest (Mulchandani, 1998). Some commonly optical transducers are described below:

The bioluminescence detection method is related to the emission of light by living microorganisms, and plays an essential role in real time prose monitoring. For example, the bac-

terial *lux* gene has been mostly used as a reporter either in an inducible or constitutive manner. In the constitutive manner, the *lux* gene product exists constitutively in the biological component and the bioluminescence will be altered directly with the supplementation of the chemical target. In the inducible manner, the reporter *lux* gene is fused to a promoter which is transcriptionally regulated by the concentration of a compound of interest. Therefore, concentrations can be quantitatively determined by measuring the bioluminescence intensity (Belkin, 2003; Lei, 2006; Rensing and Maier, 2003).

Fluorescence spectroscopy has been popularly applied in analytical chemistry. Through the illumination by a light source, normally ultraviolet light, compounds are excited and start to emit light. Fluorescent biosensors normally come in two types, either using fluorescent materials or employing green fluorescent protein for their constructions (Lei *et al.*, 2006; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Tran, 1993; Turner *et al.*, 1992).

Colorimetric biosensors involve the fabrication of the colour product which can be measured and correlated with the concentration of the chemical of interest. The detection principle is generally based on the measurement of the intensity of colour products under certain wavelengths.

Calorimetric

Calorimetric transducers measure the heat of a biochemical reaction at the biological component (Reyes-De-Corcuera and Cavalieri, 2003). Since many enzymatic reactions are exothermic, the analyte concentration can thus be quantified. Calorimetric devices are classified according to the way heat is transferred. Isothermal calorimeters maintain the reaction cell at a stable temperature using Joule heating or Peltier cooling and measure the amount of energy required. Heat conduction calorimeters measure the temperature difference between the reaction vessel and an isothermal heat sink surrounding it. Most widely used is the isoperibol calorimeter that also measures the temperature difference between the reaction cell and an isothermal jacket surrounding it (Reyes-De-Corcuera and Cavalieri, 2003).

1.7. Microbial biosensor

Definition of the mircobial biosensor

A microbial biosensor is an analytical device that combines microorganisms (microbial component) with a transducer to assure rapid, accurate and sensitive detection of target

analytes with applications in medicine, environmental monitoring, food processing and safety assays (Lei *et al.*, 2006).

Advantages of microbial biosensor

Among biosensors, microbial biosensors are especially suitable for the environmental monitoring. They are able to measure direct effects on living cells, e.g. their respiratory activity and the changes caused by environmental pollutants (Riedel *et al.*, 2002). Furthermore, they can recognize analogous substances simultaneously. This ability allows them to measure complex effects like sum parameters (Riedel *et al.*, 2002). Since there are numerous substances in wastewater samples, sum parameters often permit a better evaluation of the status of the environment than the determination of the concentration of individual substances. Therefore, sum parameters have become an essential part of wastewater monitoring systems (Riedel *et al.*, 2002). Additionally, microbial biosensors also bring the possibility to follow multi-step transformations, which is difficult to achieve with biosensors based on enzymatic sensing systems. Moreover, microbial biosensors require a simple preparation procedure and are less time-consuming (Lei *et al.*, 2006; Riedel, 1998; Riedel *et al.*, 2002). A very important advantage of microbial biosensors is their higher stability. Since their microorganism component is a living system, they can be fed and kept alive for a long period of time. The inexhaustible sources of microorganisms with their broad spectrum of different metabolic types provide a larger field of applications for microbial sensors in environment monitoring (Riedel *et al.*, 2002).

Types of microbial biosensors

Microbial biosensors are classified based on the transducers. The initial microbial biosensors employed the respiratory and metabolic functions of the microorganism to detect a compound which is related to these processes. Currently, genetically modified microorganisms play an increasingly important role in improving the capacity of microbial biosensors (Lei *et al.*, 2006). Although bioluminescence (Bechor *et al.*, 2002; Horsburgh *et al.*, 2002; Philp *et al.*, 2003; Rasmussen *et al.*, 2000; Tiensing *et al.*, 2002; Tom-Petersen *et al.*, 2001) and fluorescence microbial biosensors (Casavant *et al.*, 2003; Chee *et al.*, 2000; Dai *et al.*, 2004; Joyner and Lindow, 2000) have been highlighted since 2000, electrochemical and optical types of transducers have been still the most commonly used in microbial biosensors. Electrochemical microbial biosensors come in three types: amperometric (Chan *et al.*, 1999;

Lehmann *et al.*, 2000; Riedel *et al.*, 1998; Tag *et al.*, 2000a, b; Subrahmanyam *et al.*, 2000), potentiometric (Hans *et al.*, 2002; Mulchandani *et al.*, 1998; Rotariu *et al.*, 2003) and conductometric (Bhatia *et al.*, 2003; Mulchandani and Rogers, 1998; Tran, 1993;Turner *et al.*, 1992). Additionally, the microbial fuel cell (MFC) is also a feasible and promising technology (Su *et al.*, 2011).

1.8. Cell immobilization

Advantages of cell immobilization

Cell immobilization is a process of enclosing the microorganism in a suitable and stable matrix. It generally is an essential step in the fabrication of microbial biosensors because the microorganism (microbial component) must be in close contact with the transducer to assure it can obtain a high portion of the chemical signal. Moreover, the matrix used for immobilization must protect the cells from shear forces and prevent them from diffusing into the surrounding medium while still allowing mass transfer of nutrients and metabolites. Immobilization should not harm the microorganism. Ideally, it should also enhance stability of the microorganisms during continuous monitoring and protect them from contamination (Arikawa *et al.*, 1998). Through the immobilization process microscopic cell structures are concentrated and packed into macroscopic particles facilitating their handling. Properly immobilized cells can be used under non-sterile conditions and can be reused by simple washing with water, buffer solution or medium after each assay. Hence, after an application it is not necessary to recultivate and prepare new cells. This recycling property of microbial sensors makes them highly feasible in *online*, automatic and continuous systems.

Methods used for cell immobilization

Microorganisms can be immobilized on transducer or support matrices by chemical or physical methods (Blum and Coulet, 1991; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Nikolelis *et al.*, 1998; Tran, 1993; Turner *et al.*, 1992). Chemical methods include covalent binding and cross-linking (Blum and Coulet, 1991; D'Souza, 2001; Lei *et al.*, 2006; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Nikolelis *et al.*, 1998; Tran, 1993; Turner *et al.*, 1992). Since chemical methods often damage the cell membrane and decrease the biological activity, those methods have rarely been successfully applied (Blum and Coulet, 1991; D'Souza, 2001; Mikkelsen and Cortón, 2004; Mulchandani and

Rogers, 1998; Nikolelis *et al.*, 1998; Nomura and Karube, 1996; Tran, 1993; Turner *et al.*, 1992). The most widely used methods of immobilization are physical adsorption or entrapment (Arikawa *et al.*, 1998; Blum and Coulet, 1991; D'Souza, 2001; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Nikolelis *et al.*, 1998; Nomura and Karube, 1996; Tran, 1993; Turner *et al.*, 1992). The advantage of these methods is that they do not involve covalent bond formation with microorganism and thus cause relatively small perturbation of microorganism native structure and function (Arikawa *et al.*, 1998; D'Souza, 2001; Matrubutham and Sayler, 1998; Riedel, 1998; Simonian *et al.*, 1998).

Physical adsorption is the simplest and the oldest method of immobilizing microorganisms. For adsorption, the microorganisms are held to the surface of the carriers by physical forces (van der Waals forces) (Hartmeier, 1986) (Fig. 1-5A). Typically, a cell suspension is incubated with the electrode or an immobilization matrix such as alumina and glass bead (D'Souza, 2001; Mikkelsen and Cortón, 2004) followed by rinsing with buffer to remove unadsorbed cells. The microorganisms are immobilized due to adsorptive interactions such as ionic, polar or hydrogen bonding and hydrophobic interaction. However, immobilization using adsorption alone generally leads to poor long-term stability because of desorption of microorganism.

Physical entrapment is the preferred technique for the immobilization of whole cells and active cell components (organelles). In this method, the microorganisms are embedded in natural or synthetic polymers, mostly of a gel-like structure (Fig. 1-5B). A disadvantage of entrapment immobilization is the diffusion resistance offered by the entrapment material, which results in lower sensitivity and higher detection limit. Immobilization of microorganisms by entrapment can be achieved by either retention of the cells in close proximity of the transducer surface using dialysis or filter membrane or in chemical/biological polymers/gels. The chemical/biological polymers/gels include: alginate (Final *et al.*, 2006; Hannoun and Stephanopoulos, 1986; Kurosawa *et al.*, 1989), agar gel (Okada *et al.*, 1981), carrageenan, chitosan, collagen, polyacrylamide, poly(ethylene glycol), polyurethane (Arikawa *et al.*, 1998; D'Souza, 1997; Mikkelsen and Cortón, 2004; Mulchandani and Rogers, 1998; Tran, 1993; Turner *et al.*, 1992) or polyvinylalcohol (PVA) (Bezbradica *et al.*, 2007; Fine *et al.*, 2006; Jahnz *et al.*, 2002; Kumar and D'Souza, 2008; Nunes *et al.*, 2010).

Alginate gel itself is not toxic but the presence of chelators (e.g., phosphate buffer), may affect embedded cells. In addition, alginate is highly biodegradable, reducing its value for long-term applications (Chan, 1999; Hartmeier, 1986; Schlieker and Vorlop, 2006). Most

synthetic polymers like polyacrylamide and polyurethane are not particularly suitable for matrix entrapment of living organisms because of their toxicity. An exception is polyvinyl alcohol (PVA), a hydrophilic polymer which is non-toxic to all biological material investigated so far. PVA is mechanically stable and since most organisms cannot use it as a carbon source, it is hardly biodegradable which is especially important for the use under non-sterile conditions. Additionally, its geometry is supposed to reduce mass transfer resistance in the matrix. Many studies using cells, including yeast cells, immobilized in PVA matrix have supported these advantages (Bezbradica *et al.*, 2007; Fine *et al.*, 2006; Parascandolaa *et al.*, 2006; Shindo and Kamimura, 1990; Ting and Sun, 2000). PVA (used under the trade-name Lentikats® developed by the department of technology, Federal Agricultural Research Centre, Germany (Genialab®BioTechnologie, Braunschweig) was also utilized in the current study for immobilizing *A. adeninivorans* cells. The Lentikats obtained after immobilization are lenticular-shaped with a maximum thickness of approximately 0.2 to 0.4 mm. This improves diffusion characteristics (Jekel *et al.*, 1998).

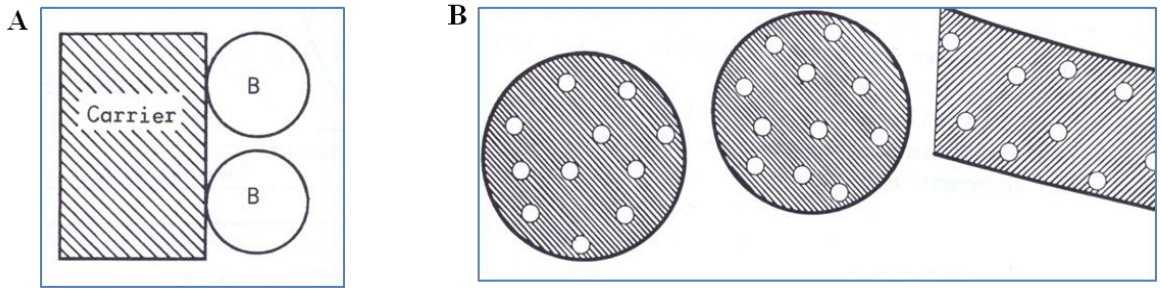

Fig. 1-5. (A) Biocatalysts-B bound to a carrier by absorption. (B) Matrix-entrapped biocatalysts in most common spherical and fiber forms (Hartmeier, 1986).

1.9. *A. adeninivorans*–microbial component of the EstraMonitor

The wide-type strain *A. adeninivorans* LS3 (Fig. 1-6) was isolated from wood hydrolysates in Siberia (Russia) (Kunze and Kunze, 1994). *A. adeninivorans* is a haploid, non-pathogenic, thermo-resistant (Wartman *et al.*, 1995), osmo-tolerant (Böer *et al.*, 2004; Yang *et al.*, 2000) and dimorphic yeast with unusual biochemical characteristics (Steinborn *et al.*, 2007a, b).

The thermo-resistant character of *A. adeninivorans* refers to its temperature-dependent dimorphism. Below a temperature of 42 °C this yeast will grow as budding cells, above 42 °C it forms mycelia (Wartmann *et al.*, 1995, 2000; Wartmann and Kunze, 2000). Additionally, gene expression pattern correlates with the mophological status (Rösel and Kunze, 1995; Stoltenburg *et al.*, 1999; Wartmann *et al.*, 2002).

Particularly, the osmo-tolerance of *A. adeninivorans* is an advantage for many applications especially for monitoring environmental samples. The yeast can grow in minimal as well as rich media supplemented with up to 20% NaCl without any changes in physiology (Wartmann and Kunze, 2000; Wartmann *et al.*, 1995). However, NaCl concentration above 10% results in a decrease in the specific growth rate, a longer adaptation phase and a lower cell count during stationary growth phase (Wartmann and Kunze, 2000).

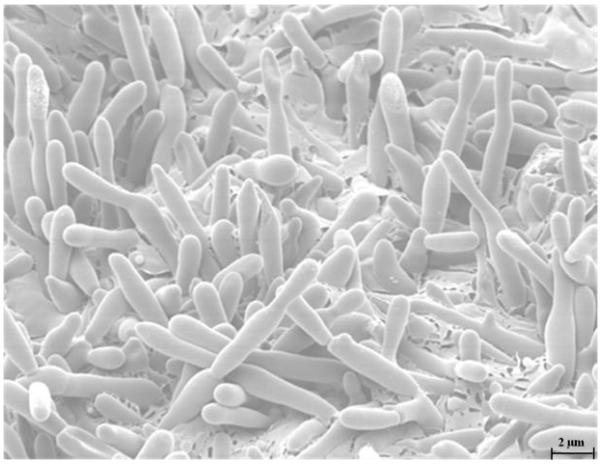

Fig. 1-6. *A. adeninivorans* cells. The *A. adeninivorans* wild-type strain LS3 was the parent strain for the auxotrophic mutants *A. adeninivorans* G1212 [*aleu2 atrp1::ALEU2*] and G1214 [*aleu2 aura3::ALEU2*] used in this study. Bar = 2 µm

The ability of *A. adeninivorans* to utilize a wide variety of organic compounds (including adenine, uric acid and tannic acid) as a carbon source (Wartmann *et al.*, 1995) was exploited in developing a biosensor which could detect gallic acid, naphthalene, di-butyl phthalate and other environmental contaminants (Chan, 1999; Gurazada, 2008; Kaiser *et al.*, 2010; Riedel *et al.*, 1998; Tag *et al.*, 2000a, b).

During cultivation, *A. adeninivorans* produces and secretes several extracellular enzymes including RNase, protease, glucoamylase, acid phosphatases and phytase (Wartmann *et al.*, 2000). This makes *A. adeninivorans* also attractive as a producer of recombinant enzymes without the need of cell lysis.

Its properties make *A. adeninivorans* an attractive microorganism for biosensor applications.

1.10. Xplor 2-transformation/ expression platform for the recombinant protein production in *A. adeninivorans*

The Xplor 2 system has been successfully established as a common transformation system in *A. adeninivorans* (Böer *et al.*, 2009). The procedure allows for the construction of

resistance marker-free transformants and thus increases the acceptance of the system. The Xplor 2 system permits the construction of expression plasmids with up to 5 modules for selection and expression of proteins of biotechnological interest. Furthermore, it also allows the integration of small vector cassettes containing yeast DNA sequences (Böer *et al.*, 2009). The system is based on a vector backbone (Fig. 1-7) into which yeast modules such as selection and expression modules can be inserted. The multi-cloning sites (MCSs) for insertion of these modules are located between two d25S rDNA (Δ25S rDNA) segments which are arranged in the same orientation but in opposite order to the genomic rDNA. After insertion of the relevant modules, the *E. coli* part can be excised by restriction with *Asc*I. The resulting linearized fragment is targeted to rDNA, permitting it to be used as "Yeast rDNA Integrative expression Cassettes" (YRCs).

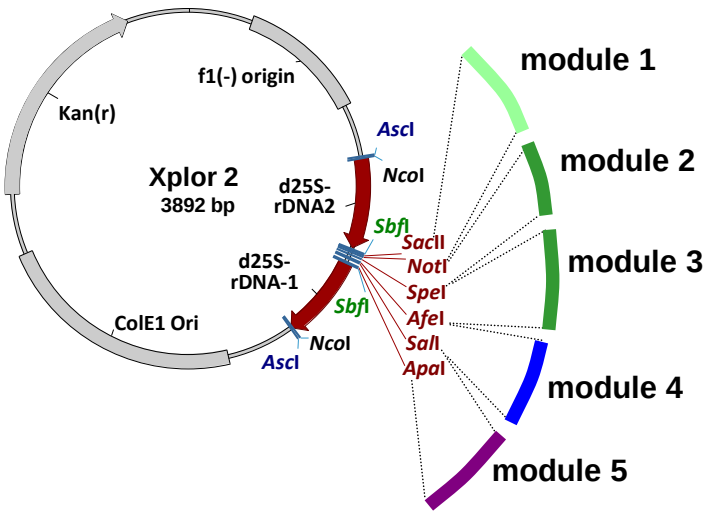

Fig. 1-7. Design and functional components of the Xplor 2 vector. The basic vector is composed of bacterial sequence elements necessary for propagation in the *E. coli* system, two d25S rDNA segments interrupted by a MCS allowing the insertion up to 5 modules for selection and expression (Böer *et al.*, 2009).

1.11. The EstraMonitor- principle and processes

The EstraMonitor, introduced in this study, is based on the principle of a microbial biosensor. The EstraMonitor employs immobilized *A. adeninivorans* cells in combination with an amperometric detection method for *semi-online* estrogenic detection.

The microbial component includes the measuring strain *A. adeninivorans* G1212/YRC102-hERα-phyK and the control strain *A. adeninivorans* G1212/YRC102-phyK

(Fig. 1-8A, B, respectively). These strains were constructed by Kaiser *et al.* (2010) using the Xplor 2 system. The *ATRPm* selection module, *hERα* expression module and reporter *phyK* expression module were inserted into the Xplor 2 vector between two d25S rDNA segments. The plasmids were linearized by restriction with *Asc*I to remove *E. coli* sequences prior to transformation into *A. adeninivorans* G1212 [*aleu2 atrp1::ALEU2*] (Steinborn *et al.*, 2007b).

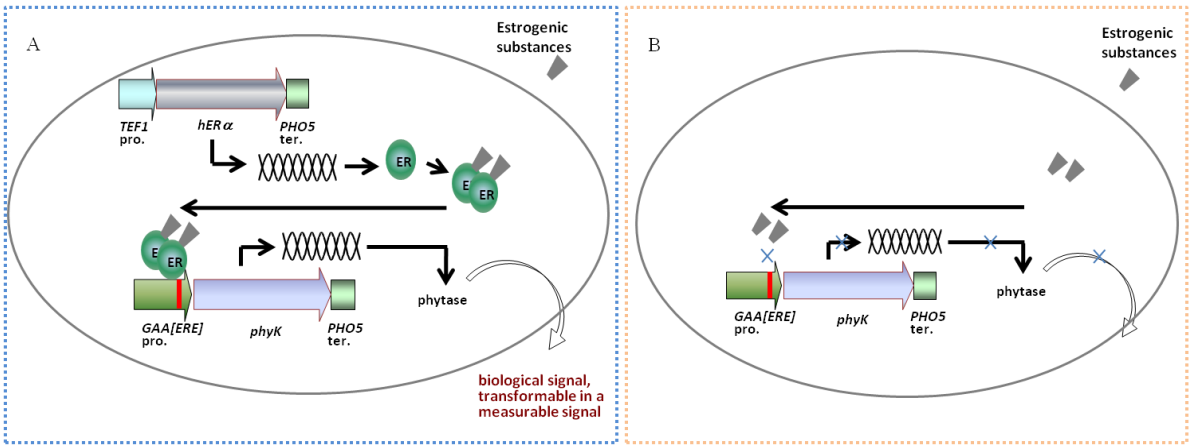

Fig. 1-8. Schematic illustration of the microbial component of the EstraMonitor apparatus. (A) Measuring strain (*A. adeninivorans* G1212/YRC102-hERα-phyK) with *hERα* receptor and *phyK* reporter gene modules. In the presence of estrogenic compounds, ER, constitutively expressed by *hERα*, interacts with these, dimerizes and binds to EREs in the *GAA* promoter region. This activates *phyK* gene leading to synthesis and secretion of phytase which acts as the biological signal; (B) Control strain (*A. adeninivorans* G1212/YRC102-phyK) with *phyK* reporter gene module only. In the presence of the estrogenic substances, *phyK* gene is not activated and phytase is not synthesised.

The measuring strain (*A. adeninivorans* G1212/YRC102-hERα-phyK) contains the constitutively expressed *hERα* gene and the phytase (*phyK*) reporter gene under control of a promoter with estrogen responsive elements (EREs). In the presence of estrogenic compounds, the hERα interacts with these, dimerizes and binds to the ERE in the promoter region of the *phyK* reporter gene cassette. Under this condition, the *phyK* gene is expressed and phytase is synthesized and secreted into the medium. The control strain (*A. adeninivorans* G1212/YRC102-phyK) was designed to eliminate or reduce an activation of the reporter gene cassette by non-estrogen substances. It was created to contain only the reporter gene cassette (without receptor gene cassette). In contrast to the estrogen measuring strain, which contains both cassettes, the control strain is not able to synthesize phytase in presence of estrogens.

The principle of the EstraMonitor is blending microbiology, biochemistry and electrochemistry as shown in Fig.1-9. Immobilized *A. adeninivorans* cells are incubated with E2 and YMM-maltose in the measuring chambers of the EstraMonitor at 37 °C for 4 h. All liquid ex-

changes are performed automatically by pump modules within the EstraMonitor. After 4 h incubation the substrate is added into the measuring chambers followed by an amperometric detection for 10 min. The electric current detected is proportional to the phytase activity in response to estrogenic compounds.

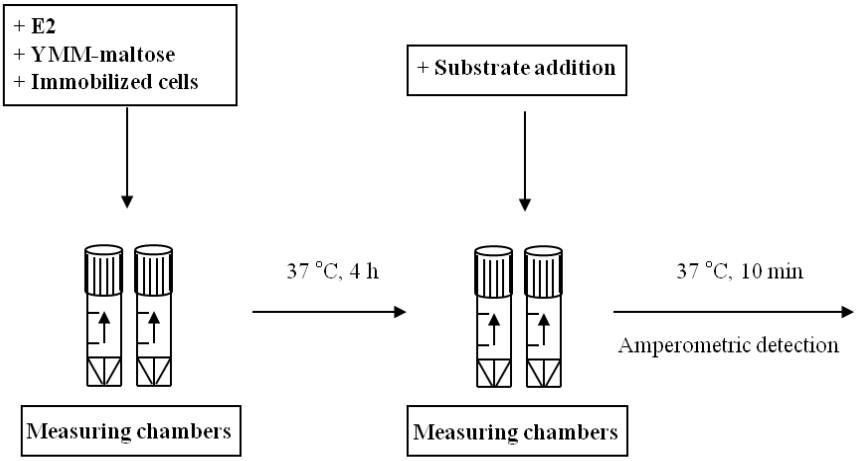

Fig. 1-9. Schematic diagram showing the working principle of the EstraMonitor for amperometric detection of estrogenic activity. Immobilized cells are incubated with E2 in YMM-maltose at 37 °C for 4 h in the measuring chambers of the EstraMonitor. Enzyme substrate (*p*-APP) is subsequently added and incubated at 37 °C. Signals are obtained by amperometric detection for 10 min after substrate addition.

Fig. 1-10 describes how the biological signal (phytase enzyme) is transformed into a measurable signal. Once phytase is secreted into the medium, it converts the substrate *p*-aminophenyl phosphate (*p*-APP) into the intermediate product *p*-aminophenol (*p*-AP). Because *p*-AP is electrochemical active it can then be oxidized at the electrode. The current is amperometrically detected and the phytase activity (and thus estrogenic activity) can be calculated on basis of the electric current.

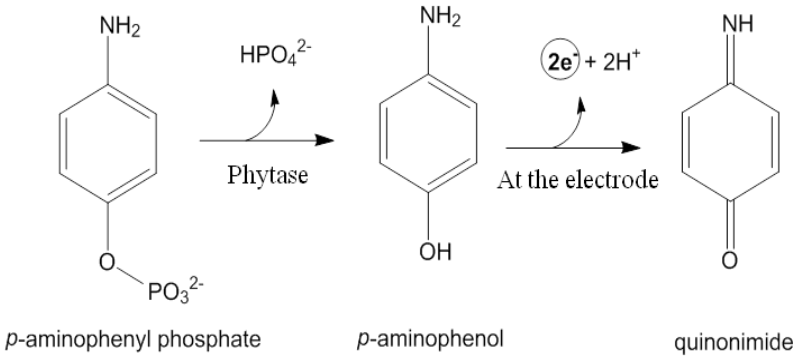

Fig. 1-10. Schematic illustration of biochemical and electrochemical reactions in the measuring chambers of the EstraMonitor. Recombinant phytase secreted by immobilized cells transforms the

substrate (*p*-APP) into *p*-AP. This intermediate product is then oxidized at the electrode and releases electrons which are detected by amperometric method.

1.12. The aims of the current study

Although there are numerous biosensors for estrogenic detection, the combination of microbial component and amperometric transducer in a single system would presents a significant improvement in this field. Therefore, in co-operation with the companies quo-data GmbH, Dresden and Prolatec GmbH, Radebeul the main purpose of this study was to establish the microbial, biochemical and electrochemical components for the EstraMonitor system.

The first investigations concerned the suitability of the transgenic yeast strains *A. adeninivorans* G1212/YRC102-hERα-phyK and *A. adeninivorans* G1212/YRC102-phyK for this EstraMonitor system and to survey the optimum conditions for the production of reporter enzyme and substrate admission to the system.

The second aim was to produce immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells, to characterize their properties in relation to activity, stability and reusability, and to investigate their capacity in analyzing real environmental samples in application with the EstraMonitor system.

The third task was to study alternative microbial component and substrate for amperometric detection method in order to enhance the sensitivity of the sensor, reduce measuring time and lower the cost for the EstraMonitor system.

2 Materials and Methods

2.1. Materials

2.1.1 EstraMonitor

The EstraMonitor shown in Fig. 2-1 uses *A. adeninivorans* G1212/YRC102-hERα-phyK (measuring strain) and G1212/YRC102-phyK (control strain) cells to semi-continuously measure estrogenic activities in wastewater. Data assessment and interpretation is performed with the EstraMonitor software version 1.5.2.1 (quo data GmbH, Dresden, Germany). The EstraMonitor is an automated system including sample cycling, measuring and calibration processes and an alarm function. The system is supplied with a waste treatment system (decontamination system) using high temperature to ensure that no viable transgenic yeast cells are released into the environment.

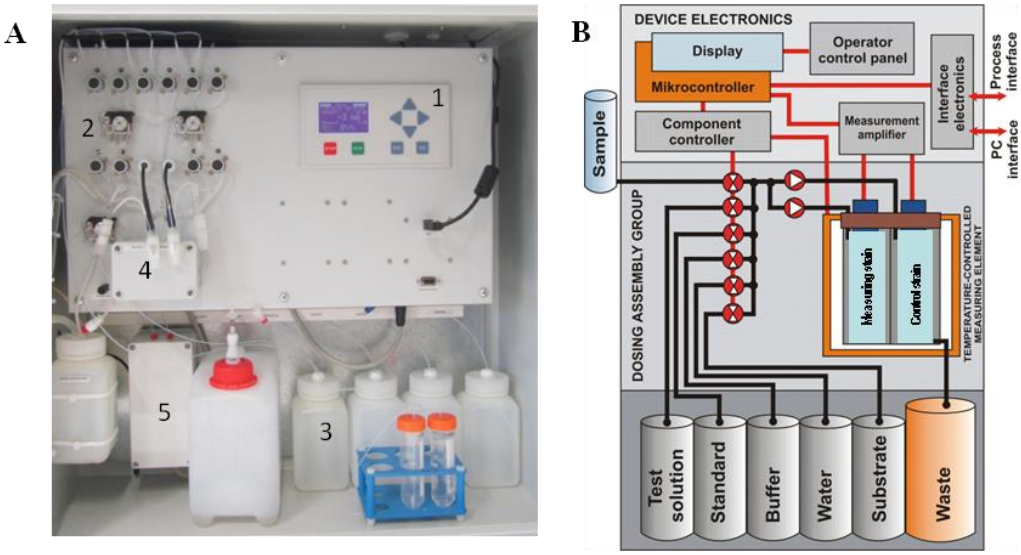

Fig. 2-1. (A) EstraMonitor apparatus. 1-amperometric transducer, 2-pump module for waste and nutrient exchange, 3-reservoir, 4-measuring chamber with immobilized yeast cells, 5-decontamination system. **(B) Structural detail of the EstraMonitor**.

2.1.2 Enzymes, chemicals, equipments, kits and softwares

- Restriction and DNA modification enzymes (e.g. restriction endonucleases and T4 DNA ligase) were obtained from Thermo Scientific company (Germany).

- Chemicals were obtained from the following companies: Roth, Sigma-Aldrich, Thermo Scientific, Qiagen, BioRad, Merck and Invitrogen. The mainly used chemicals and kits are stated in the Table 2-1.

Table 2-1. Chemicals and kits used in this study.

Chemical/ Kit	Company	Country
p-nitrophenyl phosphate disodium salt hexahydrate (*p*-NPP)	Sigma-Aldrich	Steinheim, Germany
p-aminophenylphosphate monosodium salt hydrate (*p*-APP)	Enzo Life Sciences AG	USA
17β-estradiol (E2)	Sigma-Aldrich	Steinheim, Germany
Lentikat® liquid (polyvinyl alcohol)	GeniaLab BioTechnologie	Germany
Lentikat® stabilizer	GeniaLab BioTechnologie	Germany
Nucleobond Kit XA, Midi	Machery-Nagel	Düren, Germany

The equipment and electrode were used for electrochemical experiments are listed in the Table 2-2.

Table 2-2. Electrochemical equipment and material.

Electrochemical material	Company	Country
Thick film electrode (Pt-Ag/AgCl-Pt)	BST Bio Biosensor Technology GmbH	Germany
Palmsens potentiostat	Palm Instruments BV	The Netherlands

Unless otherwise stated, the general equipments and softwares are presented in the Table 2-3.

Table 2-3. Equipments and softwares.

Equipment/ Software	Company	Country
Vortex	Scientific Industries Inc	New York, USA
Waterbath	GFL mbH	Burgwedel, Germany
Celloshaker Variospeed	Thermo Scientific	Karlsruhe, Germany
Incubation cupboard	Thermo Scientific	Karlsruhe, Germany
Sunrise® absorbance reader	Tecan Trading AG	Switzerland
Sigma Plot 11	Systat Software Inc.	Erkrath, Germany
BioVal®	quo-data GmbH	Dresden, Germany

2.1.3 Strains and vectors

2.1.3.1 Strains

E. coli and yeast *A. adeninivorans* strains listed in the following Table 2-4 and 2-5 were used for this study.

Table 2-4. *E. coli* strains.

Strain	Genotype	Reference
XL1 Blue	*endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44* F'[*::*Tn10 *proAB+ lacIq Δ(lacZ)*M15 *Amy CmR*] *hsdR17(rK⁻ mK⁺)*	Stratagene

Table 2-5. Yeast strains (*A. adeninivorans*).

Strain	Genotype/YRC	Source	Reference
G1212	*aleu2 atrp1::ALEU2*	Gatersleben, Germany	Steinborn *et al.*, 2007b
G1214	*aleu2 aura3::ALEU2*	Gatersleben, Germany	Giersberg *et al.*, 2012
P2-M-P30	YRC102-hERα-phyK	Gatersleben, Germany	Kaiser *et al.*, 2010
P2-C-N15	YRC102-phyK	Gatersleben, Germany	Kaiser *et al.*, 2010

2.1.3.2 Vectors

Vectors utilized in transformation of yeast strains are listed in the Table 2-6.

Table 2-6. Vectors.

Name	Selective marker	Reference
Xplor2-102-GAA(2ERE)^{107}-phyK	Kan^{r}	Kaiser *et al.*, 2010
Xplor2-102-hERα-GAA(2ERE)^{107}-phyK	Kan	Kaiser *et al.*, 2010
Xplor2-103-hERα-GAA(2ERE)^{107}-phyK	Kan^{r}	Giersberg *et al.*, 2012

2.1.4 Media

Media used for bacterial and yeast cultivation are presented in the Table 2-7 and 2-8, respectively.

2.1.4.1 Bacterial media

Table 2-7. Luria Bertani Media (LB-Broth).

Medium	Amount per 1 l	Source
LB-full medium powder	20 g	LB-Broth, Sigma (Steinheim, Germany)
LB-full medium Agar	35 g	LB-Agar, Sigma (Steinheim, Germany)

LB broth base was dissolved in deionized water and sterilized by autoclaving. For the selection of *E. coli* transformants the antibiotic kanamycin (Applichem, Darmstadt, Germany) was added at the final concentration of 100 µg mL^{-1}. The medium could be stored at 4 °C for up to 3 months. Solid medium for plates was prepared by adding 1.6% (w/v) agar to the liquid medium.

Table 2-8. SOB and SOC media.

Medium	Component	End concentration
SOB	Bacto-Trypton (Difco/USA)	2.0% w/v
	Yeast extract (Gibco/UK)	0.5% w/v
	NaCl	10.0 mM
	KCl	2.5 mM
	$MgCl_2$	10.0 mM
	$MgSO_4$	10.0 mM
SOC	SOB components	
	Glucose	2%

All stocks were dissolved in deionized water. After the pH of the media was adjusted to 7.5 they were sterilized by autoclaving. When stored at 4 °C media can be used for up to 3 months.

2.1.4.2 Yeast medium and supplements

The components used for preparing yeast media and supplements are listed in the Table 2-9.

Table 2-9. Yeast media and supplements.

Medium	Component per 1l	Final concentration
YPD liquid medium (non-selective medium)	50 g YPD-Broth, Fluka (Steinheim, Germany)	——
YPD agar medium	Bacto-agar	2%
Yeast minimal medium (YMM) (pH 6,1)	3.7 g $NaNO_3$	92 mM
	6.75 g KH_2PO_4	50 mM
	1.75 g K_2HPO_4	10 mM
	1 g $MgSO_4$ x 7 H_2O	8 mM
	1 ml mineral mixture	0.1%, v/v
	1 ml $FeCl_3$ x 6 H_2O (2 g L^{-1} stock solution)	0.012 mM
	1 ml $Ca(NO_3)_2$ (20 g L^{-1} stock solution)	0.12 mM
YMM-suplement 1	Carbon source (glucose, maltose, sorbitol) (20%, w/v Stock solution)	2%, w/v
YMM-suplement 2	5 ml vitamin mixture-stock solution	0.5%, v/v
Vitamin mixture stock solution	400 mg thiamine hydrochloride	1.19 mM
	4 g inositol	22.2 mM
	100 mg nicotinic acid	0.81 mM
	400 mg Ca-D-Pantothenate	1.53 mM
	400 mg pyridoxine	2.36 mM
	4 mg biotin	0.016 mM
Mineral mixture	500 mg H_3BO_4	6.4 mM
	100 mg $CuSO_4$ x 4 H_2O	0.63 mM
	100 mg KJ	0.6 mM
	400 mg $MnSO_4$ x 4 H_2O	2.65 mM
	400 mg $ZnSO_4$ x 7 H_2O	2.48 mM
	200 mg Na_2MoO_4	0.97 mM
	100 mg $CaCl_2$	0.77 mM
Amino acid/ pyrimidine base	2 ml tryptophan (10 mg mL^{-1})	0.02 mg mL^{-1}
	10 ml uracil (2 mg mL^{-1})	0.02 mg mL^{-1}

Yeast medium was prepared in deionized water and sterilized by autoclaving. After cooling down to 50 °C, medium was supplemented with a carbon source, vitamin mixture and if necessary, the appropriate amino acid/pyrimidine base. Carbon source solution was sterilized by placing it in the steamer (95 °C) for 20 min each day (repeat 3 days). After sterilization carbon source solution could be stored at 4 °C for longer use. Vitamin mixture was sterilized by filtration through a 0.22-micron filter. The mineral mixture was sterilized by autoclaving after which it could be stored at 4 °C for up to 3 months. Tryptophan and uracil were sterilized by autoclaving. Tryptophan could be stored at 4 °C and uracil at 25 °C. Solid medium for plates was prepared by adding 1.6% (w/v) agar to the liquid YMM with the respective carbon source (Rose *et al.*, 1990; Tanaka *et al.*, 1967) and appropriate amino acids if necessary.

2.2. Methods

2.2.1 Cultivation

2.2.1.1 Cultivation of *E. coli* cells

E. coli cells were grown at 37 °C on a liquid or solid LB media supplemented with 100 mg L^{-1} kanamycin. Liquid culture was incubated overnight in Erlenmeyer flasks and test tubes at 180 x g in the shaker.

2.2.1.2 Cultivation of yeast cells

Engineered yeast strains were grown at 30 °C under selective conditions in a yeast minimal medium supplemented with 2% (w/v) glucose, sorbitol or maltose (YMM-glucose, YMM-sorbitol, and YMM-maltose) for 48 h. Liquid cultures were incubated in Erlenmeyer flasks and test tubes on a shaker at 180 x g; in the 48 and 96 deep-well plates at 250 and 300 x g, respectively.

2.2.2 Storage condition of bacterial and yeast cells

For long-term storage of *E. coli* and yeast cells 100 µl of glycerol was added to 900 µl of cell culture followed by mixing on a vortex. This suspension could be stored at -80 °C (Kirsop, 1988). The yeast strain grown in YMM or YPD agar media was re-streaked every 2 to 4 weeks.

2.2.3 Methods used for work with DNA

Handling of the DNA was according to the techniques described in the book "Molecular cloning: a laboratory manual" (Sambrook & Russell, 2001).

2.2.3.1 Transformation of *E. coli* cells

For transformation purposes, 10-20 µl of DNA ligation mixture were added to 100 µl chemical competent *E. coli* cells which were then incubated on ice for 30 min. Subsequently, cells were placed at 42 °C for 90 sec and again cooled on ice. Following 200 µl of SOC medium were added and the cells were incubated under shaking for 45 min at 37 °C. Cells were spread on LB plates containing kanamycin and incubated overnight at 37 °C.

2.2.3.2 Mini isolation of plasmid DNA from *E. coli* cells

Single colonies were selected and cultivated overnight in test tubes with 2 ml liquid LB medium supplemented with ampicillin (100 µg mL^{-1}) at 37 °C. Initially overnight cultures were transferred to 1.5 ml Eppendorf tubes and centrifuged for 3 min. The supernatant was removed and the pellet was suspended in 200 µl buffer P1 (cold) by vortex. After 200 µl of buffer P2 (25 °C) was added, the suspension was incubated at 25 °C for 3 min. 200 µl buffer P3 (cold) was then added and the solution was centrifuged for 20 min at 4 °C. From the clear supernatant, 500 µl were transferred into a new 1.5 ml Eppendorf tube. The plasmid DNA was precipitated by adding 350 µl isopropanol, mixing and incubating at 25 °C for 2 min. The pellet containing the plasmid DNA was separated by centrifugation at 4 °C for 25 min. The supernatant was removed and the pellet washed with 70% ethanol (25 °C) followed by centrifugation at 4 °C for 3 min. The ethanol was removed and the pellet was dried in a vacuum dryer for 10 min. The dried pellet was suspended in 50 µl sterile water. All centrifugation steps were carried out at 12000 x g. Buffer P1, P2, P3 were obtained from "Qiagen" kit.

2.2.3.3 Midi isolation of plasmid DNA from *E. coli* cells

The isolation was performed as described in the protocol „Plasmid DNA purification (NucleoBond®Xtra Midi/Maxi)" by Macherey-Nagel (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

2.2.3.4 DNA restriction

DNA digestion with restriction enzymes was performed according to company's instructions assuming that under optimal temperature conditions 1 unit of enzyme digests 1 µg of λ-DNA in 60 min.

2.2.3.5 Agarose gel electrophoresis

DNA fragment was separated by agarose gel electrophoresis. Sample with 4 x DNA loading buffer (Table 2-10) was loaded onto 0.8% agarose gel containing ethidium bromide (0.5 µg mL^{-1}). Electrophoresis was performed at 25 °C in 1 x TB buffer (Table 2-10) with an applied voltage 8-10 V cm^{-1}. DNA markers were added for size determination of the DNA fragments obtained.

Table 2-10. Solutions used for agarose gel electrophoresis.

Solution	Content	Final concentration
4 x DNA loading buffer (100 ml)	Tris /HCl pH 7.6	10 mM
	Glycerol	60%
	EDTA	60 mM
	Bromophenol blue	0.03%
10 x TB buffer	Tris	1 M
	EDTA	25 mM
	Boric acid	0.1 M

All solutions were prepared in deionized water. Stored at 25 °C the 4 x DNA loading buffer could be used for up to 12 months; 10 x TB buffer was stored at 4 °C for up to 3 months.

2.2.3.6 DNA fragment extraction from agarose gel

Individual DNA bands were cut from the agarose gel with a razor blade under UV light. DNA was extracted using the gel extraction kit from OLS (OMNI Life science).

2.2.3.7 DNA ligation reaction

Ligation reaction was performed at 14 °C for 18 h or at 25 °C for 2-3 h. The ratio of vector to insert was 1:5 or 1:3 depending on DNA concentration and size of the DNA frag-

ment. 1 unit of T4 DNA ligase (5 U μL^{-1}, Thermo Scientific) was used in final mixture volume of 30 µl. After the reaction, the enzyme was inactivated by heating at 65 °C for 15 min and DNA was precipitated by ethanol.

2.2.3.8 Precipitation

A solution containing 6 µl of 3M Na-acetate pH 5.2 and 112 µl of 96% ethanol was added to 50 µl volume of enzyme reaction mixture. This mixture was placed at -20 °C for 10 min and afterwards centrifuged at 4 °C, 13000 x g for 10 min. The resulting DNA pellet was washed 3 times with 1 ml of 70% ethanol and 1 time with 96% ethanol. After a final centrifugation at 4 °C, 13000 x g for 10 min the pellet was air dried at 25 °C for about 10 min. The dried pellet was suspended in 30 µl sterile water.

2.2.4 Methods used for work with yeast cells

2.2.4.1 Preparation of competent *A. adeninivorans* G1214 cells

A single colony of *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*] cells was inoculated in YPD medium supplemented with 40 mg L^{-1} uracil (final concentration) at 30 °C and 180 x g for 24 h. 1 ml of this starting culture was cultivated in 200 ml YMM medium supplemented with 2% glucose and 40 mg L^{-1} uracil at 30 °C and 180 x g for about 3-4 h until the OD_{600} reached a value of approx. 1.5. Subsequently, 50 ml of cell culture was centrifuged at 25 °C, 3000 x g for 5 min. The pellet was suspended with 10 ml of 50 mM sodium phosphate buffer pH 7.5 supplied with 250 µl 1 M DDT (Table 2-11) and incubated at 30 °C for 20 min. Centrifugation was repeated, and the cell pellet was suspended in 250 µl solution A (see Table 2-11). Aliquots of 80 µl were pipetted into sterile Eppendorf tubes and stored at -80 °C.

Table 2-11. Solutions for preparation of competent *A. adeninivorans* G1214 cells.

Solution	Content per liter	Final concentration
Solution A	92.4 g sucrose	270 mM
	1.21 g tris	10 mM (pH 7.5)
	0.2 $MgCl_2$ x 6 H_2O	1 mM
1 M DTT	154 g 1.4-dithiothreitol	1 M

All solutions were prepared in deionized water, pH was adjusted to 7.5. Solution A was stored at 4 °C for up to 3 months. 1 M solution of DTT in deionized water was sterilized by filtration. Aliquots of 2 ml were stored at -20 °C for up to 12 months.

2.2.4.2 Transformation of *A. adeninivorans*

Yeast transformation was performed according to Dohmen *et al.*, (1991). First, 20 µl of DNA carrier were placed at 95 °C for 5 min in order to denature and form single strand DNA and immediately afterwards put on ice for 1 min. The 20 µl of DNA carrier were then mixed with 20 µl of DNA plasmid. The mixture was subsequently added to competent yeast cells and placed at 37 °C for 5 min under gentle shaking (cells should not be thawed until immediately before mixing with the DNA solution). Next 1.4 ml of 40% PEG 1000 (poly ethylene glycol) and 0.2 M Bicine were added and the suspension was incubated at 30 °C for 1 h without shaking. After centrifugation at 25 °C, 4000 x g for 5 min, the cellular pellet was gently dissolved in 0.15 M NaCl + 0.01 M Bicine and centrifuged again at 25 °C, 4000 x g for 5 min. Finally, the pellet was dissolved in 500 µl of 0.15 M NaCl + 0.01 M Bicine and 100 µl of this mixture was spread onto agar plate and incubated at 30 °C for 3-5 days.

2.2.4.3 Cultivation of *A adeninivorans* after transformation (stabilization)

Stabilization step after the yeast transformation serves to maintain the plasmids or cassettes which were integrated homologously in the yeast genome during large-scale culture. This is important not only to attain a high level of production but also to guarantee the quality of the desired proteins (Kuriyama *et al.*, 1992). A modified protocol for the sequence of passages on selective and non-selective media was used (Klabunde *et al.*, 2003). In this protocol the stabilization period lasted 3 weeks and was carried out as follows: Single colonies from transformation agar plates were picked up and inoculated separately in 96 deep-well plates (riplateSW® 2.5 ml of bio-HJ, Mönchengladbach, Germany) with 500 µl of YMM containing glucose (2% w/v) as carbon source and, if required, an appropriate amino acid source. 10 µl of each culture were re-cultivated every 48 h for six times. After these steps, the cells were continuously cultivated in 500 µl of YPD twice for 24 h. Final cultivation was in 500 µl YMM supplemented with glucose (2% w/v) for 48 h. After that 3 µl of each culture were dripped onto the appropriate YMM agar plate and incubated for 48 h. Finally, yeast strains growing in solid media can be used for starting culture.

2.2.4.4 Isolation of chromosomal DNA from *A. adeninivorans* cells

Isolation of chromosomal DNA from yeast cells was performed according to a modified method of Ledeboer *et al.* (1985). A single colony from an YMM agar plate was picked and inoculated in 5 ml YPD at 30 °C, 180 x g for 24 h. Following the cell pellet was collected in a SS34 tube by centrifugation at 4 °C and 5000 × g for 5 min. The pellet was transferred into a 2 ml tube, washed twice with 1 ml distilled water and centrifuged again at 4 °C, 5000 × g for 5 min and suspended and incubated in 500 µl freshly prepared lysis solution (Table 2-12) for 1 h at 37 °C. After another centrifugation at 4 °C, 5000 x g for 10 min, the pellet was suspended in 500 µl of EDTA 0.05 M, pH 7.5 and 50 µl of 10% SDS (Table 2-12). This mixture was incubated at 65 °C for 30 min to degrade residual proteins in the cell lysate. Subsequently, the lysate was placed on ice, supplemented with 200 µl of cold 5M potassium acetate (Table 2-12) and chilled for 1 h on ice. After centrifugation at 4 °C, 13000 × g for 5 min, the clear supernatant was transferred into a new 2 ml Eppendorf tube, mixed with 750 µl isopropanol and placed at 25 °C for 5 min. The isopropanol was removed by centrifugation at 4 °C, 13.000 × g for 15 min. The chromosomal DNA pellet was washed twice with 150 µl of 70% ethanol and dried in the SpeedVac® for 10 min.The dried pellet was suspended in 40-50 µl distilled water.

Table 2-12. Solutions used for isolation of yeast chromosomal DNA.

Solutions	Contents
Lysis solution	In volume of 20 ml 50 mg zymolase (4 °C) 20 ml sodium phosphate buffer pH 7.5; 0.05 M 20 µl mercaptoethanol (4 °C)
10% SDS	In volume of 500 ml 50 g SDS
5M potassium acetate	In volume of 100 ml 49.07 g potassium acetate

All solutions were prepared in deionized water. Lysis solution was always prepared freshly, 10% SDS was stored at 25 °C for up to 3 months and 5 M potassium acetate was stored at 4 °C for up to 3 months.

2.2.5 Integration of the cassette containing *AURA3mm* selection marker module in the auxotrophic uracil mutant *A. adeninivorans* G1214

2.2.5.1 Construction of the plasmid Xplor2-103-hERα-GAA2ERE107-phyK

The plasmid with *AURA*3*mm* selection marker module was obtained after exchange of the *ALEU2* promoter-*ATRP1m* selection marker module from plasmid Xplor2-102-hERα GAA(2ERE^{107})-phyK (Kaiser *et al.*, 2010) by the *AURA3mm* selection marker module. In a first step the backbone plasmid was created by removing the *ALEU2* promoter-*ATRP1m* from the plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK by restriction with *Sal*I and *Afe*I. Simultaneously, the *AURA*3*mm* gene of the plasmid pCR4-AURA3mm-13 which is derived from *Arxula* plasmid cDNA library (YEP112A1NEXs-cDNA) (Wartmann *et al.*, 2003), was selected by digestion with the same *Sal*I and *Afe*I restriction enzymes. The *AURA3mm* gene fragment was subsequently ligated into the backbone plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK by T4 DNA ligase. As a result, the plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK was created. Design and physical map of these processes are shown in Fig. 2-2. Proper orientation of the ligated *phyK* fragment in the plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK was confirmed by digestion with *Eco*RI restriction enzyme.

2.2.5.2 Transformation of auxotrophic uracil mutant *A. adeninivorans* G1214 with the YRC containing *AURA3mm* selection marker module

Since plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK was based on the Xplor 2 system (Kaiser *et al.*, 2010), selection and expression modules of this vector system were inserted between the two d25S rDNA fragments flanked with *Asc*I restriction sites between the *E. coli* and yeast portions of the plasmid. Therefore, to assemble the final construct used for integration, the obtained plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK was digested with *Asc*I to remove the *E. coli* sequences including the kanamycin resistance marker. The resulting cassette with the combined modules flanked by the two d25S rDNA fragments was introduced into auxotrophic uracil mutant *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*] (Giersberg *et al.*, 2012) according to the transformation method described by Dohmen *et al* (1991) (see section 2.2.4.1). In contrast to the auxotrophic mutant *A. adeninivorans* G1214, which is not able to grow in YMM medium without uracil, the transformant *A. adeninivorans* G1214/YRC103-hERα-phyK carring the YRC with *AURA3mm* selection marker module can grow in YMM without uracil supplementation.

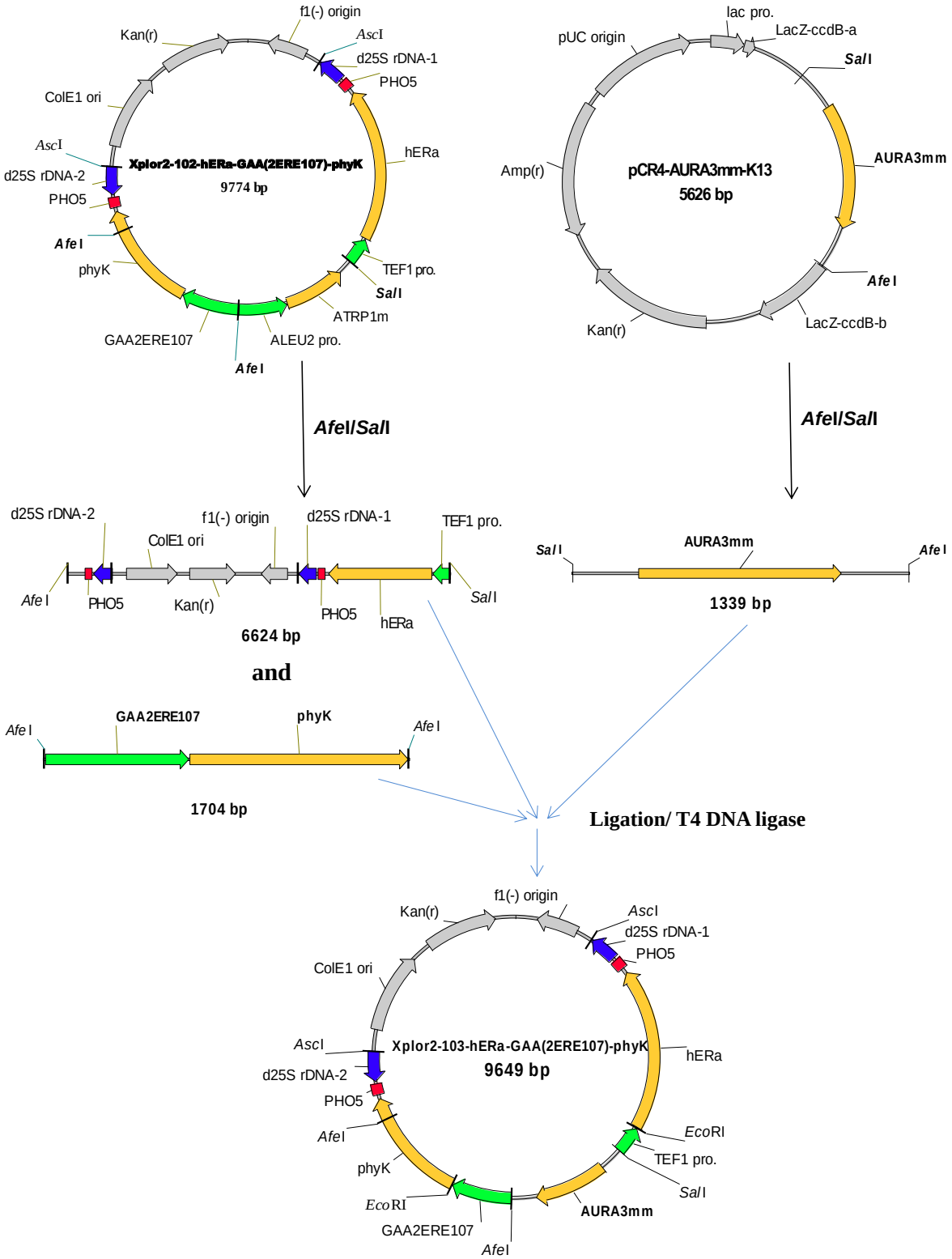

Fig. 2-2. Design and physical map of the plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK. Plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK was constructed as described in section 2.2.5.1. Since the basis of this plasmid construction was the Xplor 2 vector, plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK contains the following elements: *E. coli* elements (ColE1 ori; Kan(r); f1(-) origin) for propagation and selection in the *E. coli*; module for selection (*AURA3mm* fused to its promoter); the *phyK* expression module (*A. adeninivorans*-derived *TEF1* promoter-*phyK* reporter gene-*S.cerevisiae*-derived *PHO5* terminator; *hERα* expression module (*A. adeninivorans*-derived

GAA(2ERE^{107}*)* promoter-*hERα* receptor gene); the two d25S rDNA-2 target sequences disrupted by the insertion of modules; the *Asc*I restriction sites between *E. coli* part and the two d25S rDNA target sequences help to create yeast rDNA integrative cassette (YRC) by simple restriction with *Asc*I.

2.2.6 Immobilization of the yeast cells

2.2.6.1 Immobilization method

A modified protocol for Lentikat® production was used (Fine *et al.*, 2006; Jahnz *et al.*, 2002; Jekel *et al.*, 1998) to entrap yeast cells in polyvinyl alcohol (PVA). The process of immobilization method and illustration of the Lentikat are presented in the Fig. 2-3.

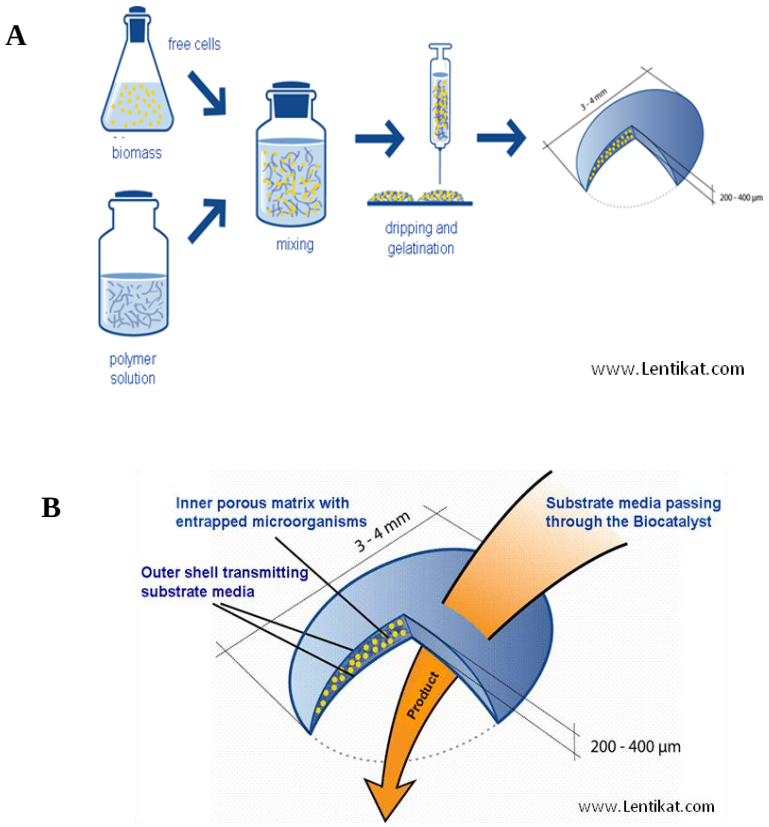

Fig. 2-3. (A) Process of immobilization method. The cell suspension was mixed throughout with Lentikat® liquid–PVA at a volume ratio of 1:4. This mixture was dripped onto the surface of the petri dish. Gelation by air-drying reduced the original mass of the droplets by 70%. **(B) Illustration of the Lentikat.** After immobilization, the obtained lenticular Lentikat consists of cells entrapped inside a porous matrix which helps to stably immobilize cells while still allowing a mass transfer of substrates and nutrient exchange.

Cells of *A. adeninivorans* G1212/YRC102-hERα-phyK and G1212/YRC102-phyK were collected by centrifugation at 4000 x g after 48 h growth in YMM-sorbitol. Pellets were

suspended in 0.5% NaCl to a cell titer of 15 x 10^9 cells mL^{-1}. This cell suspension was mixed thoroughly with Lentikat® liquid–PVA at a volume ratio of 1:4 to obtain a final cell density of 3 x 10^9 cells mL^{-1}. Small uniformly shaped 4 mm diameter droplets were formed by dripping aliquots of approx. 25.6 µl onto the surface of a petri dish lid using a sterile syringe with an inner diameter of 0.9 mm. Droplets were dried in a laminar airflow biohazard cabinet until their original mass was reduced by 70%. The resulting lens-shaped 'Lentikats' were then rehydrated in YMM-sorbitol containing 1.5% Lentikat® stabilizer.

2.2.6.2 Activation, stability and reusability of the immobilized cells

In order to adapt the immobilized cells to the new environment inside the matrix, they were activated in YMM-sorbitol at 30 °C for 12-48 h.

To establish the stability of the immobilized cells after activation, cells were stored at 4 °C in YMM-sorbitol and samples were taken over time to check the level of phytase secreted by the immobilized cells.

To demonstrate how many times the immobilized cells can be reused, the Lentikats were assayed according to a modification of the nAES-P assay (Kaiser *et al.*, 2010, see also section 2.2.8.2). After each experiment, Lentikats were washed twice with distilled water before reuse.

2.2.7 Electrochemical methods

2.2.7.1 Cyclic voltammetry

Cyclic voltametry (CV) is applied to investigate the electrochemical properties of the product (*p*-AP, AA) of the enzyme reaction. The studies were carried out using Palmsens potentiostat (Palm Instruments BV, The Netherlands, http://www.palmsens.com/home/) and PS lite software. CVs were performed with thick film electrodes, consisting of working, reference and counter electrodes (Pt-Ag/AgCl-Pt). Standard dimensions of the electrodes are 25.4 mm x 7 mm x 0.635 mm. 1 ml total volume of each the homologous solution (*p*-AP or AA) was held in the cryovial tube used for measuring. CVs of *p*-AP used scan rate of 50 mV s^{-1} and scanning from −200 mV to 200 mV. CVs of AA utilized scan rate of 50 mV s^{-1} and scanning from −200 mV to 600 mV.

2.2.7.2 Hydrodynamic voltammetry

Hydrodynamic voltammetry was used to select the optimal applied potential for the working electrode for the detection of electrochemical substrate (*p*-APP) and the intermediate product (*p*-AP). All measurements were made using thick film electrodes (see section 1.2.7.1). Measurements were made at potentials from 0 to 400 mV in 50 mV increments and in stirred solutions. The background signal (measured with citrate buffer) was allowed to reach steady state after which *p*-APP (0.1 mM) and *p*-AP (0.1 mM) were added and the signal measured for 5 min. All experiments were performed in triplicate.

2.2.8 Measurement of estrogenic activity

2.2.8.1 Amperometric detection

Non-immobilized yeast cells were first incubated with different E2 concentrations, YMM-maltose and 1.5 mM *p*-APP (prepared in 0.1 M sodium citrate buffer pH 3.9) in 48-deep well plates at 37 °C for certain time periods with orbital shaking (220 x g). Cells were removed by centrifugation at 5000 x g for 10 min. Supernatants were transferred to the measuring chambers of the EstraMonitor to amperometrically measure the current for 10 min at an optimal potential obtained from hydrodynamic voltametry experiments. To confirm the electrochemical analysis, the same supernatants were measured for 10 min using a 'Palmsens' potentiostat (Palm Instruments BV, The Netherlands, http://www.palmsens.com/home/).

Immobilized yeast cells (Lentikats) or non-immobilized cells were incubated with E2, YMM-maltose at 37 °C, 220 x g for 4 h in 48 deep-well plates. Following the Lentikats were allowed to settle and the supernatant was transferred into cryovial tubes (Cryo TubeTM Vials, Denmark). After 1.5 mM *p*-APP (prepared in 0.1 M sodium citrate buffer, pH 3.9) (final concentration) was added, the cryovial tube were transferred to the measuring chambers of the EstraMonitor. Amperomtric measurements were performed at 37 °C for 10 min.

For determination of estrogenic activities by the EstraMonitor, 8 'Lentikats' with immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK and G1212/YRC102-phyK cells were added to each measuring chamber along with 290 µl of YMM-maltose (1.8 x) and 236 µl of E2 solution (111 ng L^{-1}). Cells were incubated at 37 °C for 4 h with air sparging. After incubation, 186 µl of *p*-APP (6 mM prepared in 0.1 M sodium citrate buffer, pH 3.9) were added to each chamber and then further incubated at 37 °C for 10 min. Subsequently, amperometric signals were recorded for 10 min by the EstraMonitor software. After measure-

ment, the solutions in the measuring chambers were removed and distilled water was added to wash the Lentikats. 800 µl of YMM-maltose were then added and the cells were incubated for 2 min at 37 °C to re-establish background activity. Background signals were recorded for 5 min to demonstrate linearity. Note: all manipulations of the solutions were performed by the pump module of the EstraMonitor.

Table 2-13. Buffer solution for determining phytase activity

Solutions	Contents (in 1 liter)
0.1 M sodium citrate buffer (pH 3.9)	52.54 g citric acid x 1 H_2O 73.53 g tri-sodium citrate x 2 H_2O

All the contents were dissolved in deionized water, pH was adjusted to 3.9. Buffer solution was stored at 4 °C for up to 3 months.

2.2.8.2 Biochemical detection

A modification of the nAES-P assay (Kaiser *et al.*, 2010, Hahn *et al.*, 2006) was used.

Non-immobilized yeast cells (free cells) were incubated with E2 in YMM-maltose in a 48-deep well plate at 37 °C and at 220 x g for 4 h. After separation of the cells from the incubation medium by centrifugation (5000 x g, 10 min), 40 µl of the supernatant were incubated with 40 µl 3.5 mM *p*-NPP (prepared in 0.1 M sodium citrate buffer, pH 3.9) in 96-well plates (Nunc MaxiSorp® flat-bottom 96 well plate) at 37 °C for 60 min. The enzymatic reaction was stopped by adding 80 µl of 3 M NaOH and the absorbance of the solution measured at 405 nm with a Sunrise® absorbance reader (Tecan Trading AG, Switzerland).

Immobilized cells, Lentikats with yeast cells, were incubated with E2, YMM-maltose at 37 °C, 220 x g for 4 h in 48 deep-well plates. Then the Lentikats were allowed to settle. 40 µl of the supernatant was incubated with 40 µl 3.5 mM *p*-NPP (prepared in 0.1 M sodium citrate buffer, pH 3.9) in 96-well plate (Nunc MaxiSorp® flat-bottom 96 well plate) at 37 °C for 60 min. The enzymatic reaction was stopped by adding 80 µl of 3 M NaOH and absorbance of the solution was measured at 405 nm with a Sunrise® absorbance reader (Tecan Trading AG, Switzerland).

2.2.9 Calculation of the estrogenic activity

Scatter plots of M-E/I values versus E2 concentrations were constructed and the best-fit approach used for analysis (Scholze *et al.*, 2001; Motulsky and Christopoulus, 2004) based on M-E_{405nm} (phytase extinction) or I (current) from values of recombinant enzyme activities of the transgenic *A. adeninivorans* strains in pure water. The following four-parameter model, which is equivalent to the Hill-Slope model (Hill *et al.*, 1910) was used for modelling the M-E/I values depending on the E2 concentrations:

$$f(x_i) = y_{ij} = \frac{A-D}{1+\left(\frac{x_i}{C}\right)^B} + D + \varepsilon_{ij}$$

Here, y_{ij} denotes the M-E_{405nm}/I values (corrected extinction/current values) corresponding to the j^{th} measurement at concentration level x_i (concentration test agent/sample), and ε_{ij} describes the random error with mean zero and standard deviation σ. The four parameters *A*, *B*, *C* and *D* denote the unknown model parameters, which have to be estimated. *A* represents the mean value of the corrected extinction/current effects y_{ij} without estrogenic effects (at spike level 0, bottom curve point), where *D* is the mean value of y_{ij} with maximum estrogenic effect (curve plateau). *C* describes the mean effect concentration (curve point of inflection – EC_{50}) at which the estrogenic effect attains half of its maximum (*A*+*D*)/2, and the parameter *B* is proportional to the slope at *C*.

Calculation of these parameters has been carried out using the BioVal® software (QuoData GmbH, Dresden/Germany). In addition, the limit of detection (LoD) has been calculated according to DIN 32645, 1994. It describes the lowest analyte concentration likely to be reliably distinguished from zero level.

$$x_{LoD} = s_{x_0} \cdot t_{f,\alpha} \cdot \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\overline{X}^2}{Q_x}}$$

Where, s_{xo} : relative standard deviation (quotient of relative standard deviation of residual and slope); n : number of calibration points; m : number of measurements; Qx : sum of the mean square; $t_{f,\alpha}$:quantile of the t-distribution with one-sided issue for the error type 1; f: number of degrees of freedom; α: significant level; $\overline{x}$: mean value

In its calculation the uncertainty of the underlying four-parameter model has been taken into account (here the algorithm in the BioVal® software has been used). Relative residual standard deviations (RSD) for all calibration curves were calculated, giving a range of values from 4.4–29.1%.

The total estrogenic activities, 17β-estradiol equivalent (EEQ) of a wastewater samples which were assayed using *A. adeninivorans* G1212/YRC102-hERα-phyK cells and biochemical/ amperometric detection method were calculated by BioVal® software (QuoData, GmbH). After subtracting M-E_{405nm}/I value of spike sample from that of unspike sample, the EEQ-x was interpolated from equation 1 which was obtained from a calibration curve of the standard E2.

2.2.10 Statistical analyses

In order to analyze the results, the recombinant phytase activities were monitored in three separate experiments with at least three measurements per experiment. Modelling the calibration curve and statistical analysis of LoD and EC_{50} was performed using BioVal® software (quo data GmbH, Dresden, Germany).

For analysis, all data were tested for normality by the Kolmogorov Smirnov test. For normally distributed data, we calculated the mean and standard deviation of all replicates and checked them for significant differences compared to the solvent control using one-way analysis of variance (ANOVA α=0.05, two tailed) followed by Dunnett's post hoc test. In case of non-normally distributed data, the Kruskal-Wallis test followed by Dunn's post hoc test was used. Nonparametric Wilcoxon–Mann–Whitney tests (α=0.05, two-tailed) were applied to compare the medians of data sets. Outliers were excluded by using the Grubbs test (α=0.05, two tailed) (Motulsky, 2010). When no standard deviation is given, it can be assumed that the RSD value (based on the calibration curve) was less than 12%, which is the criterion for an accepted calibration curve.

3 Results

3.1. Electrochemical characterization of the components of the EstraMonitor

The intermediate product *p*-AP generated by the enzyme substrate reaction, is electrochemical active. Oxidation of the *p*-AP releases electrons which can be measured as an electric current by the amperometric detection method. In order to perform amperometric measurements, it is necessary to establish the electrochemical behaviour of the *p*-AP and the potential that has to be applied for the amperometric detection method.

3.1.1 Electrochemical behaviour of *p*-AP

Cyclic voltammetry was applied to examine the electrochemical properties of stirred solution of 1 mM *p*-AP (99.99%) in 0.1 M sodium citrate buffer pH 3.9. The line in blue in Fig. 3-1 shows the typical cyclic voltammogram of *p*-AP at a Pt-Ag/AgCl-Pt electrode, using a scan rate of 50 mV s^{-1} and a scan amplitude from -200 to +200 mV. The results demonstrate that *p*-AP exhibited a chemically reversible behaviour as indicated by similar sized peaks for the forward and reverse scan. The anodic current peak corresponding to the oxidation of *p*-AP was observed at about 150 mV versus Ag/AgCl. In contrast to this, the CV of the sodium citrate buffer shown in the red did not illustrate an oxidation or reduction peak.

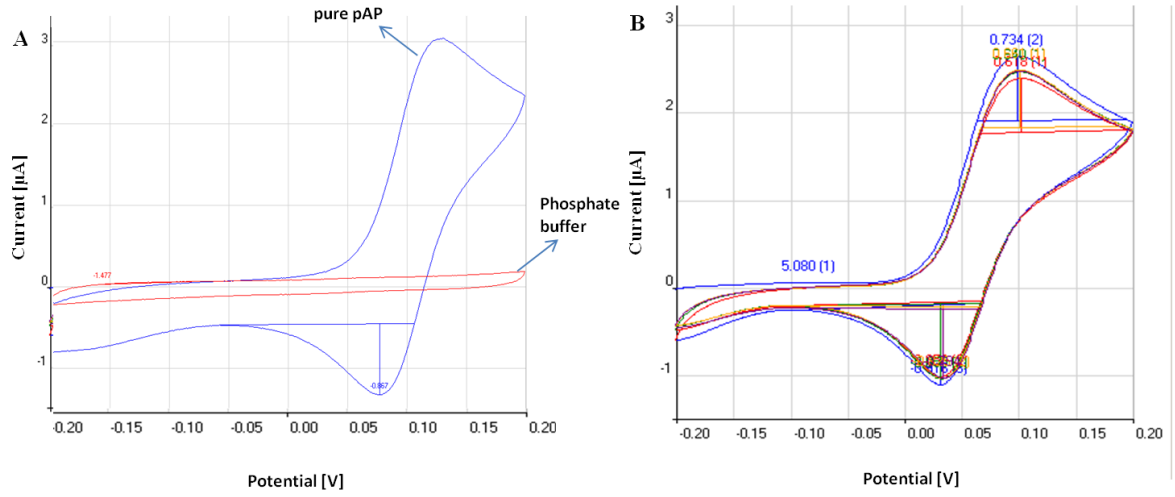

Fig. 3-1. Cyclic voltammogram of 1 mM *p*-AP in sodium citrate buffer (pH 3.9). (A) Scan rate 50 mV s^{-1}, CV of the sodium citrate buffer shown in red, CV of 1 mM *p*-AP shown in blue. (B) Results of five consecutive scans depicted in different colours. A Pt-Ag/AgCl-Pt electrode was used in all scans.

As a control five repetitive scans of *p*-AP by cylic voltammetry were performed, which gave near to identical curves (Fig. 3-1B). This proves that over the course of five scans *p*-AP does not cause any noticeable contamination on the surface of electrodes.

3.1.2 Optimal potential for the working electrode

The optimal applied potential of the working electrode (Pt-Ag/AgCl-Pt) was established for the electrochemical substrate *p*-APP and the intermediate product *p*-AP by performing hydrodynamic voltammetry at 50 mV intervals from 0 mV to 400 mV. As shown in Fig. 3-2A, the electro-oxidation current response (ΔI) of the intermediate product *p*-AP increases as a function of the applied potential to reach a maximum at 150 mV (versus Ag/AgCl) and remain at a steady state thereafter. At 150 mV potential *p*-APP was not oxidized and the ΔI therefore resulted from the oxidation of 0.1 mM *p*-AP only. Consequently, the potential of 150 mV was used in all subsequent studies.

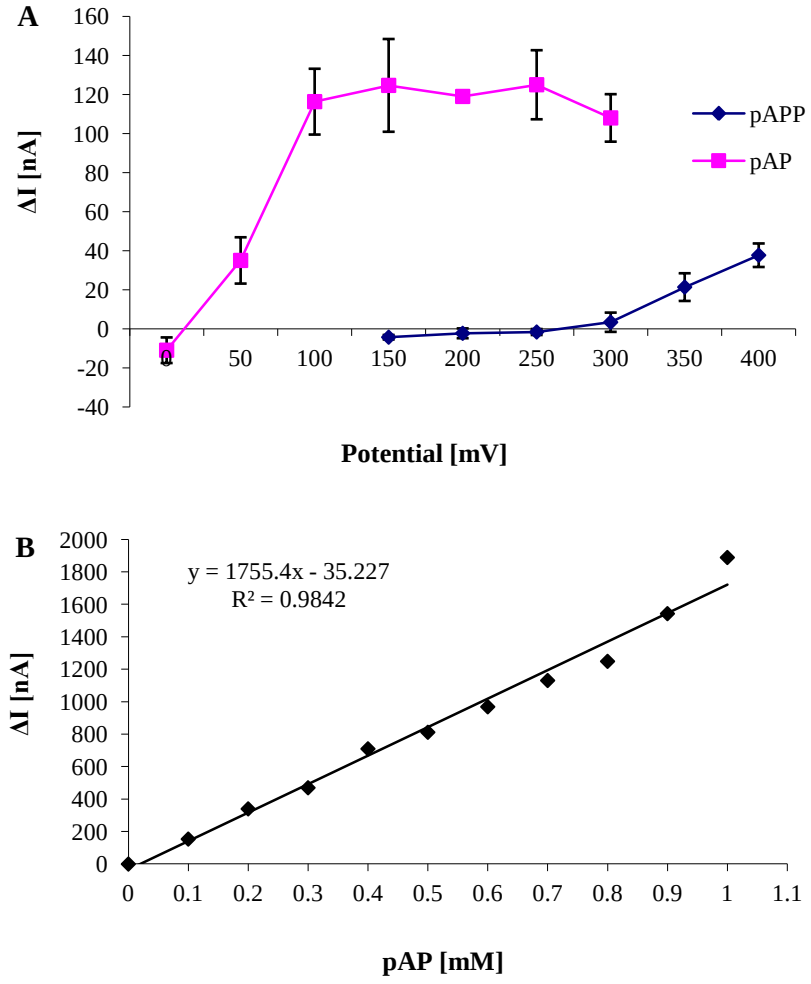

Fig. 3-2. (A) Values for hydrodynamic voltammograms measured at various potentials of 0.1 mM *p*-APP and 0.1 mM *p*-AP in 0.1 M sodium citrate buffer (pH 3.9) at 37 °C. ΔI values were calculated

as ΔI = IP - IB (IP = value for 0.1 mM *p*-APP or 0.1 mM *p*-AP; IB = value for citrate buffer). **(B) Correlation of *p*-AP concentration to current (ΔI).** Equation was obtained by regression analysis.

The electro-oxidation current density (ΔI) correlated directly to the *p*-AP concentration in a range from 0.1 mM to 1.0 mM. Regression analysis showed a linear relationship between both parameters with a high correlation coefficient (R^2 = 0.9842). The equation (y = 1755.4x – 35.227) can be used to calculate the phytase activity, and thus the E2 concentration (Fig. 3-2B).

3.2. Studies on the microbial component of the EstraMonitor

A. adeninivorans G1212/YRC102-hERα-phyK (measuring strain) and *A. adeninivorans* G1212/YRC102-phyK (control strain) constructed by Kaiser *et al.* (2010) were designed as the microbial components of the EstraMonitor. *A. adeninivorans* G1212/YRC102-hERα-phyK was engineered to co-express the human estrogen receptor (*hERα*) gene and the inducible phytase (*phyK)* reporter gene under the control of a promoter with estrogen response elements (*EREs*). Control strain *A. adeninivorans* G1212/YRC102-phyK only contains the reporter gene cassette. Before these microbial components were applied into the EstraMonitor, phytase production and suitability of the microbial components for the system were investigated.

3.2.1 Influence of temperature on phytase production

The effect of incubation temperature on the phytase production signal (M-E_{405nm}) using *A. adeninivorans* G1212/YRC102-hERα-phyK as the non-immobilized microbial component was performed using the nAES-P assay. The measuring signal showed a temperature dependent increase to reach a maximum of EC_{50} value of 115 ng L^{-1} and a LoD of 31 ng L^{-1} at 37 °C. At temperatures greater than 37 °C the signal decreased. At 42 °C the response was reduced by up to 25% and at higher temperatures the effect was more severe (Fig. 3-3). Therefore, a temperature of 37 °C must be considered optimal for the detection of estrogenic activities.

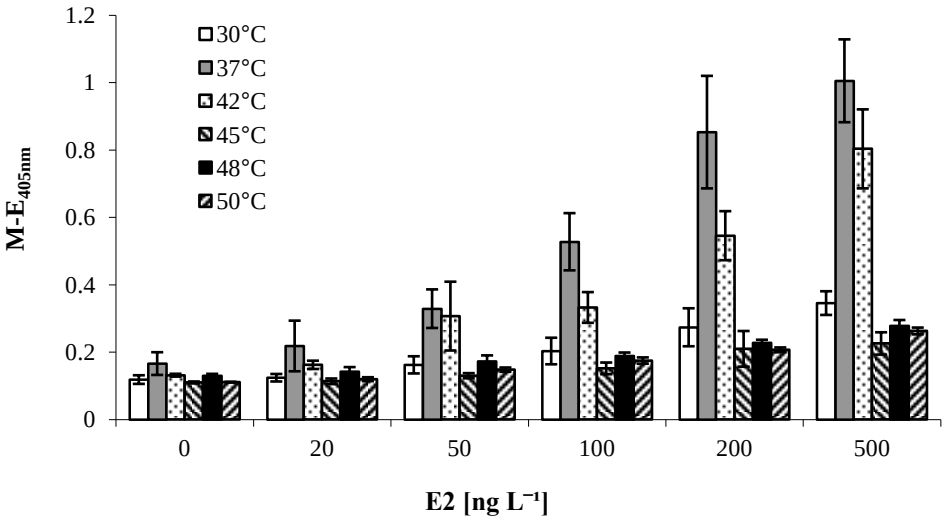

Fig. 3-3. Influence of temperature on the measuring signals (M-E_{405nm}) using *A. adeninivorans* G1212/YRC102-hERα-phyK. Graphs show the relative phytase activity after 4 h in relation to concentration of E2 at incubation temperatures of 30, 37, 42, 45, 48 and 50 °C.

3.2.2 Suitability of the microbial component for the EstraMonitor system

The working principle of the EstraMonitor is to use an amperometric method to detect the signals provoked by estrogenic substances. To evaluate the suitability of the microbial component for the amperometry, non-immobilized cells of *A. adeninivorans* G1212/YRC102-hERα-phyK (measuring strain) were incubated for up to 24 h with substrate (*p*-APP), YMM-maltose and E2 at 37 °C under orbital shaking conditions. At regular time points samples were transferred to the measuring chambers of the EstraMonitor for analysis.

The results showed a positive correlation between the length of the incubation period and concentration of E2 on one side and the intensity of the signals measured (Fig. 3A). This demonstrated the suitability of this new microbial biosensor system for quantitative measurements. The strongest signals were measured after 10 h incubation time. Longer incubation periods, however, caused an increase in control signals relative to the measuring signals e.g. an increase in the signal to noise ratio. An incubation period of 2 h was required to detect an E2 concentration of 20 ng L^{-1}, whereas higher concentrations (200 ng L^{-1} E2) could be detected within 60 min (Fig. 3-4A).

To investigate possible contaminations in the samples might influence the amperometric signals, negative controls without substrate, cells or dead cells, were included in the experiments. In contrast to the signals with a measuring strain, control samples produced a very low (<10 nA) background signal only (Fig. 3-4 B-D). These observations

demonstrate that neither samples, incubation medium (YMM) or cells affect the amperometric reaction and that the signals measured are a direct consequence of the phytase activity induced by E2.

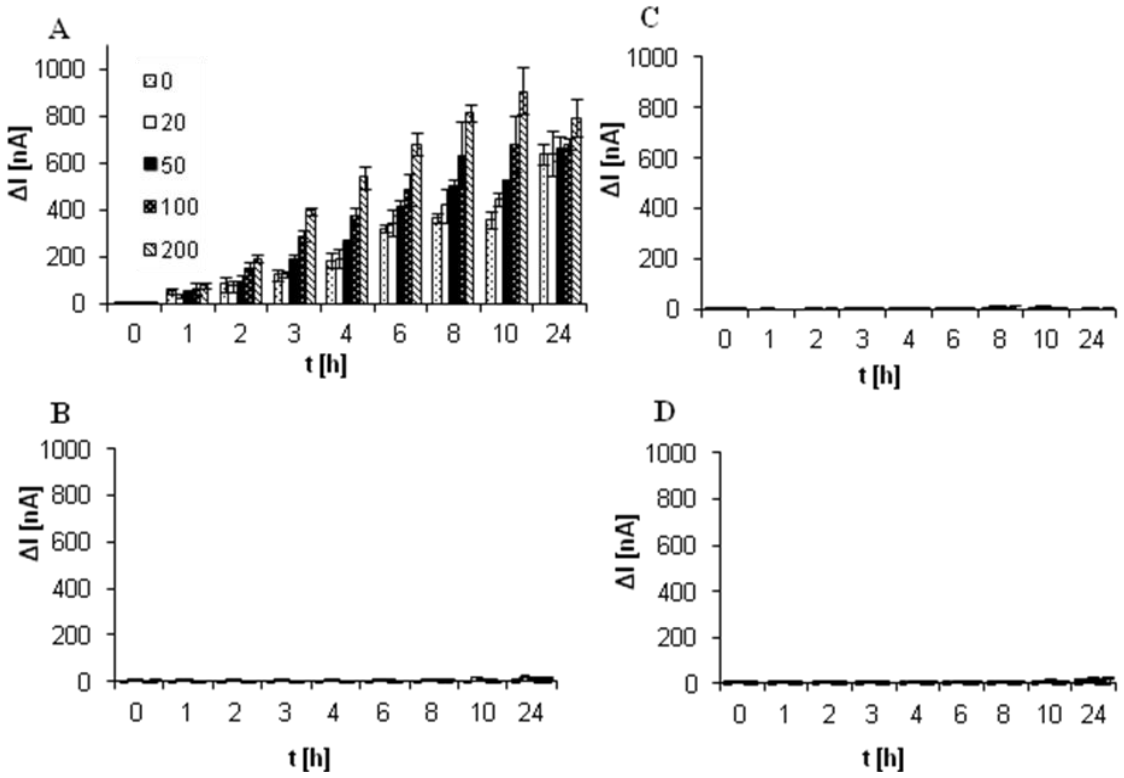

Fig. 3-4. Influence of E2 concentration and incubation time on the amperometric signals (ΔI) of the EstraMonitor with non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK as the microbial component. (A) Yeast cells were incubated with substrate, YMM-maltose and E2 at 37 °C with orbital shaking up to 24 h. (B) Negative control 1 – yeast cells with YMM-maltose and E2 but without substrate. (C) Negative control 2 – substrate, YMM-maltose and E2 without cells. (D) Negative control 3 - dead cells with YMM-maltose and E2. ΔI values were calculated as ΔI = IS - IC (IS = measuring value sample; IC = measuring value control without E2).

3.2.3 Standard curves for E2 using the EstraMonitor and Palmsens potentiostat

Measurements obtained with the EstraMonitor system were compared with those obtained with a commercially available potentiostat (Palmsens) using non-immobilized measuring and control strains (*A. adeninivorans* G1212/YRC102-hERα-phyK and *A. adeninivorans* G1212/YRC102-phyK respectively). The cells were incubated under standard procedures. For both methods performance characteristics were calculated (see Fig. 3-5) including A = I without estrogenic effects, B =slope at C, C =EC_{50} values, D = I with maximum estrogenic effect for E2. The EstraMonitor and the Palmsens analysis resulted in similar experimental values of

EC_{50} of 69.9 ng L^{-1} and 78.1 ng L^{-1}, respectively and LoD values of 44.5 ng L^{-1} and 43.9 ng L^{-1}, respectively (Fig. 3-5). The signal from the control strain remained static under increasing E2 concentration regardless of the analytical device used.

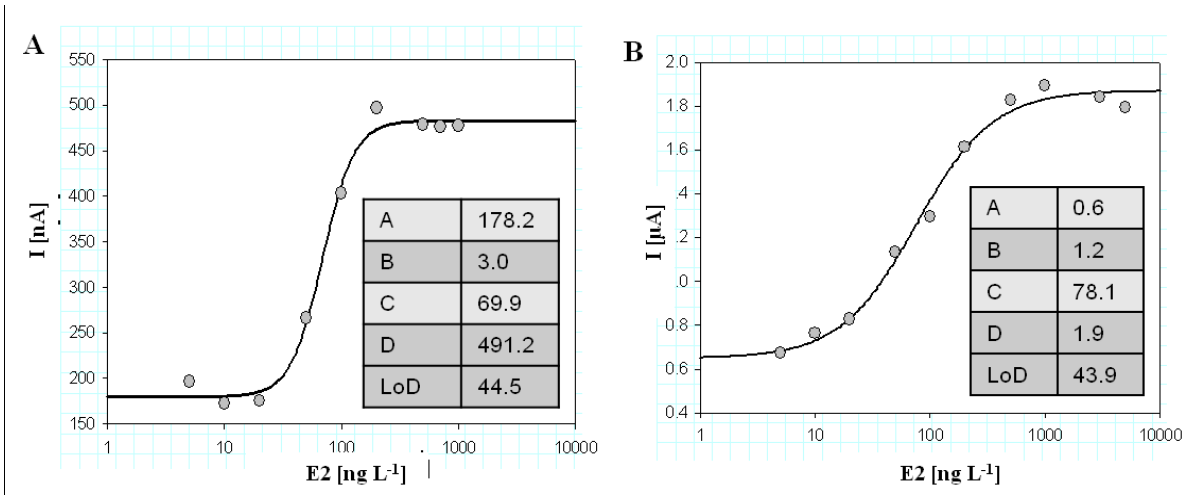

Fig. 3-5. Standard calibration curves of amperometric measurements with (A) EstraMonitor and (B) Palmsens potentiostat. Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (final OD_{600nm} = 30) were incubated with different E2 concentrations and 1.5 mM *p*-APP (pH 3.9) for 4 h. Supernatants were amperometrically measured for 10 min with each monitor. A represents mean I value without estrogenic effects, D implies the I value with maximum estrogenic effect, C is the concentration at which the I value equals (A+D)/2 and B is the slope which is proportional to C.

3.3. Studies on substrate used for amperometric measurement by the EstraMonitor

p-APP was applied as the enzyme substrate for amperometric detection. The phytase activity of the microbial component with respect to E2 was determined by using *p*-APP. The generated *p*-AP by enzyme strubstrate reaction was detected amperometrically at the electrode. For the purpose of utilizing *p*-APP in the EstraMonitor system, the following tasks were investigated.

3.3.1 Degradation of the substrate

In order to convey whether the substrate was degraded under presence of the cell culture, non-immobilized cells of *A. adeninivorans* G1212/YRC102-hERα-phyK were incubated with 1.5 mM *p*-APP (pH 3.9) and E2 concentrations ranging from 0 to 200 ng L^{-1}. The line in blue in Fig. 3-6 shows that in the present of 200 ng L^{-1} E2, the expected signals directly correlate to

the incubation time. In theory, in the absence of E2, phytase should not be synthesized and secreted into the media and therefore the substrate (*p*-APP) should not be transformed into the intermediate electrochemical active product (*p*-AP) and consequently no current should be generated. However, the results in Fig. 3-6 (pink line) show a gradual increae in current in samples containing no E2. The reason might be a substrate activated native phytase production or maybe the substrate was degraded into other substances, which were easy oxidized and released electrons (background signals).The higher signals in the samples incubated with E2 (blue line) when compared with those without E2 became clear that these signals must result from both substrate degradation and oxidation of the generated electrochemical active product *p*-AP. This observation suggests that the substrate must be added after incubation of the cells with E2 to avoid a high background signals.

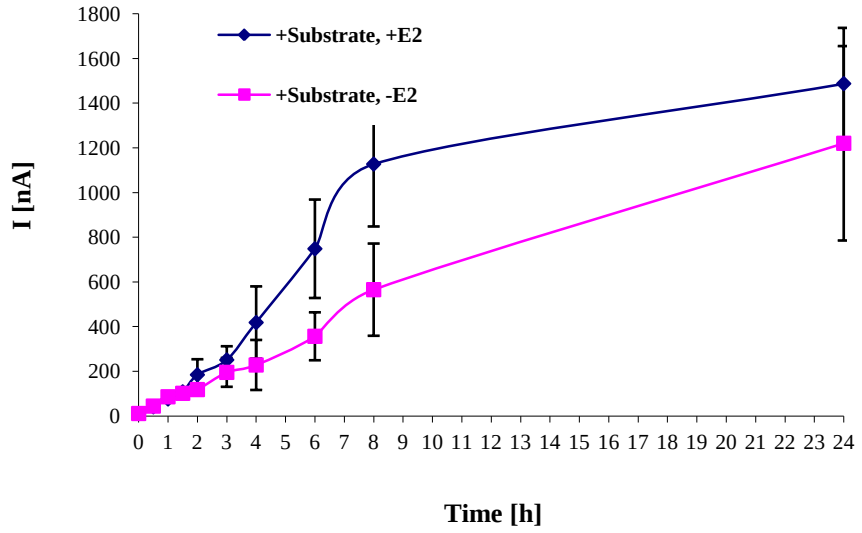

Fig. 3-6. Effect of substrate incubation condition on its degradation. Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with 1.5 mM *p*-APP (pH 3.9), YMM-maltose, 200 ng L^{-1} and 0 ng L^{-1} E2 at 37 °C, 220 x g in the 48 deep-well plate up to 24 h. Supernatants were taken for amperometric detection within 10 min at certain time periods (0, 1, 2, 3, 4, 6, 8 and 24 h).

3.3.2 Substrate incubation and measuring time

To investigate the substrate incubation time and the measuring time after addition of the substrate, the non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 concentrations (0, 5, 20, 50, 100, 200, 500 and 1000 ng L^{-1}) for 4 h. Amperometric measurements were performed directly after adding 1.5 mM substrate (pH 3.9). The signals were recorded after 5, 10 and 15 min. As shown in Fig. 3-7 the signals corre-

late with the E2 concentrations and attain their maximal value after 10 min of incubation. Since 10 min appeared sufficient for optimal signal measurement with the EstraMonitor system, a 10 min incubation time became standard in all following amperometric detection experiments.

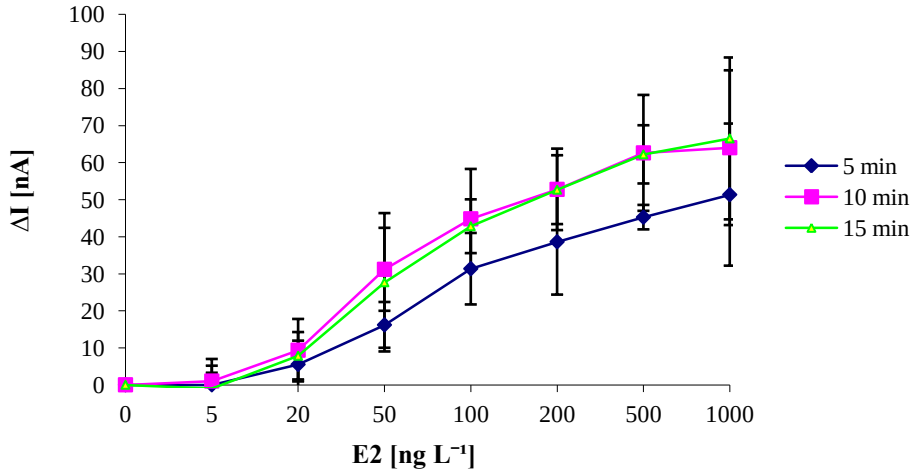

Fig. 3-7. Influence of substrate incubation time on signal intensity. Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 concentrations (0, 5, 20, 50, 100, 200, 500 and 1000 ng L^{-1}) at 37 °C, 220 x g in 48 deep-well plate for 4 h. After 1.5 mM *p*-APP (pH 3.9) was added, phytase activities were amperometrically measured for 15 min. ΔI was calculated as $\Delta I = I_E - I_0$ (I_E = electric current at different E2 concentrations; I_0 = electric current at E2 of 0 ng L^{-1}).

3.3.3 Substrate stability

If the EstraMonitor is to be used in an automated and *semi-online* mode in sewage treatment plants, the substrate for the amperometric detection must be incorporated within the apparatus under unaccompanied temperature control. Therefore, the long-term stability of the substrate was investigated after several days of storage at 25 °C and 4 °C. To this purpose, 7.5 mM substrate (*p*-APP) was dissolved in 0.1 M sodium citrate buffer (pH 3.9) and stored at 25 °C and 4 °C. The substrate solution was taken at day 1, 2, 3, 4, 5 and 6, incubated with the enzyme phytase, and subsequently amperometrically measured. Fig. 3-8A shows that the amperometric measurements of samples after the enzyme substrate reaction generate much higher signals compared to samples with substrate only. Obviously, in samples containing substrate and phytase, the enzyme transforms the substrate (*p*-APP) into the intermediate product (*p*-AP) which is subsequently oxidized, resulting in the release of electrons, which are detected as an electric current. In samples containing substrate only no intermediate products

are formed and thus no current generated. The results in Fig. 3-8A further show signals for substrate and enzyme substrate solutions stored at 4 °C remain stable for over 6 days and only slight increase when stored at 25 °C. However, when the signals of the control samples, measuring substrate only, are subtracted from the signals of the enzyme substrate reaction, it turns out that signal differences remain stable for 6 days (Fig. 3-8B). Therefore, substrate stability seems guaranteed for up to 6 days regardless if stored at or 4 °C or 25 °C.

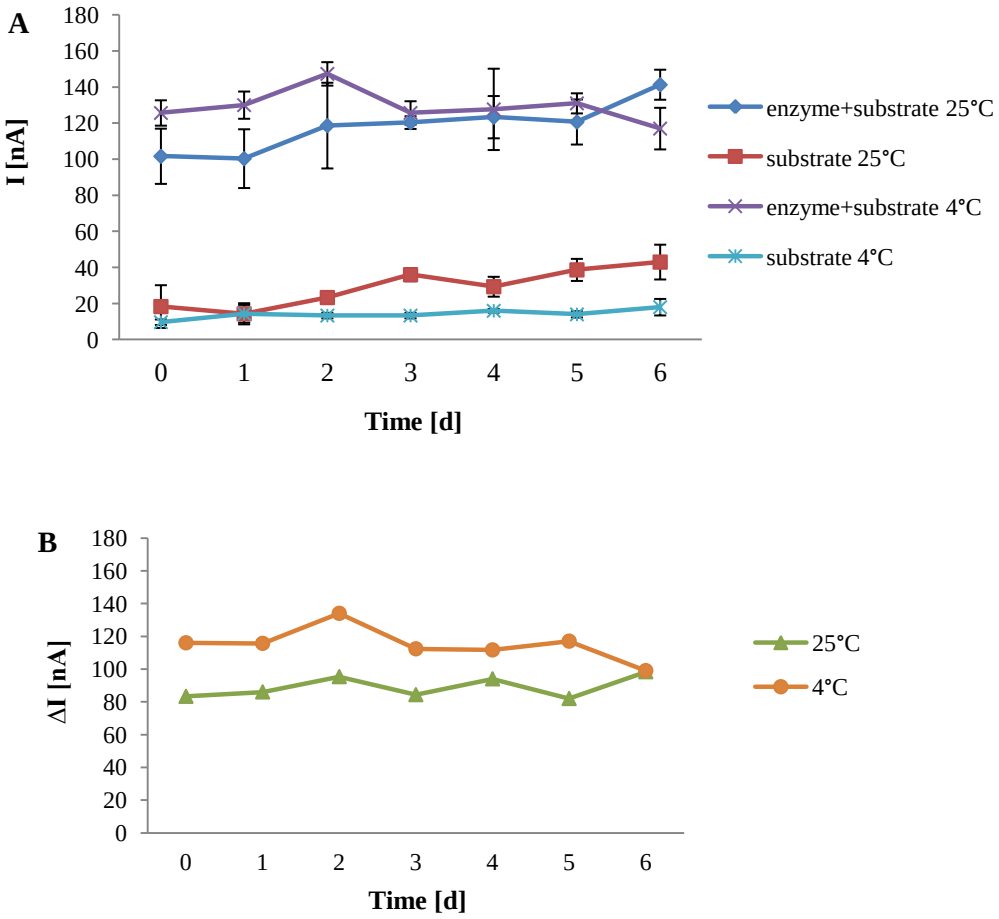

Fig. 3-8. Stability of the substrate *p*-APP. (A) Real values obtained from the amperometric detection. (B) Substracted values ($\Delta I = \text{value}_{\text{enzyme+substrate}} - \text{value}_{\text{substrate}}$). 7.5 mM *p*-APP was dissolved in 0.1 M sodium citrate buffer (pH 3.9) and stored at 25 °C or 4 °C. Enzyme reactions with phytase were carried on daily basis. Signals were amperometrically measured 5 min.

3.4. Working conditions for the EstraMonitor

3.4.1 Influence of air sparging on the measuring signals

The prototype of the EstraMonitor was designed without a shaking system for the microbial component. As an alternative to shaking, the efficiency of the microbial component

was examined in the absence and presence of air sparging. Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 concentrations at 37 °C for 4 h with and without air sparging (oxygen supplying system). Fig. 3-9 reveals that the electric currents from samples that were incubated with air sparging are 3 times higher than the signals from samples without sparging. This result clearly demonstrates the necessary of an air sparging system in the EstraMonitor.

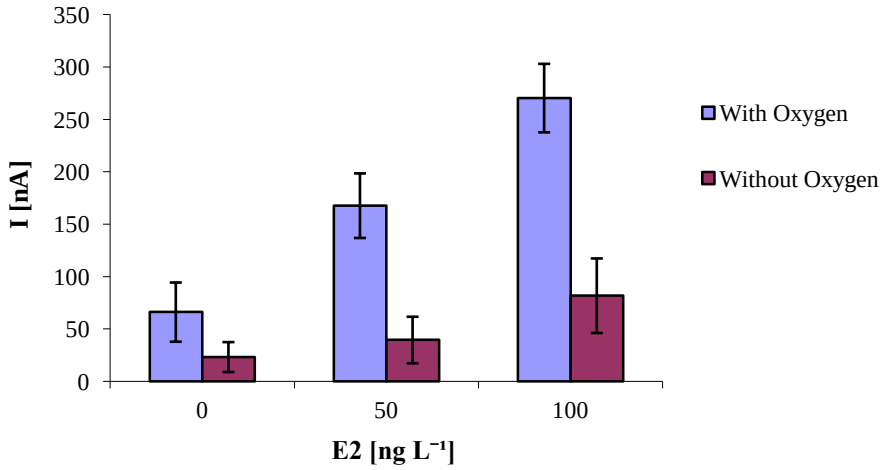

Fig. 3-9. Influence of air sparging on phytase production of non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells. Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 concentrations (0, 50 and 100 ng L^{-1}) at 37 °C for 4 h with and without air sparging. Phytase activities of these samples were amperometrically measured for 10 min after 1.5 mM *p*-APP (pH 3.9) addition.

3.4.2 Decontamination of the EstraMonitor

To eliminate the transgenic yeast cells, the EstraMonitor apparatus contains a decontamination system, which uses high temperature 80-85 °C for 4 min and 45 sec to treat the fluid waste. Before discharging into the waste reservoir, all waste will pass through this decontamination system. Samples taken before and after treatment were tested by determining the CFU (colony forming unit). The results in Fig. 3-10B indicate that after decontamination, no viable transgenic cells remain. Without decontamination a high number of cells survive (Fig. 3-10A).This additional function ensured that the contents of the yeast cell biosensor cannot escape into the environment.

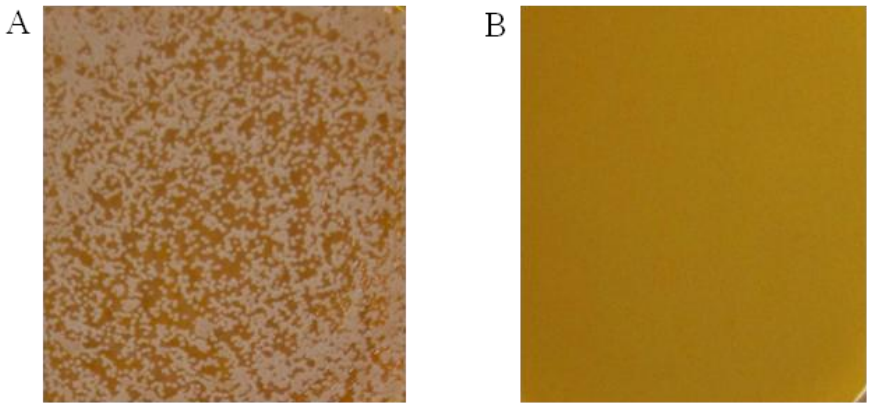

Fig. 3-10. EstraMonitor decontamination system eliminating the transgenic yeast cells. The CFU of 100 µl fluid waste before and after going through the decontamination system were determined on YMM agar plates. (A) Waste before treatment. (B) Waste after treatment by decontamination system.

3.5. Amperometric measurements using immobilized yeast cells

Immobilized cells are an important application in microbial biosensor because of their easy-handiness, long-term stability and reusability. Since the EstraMonitor designed was based on the principle of the microbial biosensor, *A. adeninivorans* G1212/YRC102-hERα-phyK, a microbial component of the EstraMonitor was also immobilized in a PVA matrix and its properties were studied.

3.5.1 Yeast cell immobilization in Lentikat® liquid (PVA)

Before their application in the EstraMonitor system, the estrogenic detection performance of the PVA-immmobilized yeast cells was characterized. For entrapment, a cell suspension of yeast *A. adeninivorans* G1212/YRC102-hERα-phyK was throughout mixed with Lentikat® liquid at a ratio of 1:4 (v/v). Subsequently this mixture (approx 25.6 µl) was dripped carefully but rapidly on the lid of a plastic petri dish using a syringe with an inner diameter of 0.9 mm. This approach yields uniform lenticular shaped particles (Lentikats) approx. 4 mm in diameter and 800 µm in height (Fig. 3-11).

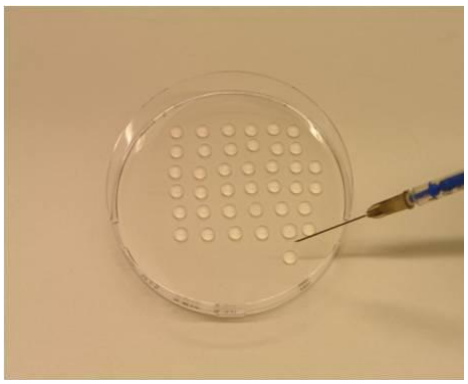

Fig. 3-11. Preparation of Lentikats. Lenticular Lentikats were obtained by dripping a homologous mixture of *A. adeninivorans* G1212/YRC102-hERα-phyK cell suspension and Lentikat® liquid (ratio 1:4 v/v) onto the surface of the lid of a plastic petri dish using a syringe with inner diameter of 0.9 mm.

Distribution of the cells inside the Lentikats was analyzed by means of the GFP (green fluorescent protein) expressing *A. adeninivorans* strain G1211/WRIV-ALEU2m-ARS-GFP. This yeast strain harboured a wide-range yeast integration expression vector (WRIV) containing a *GFP* reporter gene under control of the strong constitutive *A. adeninivorans*-derived *TEF1* promoter (Terentiev *et al.*, 2004), an *ALEU2m* selection marker and an autonomous replication sequence (ARS) modules. Thin slices of Lentikat embedded yeast cells were analyzed in a Zeiss LSM510 confocal laser scan miroscopy (CLSM). GFP was excitated with a 488 nm laser line and emission measured with a 505-530 bandpass filter. The results confirmed an uniform distribution of *A. adeninivorans* G1211/WRIV-ALEU2m-ARS-GFP yeast cells throughout the Lentikat (Fig. 3-12).

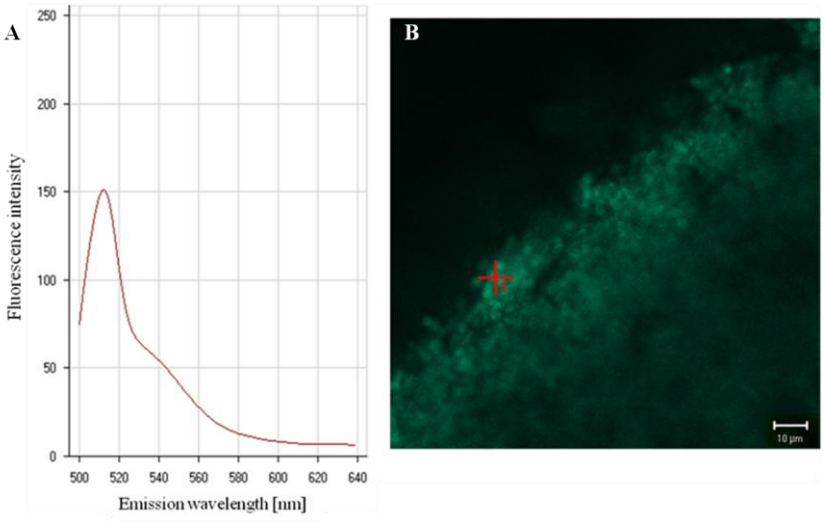

Fig. 3-12. Fluorescence-based distribution analysis of *A. adeninivorans* G1211/WRIV-ALEU2m-ARS-GFP cells in Lentikat. GFP expressing yeast cells were visualized in a CLSM using a 488 nm

laser line for excitation. (A) A photospectrometric analysis with the lambda-detector of the emission profile of the spot marked with ($+_1$) in (B) which showed an emission peak near 509 nm (A) thus confirming the presence of GFP. Bar = 10 µm.

3.5.2 Activation of immobilized cells

In order that the immobilized cells adapt to their new environment and multiply inside the PVA matrix, the activation step was done after cell immobilization. *A. adeninivorans* G1212/YRC102-hERα-phyK cells (with different cell densities) were first immobilized, followed by activation in YMM-sorbitol at 30 °C for 0, 12, 24, 30, 36, 42 or 48 h. As shown in Fig. 3-13, cell concentrations and duration of the activation period both had a clear effect on the signal measured. Optimal signal detection required a cell density of 3 x 10^9 yeast cells mL^{-1} in combination with 30 h long activation in YMM-sorbitol at 30 °C.

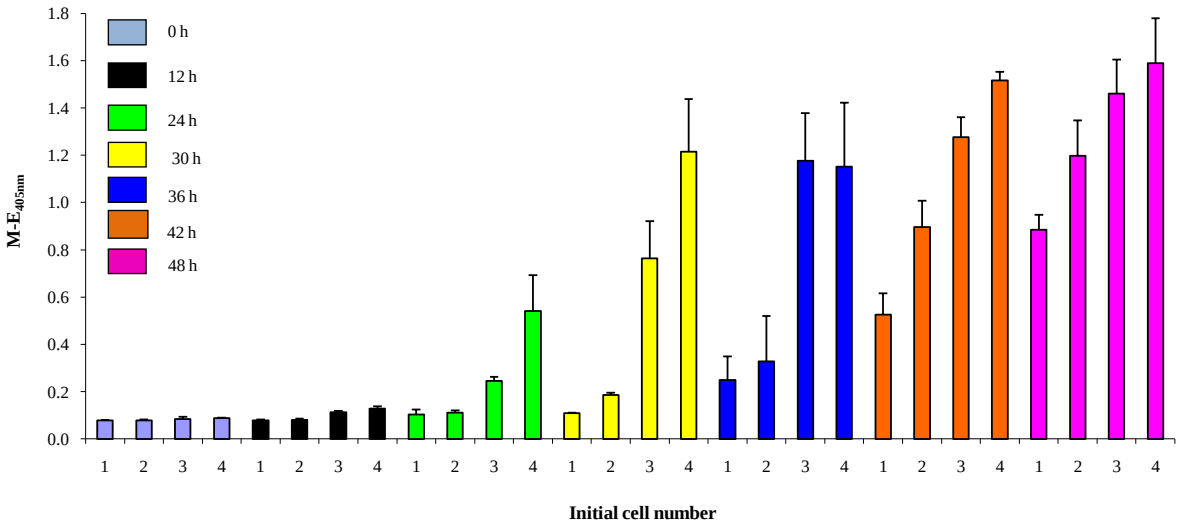

Fig. 3-13. Correlation of activation time and the cell concentration to the measuring signal intensity (M-E_{405nm}). Lentikats containing 0.75 x10^7 (1), 1.5 x10^7 (2), 3.75 x10^7 (3), 7.5 x10^7 (4) *A. adeninivorans* G1212/YRC102-hERα-phyK cells were activated in YMM-sorbitol at 30 °C for 0, 12, 24, 30, 36, 42 or 48 h. After each time interval, eight Lentikats were incubated with 100 ng L^{-1} E2, YMM-maltose for 4 h at 37 °C, recombinant phytase activity was biochemically measured by M-E_{405nm} in 0.1 M sodium citrate buffer, pH 3.9.

3.5.3 Optimal number of immobilized cells per measuring chamber

To evaluate the optimal number of immobilized cells (Lentikats) per measuring chamber of the EstraMonitor, different numbers of Lentikats with a cell density of 7.5 x 10^7 cells per Lentikat, were employed in the presence of 100 ng L^{-1} E2. Owning to the size limita-

tions of the 1.8 ml cryovial tube (Cryo tube™ Vials, Denmark) employed as measuring chamber, experiments with more than 10 Lentikats could not be performed. The results shown in Fig, 3-14 reveal a linear correlation between Lentikat numbers used and signal intensity measured. Although saturation had not been achieved, eight Lentikats were considered to be optimal for operation of the system and sufficient to give a low detection limit.

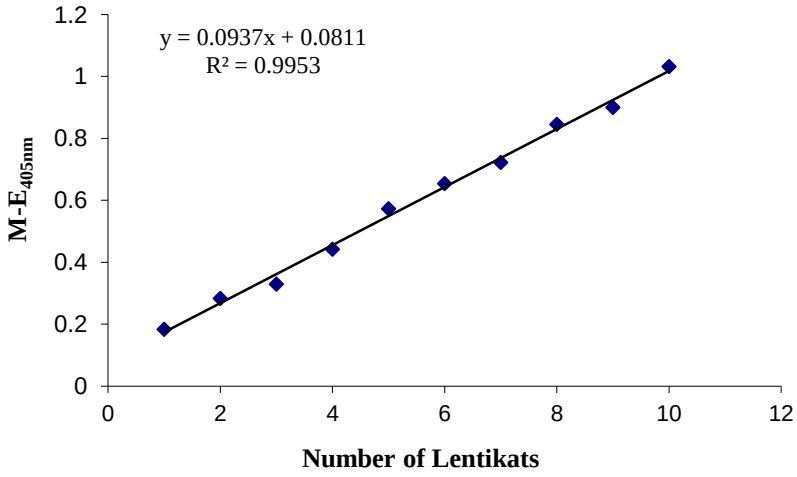

Fig. 3-14. **Effect of Lentikat number on the measuring signal intensity.** One to ten Lentikats (each containing 7.5 x10^7 cells) were incubated with 100 ng L^{-1} E2 for 4 h at 37 °C. Recombinant phytase activity was measured by M-E_{405nm} in 0.1 M sodium citrate buffer, pH 3.9.

3.5.4 Stability of the immobilized cells

Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were stored in YMM-sorbitol at 4 °C for 6.5 weeks to analyze their stability. At distinct time point, Lentikats were incubated with 100 ng L^{-1} E2 and assayed biochemically. The response over the first thirty days was constant but longer storage times caused a dramatic decrease in M-E_{405nm} (see Fig. 3-15).

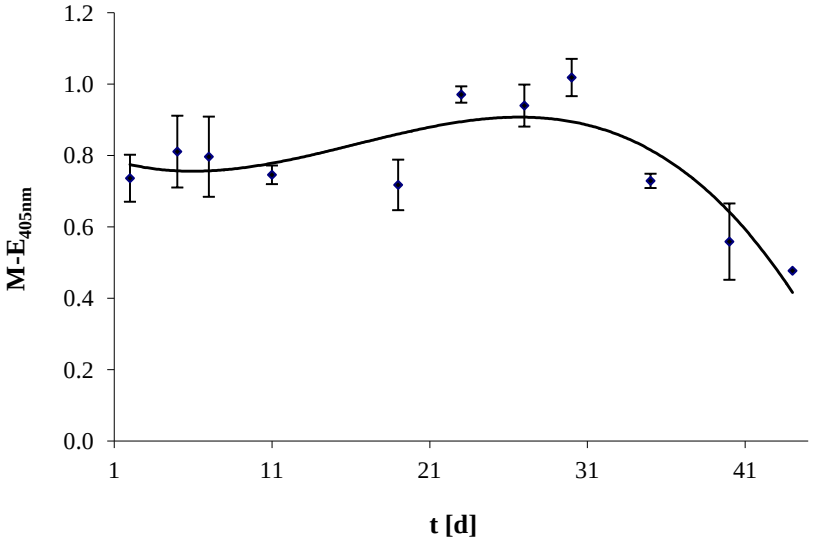

Fig. 3-15. **Stability of immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells.** Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (Lentikats) were stored at 4 °C after activation. At various time points eight Lentikats were taken from storage and incubated with 100 ng L^{-1} E2 for 4 h at 37 °C. Phytase activity was measured by M-E_{405nm} in 0.1 M sodium citrate buffer, pH 3.9.

3.5.5 Reusability of the immobilized cells

In theory, immobilized cells can be reused which is highly desirable in an *online* monitor. To confirm this ability, immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were washed after each experiment with distilled water and then reused. The subsequent measuring data (Fig. 3-16A, B, C) showed that at E2 concentrations ≤ 25 ng L^{-1} cells could be reused for up to 15 times without significant loss of their activity. At an E2 concentration of 50 ng L^{-1} (Fig. 3-16D), phytase activity maintained high until two times reuse, after which it rapidly dropped to 50% of the original activity at which it remained up to the 15th reuse. However, at E2 concentration of 100 and 200 ng L^{-1} (Fig. 3-16E, F), cells could be reused only two times, since the signal intensity from the 3rd measurement was dramatically decreased.

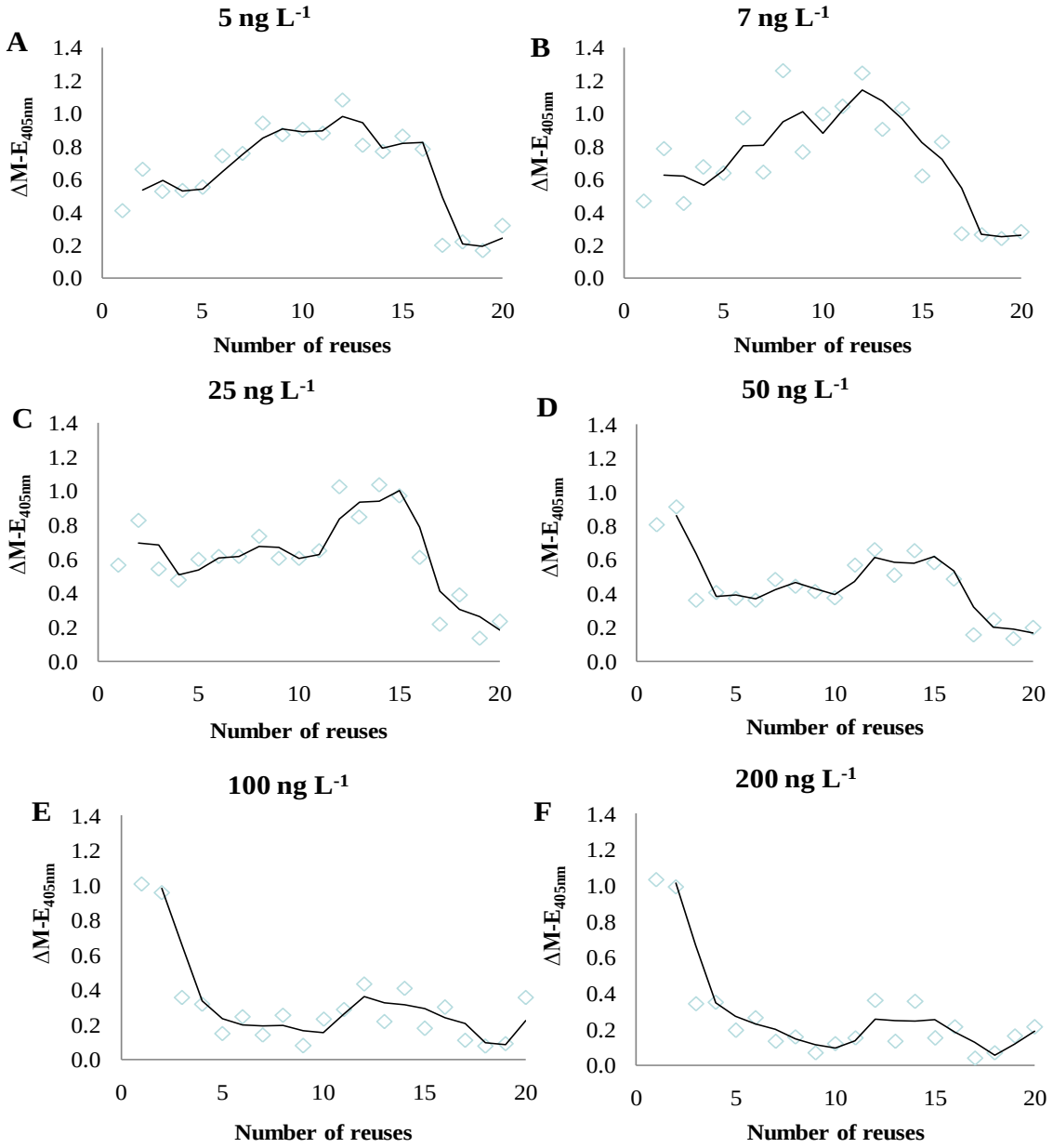

Fig. 3-16. Reusability of the immobilized cells. Immobilized *A. adeninivorans* G1212/YRC102-hER*α*-phyK cells were induced with E2 concentrations (0, 5, 7, 25, 50, 100 and 200 ng L^{-1}) for 4 h. Phytase activity was measured by M-E_{405nm} in 0.1 M sodium citrate buffer, pH 3.9. After each experiment cells were washed with distilled H_2O before reuse. ΔM-E_{405nm} = M-E_{405nm} (E2) - M-E_{405nm} (0) in which M-E_{405nm}. (0) is the value obtained in an experiment without E2; M-E_{405nm} (E2) is the value attained in the experiment with E2=5 (A), 7 (B), 25 (C), 50 (D), 100 (E) and 200 ng L^{-1} (F).

3.5.6 Comparison amperometric and biochemical detection methods of estrogenic activity using immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells

To compare the amperometric method of estrogenic detection with the existing biochemical system used in the nAES assay (Kaiser *et al.*, 2010), immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 concentrations at 37 °C

for 4 h under orbital shaking condition. Substrates (*p*-APP, *p*-NPP) were added, incubated for 10 or 60 min and phytase activities detected by amperometric measurements in the EstraMonitor and by biochemical measurements in a microtiter plate absorbance reader. The results shown in Fig. 3-17 reveal that the signals obtained by the two detection system were very similar with the amperometric method having an EC_{50} of 20.9 ng L^{-1} and a LoD of 8.3 ng L^{-1} compared to an EC_{50} value of 13.9 ng L^{-1} and a LoD value of 3.4 ng L^{-1} for the biochemical method.

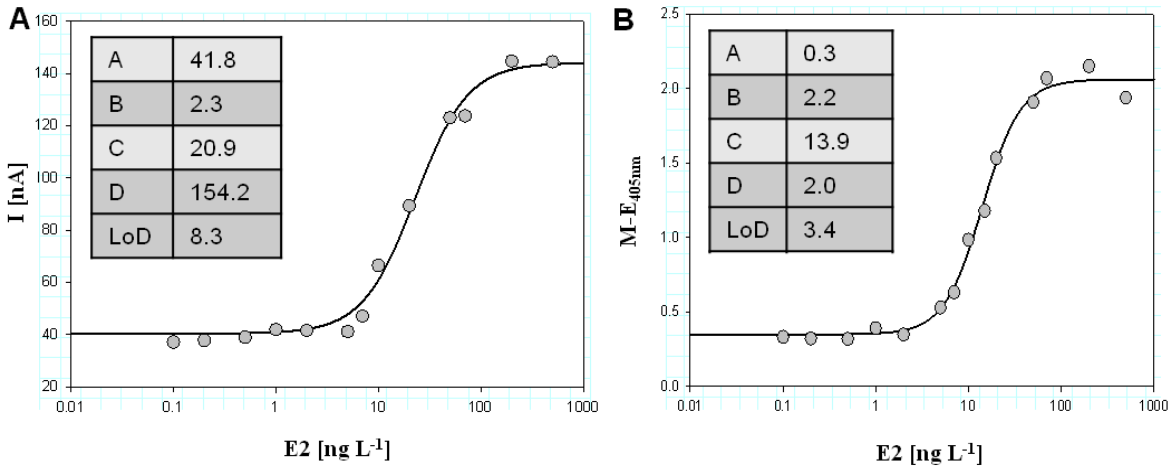

Fig. 3-17. Standard calibration curves for E2. (A) Amperometric measurements with the EstraMonitor and (B) nAES biochemical measurements. Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (8 Lentikats) were incubated with different E2 concentrations for 4 h. After substrate solutions (final concentration of 3.5 mM *p*-NPP or 1.5 mM *p*-APP, pH 3.9) were added, supernatants were measured amperometrically for either 10 min (EstraMonitor) or 60 min (biochemically). A = mean I value without estrogenic effects, D = I value with maximum estrogenic effect, C = concentration at which I value equals (A+D)/2, B = slope which is proportional to C.

3.5.7 Influence of air sparging on phytase production by immobilized cells

In order to establish to what extend phytase production by the immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells depend on air sparging, immobilized cells were incubated with different E2 concentrations (0, 50 and 100 ng L^{-1}) at 37 °C for 4 h with and without air sparging (oxygen supplying system). The results shown in Fig. 3-18 demonstrate that air sparging increases signal intensity by a factor 2 or more. It was therefore decided to improve the EstraMonitor system with air an air sparging system.

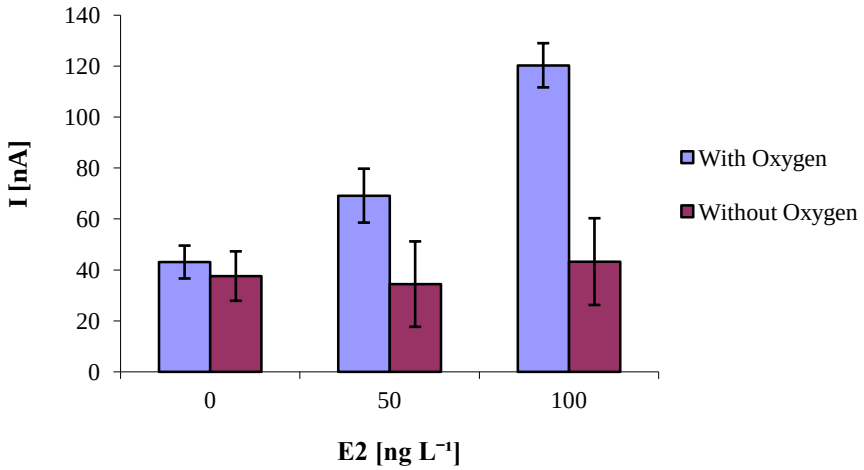

Fig. 3-18. Influence of air sparging on phytase production by immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells. Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 concentrations (0, 50 and 100 ng L^{-1}) at 37 °C for 4 h with and without air sparging. Phytase activities were amperometric measured for 10 min after addition of 1.5 mM *p*-APP, pH 3.9.

3.6. *In situ* use of the EstraMonitor

The EstraMonitor was trialed as a working device. Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (measuring strain) and G1212/YRC102-phyK cells (control strain) were loaded into the incubation chambers and incubated for 4 h with various E2 concentrations. At the end of the incubation, *p*-APP was added and the oxidation reaction was monitored amperometrically for 10 min. The measuring signals obtained from the measuring strain correlated with the E2 concentration, whereas the control strain only showed low background signals of <5 nA.

To reduce the influence of non-estrogenic compounds in samples on the analysis, a corrected measured value (y_{ij}) was determined by $y_{ij} = I_{ms} - I_{cs}$. where I_{ms} denotes the amperometric signal of the measuring strain and I_{cs} the value of the control strain signal.

The calibration curve with EC_{50} value of 33.6 ng L^{-1}, LoD of 5.3 ng L^{-1} and a measuring range between 5-100 ng L^{-1} (see Fig. 3-19) correlates well with the results obtained with the biochemical system.

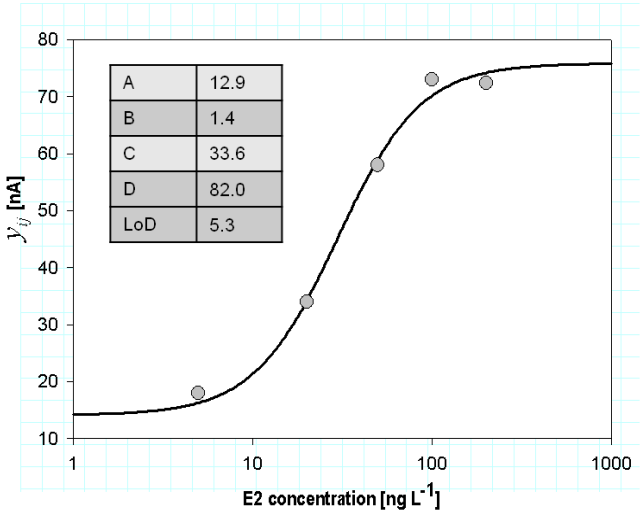

Fig. 3-19. Typical standard calibration curve of amperometric measurements with the Estra-Monitor based on corrected measured values (y_{ij}). Immobilized *A. adeninirorans* G1212/YRC102-hERα-phyK cells and G1212/YRC102-phyK cells (each 8 Lentikats) were incubated with different E2 concentration at 37 °C for 4 h. Supernatants were amperometrically measured in both measuring chambers of the EstraMonitor for 10 min. y_{ij} represents the corrected measured value (y_{ij} = I_{ms} - I_{cs}). Estrogenic activities were calculated with the BioVal® software.

3.7. Studies on oxidation of the intermediate product (*p*-AP)

3.7.1 Influence of pH on *p*-AP oxidation

The effect of pH on the oxidation of the intermediate product *p*-AP was assayed using 0.1 mM *p*-AP and different pH values ranging from 3.9 to 14. The current obtained from the oxidation of 0.1 mM *p*-AP were amperometrically detected and recorded. Fig. 3-20 shows that the signals were optimal in the pH range 3.9 to 6.1. Between pH 6.2 and 7.5 signal intensity gradually decreased to 60% of the optimal value and remained at this level until a pH of 12.5. At higher pH values signal intensity fell off dramatically. In general, the oxidation of *p*-AP is most efficient in the pH range between 3.9 and 6.1.

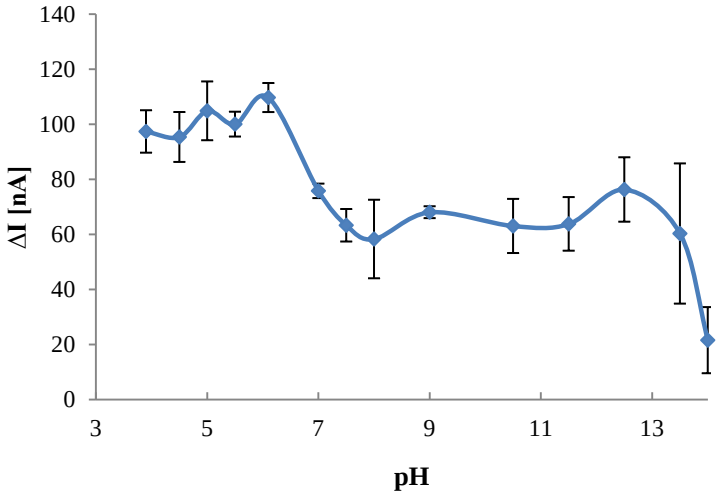

Fig. 3-20. **Influence of pH on phytase reaction product (*p*-AP).** 0.1 mM *p*-AP was dissolved in 0.1 M sodium citrate buffer with variable pH and amperometrically measured in the EstraMonitor. $\Delta I = I_{p\text{-AP}} - I_{buffer}$

3.7.2 Influence of salinity on *p*-AP oxidation

The electrode system contains 3 electrodes: working, counting and reference electrodes. The reference electrode consists of Ag/AgCl and might be influenced by salinity. Therefore, the oxidation of 0.1 mM *p*-AP was investigated in the presence of up to 2% NaCl. Amperometric measurements were recorded for 5 min. The results depicted in Fig. 3-21 reveal a stable signal intensity until a NaCl concentration of 0.6%, above this concentration the measured signal started to fall off gradually.

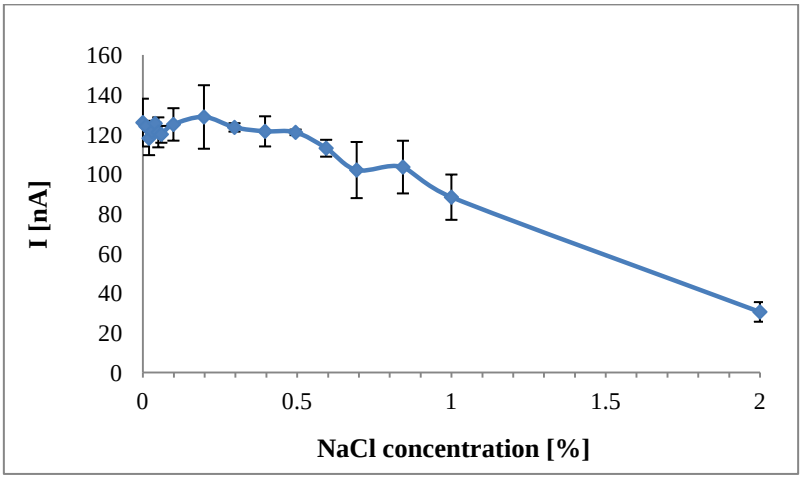

Fig. 3-21. Influence of NaCl concentration on *p*-AP oxidation. 0.1 mM *p*-AP in 0.1 M sodium citrate buffer (pH 3.9) supplemented with different NaCl concentrations (0-2%) was amperometrically measured for 5 min in the EstraMonitor.

3.8. Influence of salinity on phytase production

Salinity in wastewaters is often produced by industries involved seafood processing, food canning, pickling and chemical manufacturing. For several bioassays which monitor the environmental pollutants, salinity is a critical factor. In order to establish the functionality of the current microbial component, the salinity tolerance of the non-immobilized and immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were tested.

3.8.1 Phytase production of non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells

Salinity influences the bioavailability, cytotoxicity and the speciation of contamination in the environment samples, which can directly affect the results of the biotest (Kase *et al.*, 2009). To analyze these influences on the phytase production by non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells in response to E2, cells were incubated with different concentrations of E2 and NaCl in YMM-maltose at 37 °C, 330 x g, in 96 deep-well plates for 4 h. Phytase activity was biochemically measured by M-E_{405nm} in 0.1 M sodium citrate buffer, pH 3.9. The results shown in Fig. 3-22A illustrate that the decreased level of the absorbance resulted from the increasing salt concentrations, which led to a reduction of the binding capacity of the estrogen receptor for E2. The span between minimum and maximum measurement values was reduced dramatically in sample supplemented with 5% NaCl. Nevertheless, the results showed nice sigmoidal concentration-respond curves to E2 even under 5% NaCl (Fig. 3-22B). Interestingly, under high salinity concentrations the limit of detection values for E2 fell from 4.25 to 1.66 ng L^{-1}.

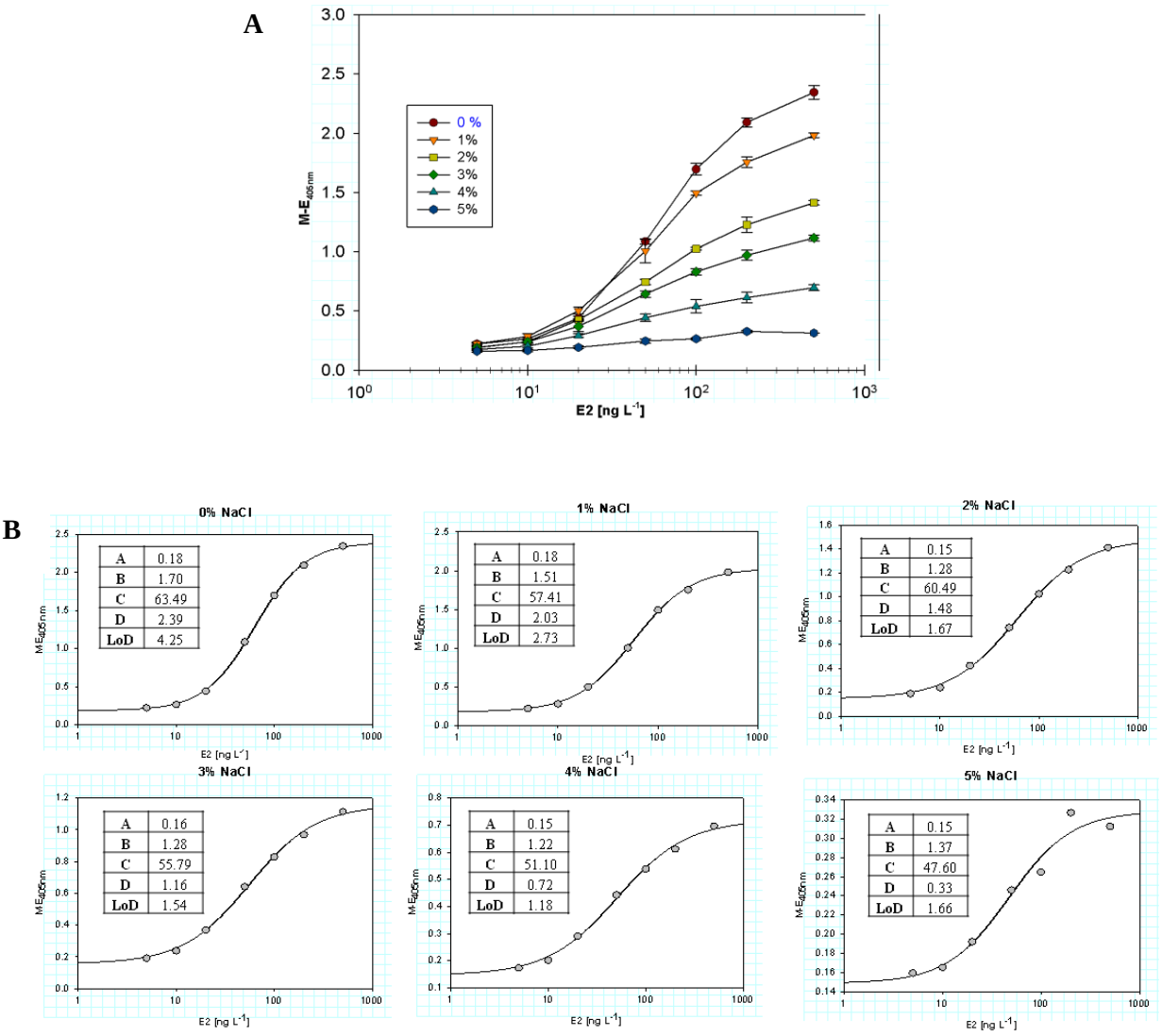

Fig. 3-22. **Influence of salinity on phytase production by non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells.** Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 (0, 5, 10, 20, 50, 100, 200 and 500 ng L^{-1}) and NaCl (1, 2, 3, 4 and 5 %) concentrations in 96 deep-well plates in YMM-maltose at 37 °C, 330 x g for 4 h. Phytase activity was biochemically measured by M-E_{405nm} in 0.1 M sodium citrate buffer, pH 3.9. (A) All measurements combined. (B) Separated measurements.

3.8.2 Phytase production by immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells

Like the non-immobilized cells, immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were affected by salinity. Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with different E2 and NaCl concentrations in YMM-maltose at 37 °C, 220 x g, in 48 deep-well plates for 4 h after that phytase activity was measured biochemically by M-E_{405nm} in 0.1 M sodium citrate buffer, pH 3.9. Fig. 3-23A demonstrates a

dose-dependent negative effect of NaCl concentrations above 1% on the levels of absorbance measured. Similar to the non-immobilized cells, the results using immobilized cells show clear sigmoidal curves with E2 under increasing salinity even to a level of 5% NaCl (Fig. 3-23B). The detection limits for E2 (from 1.52 to 1.1 ng L^{-1}) were lower than those when non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were used.

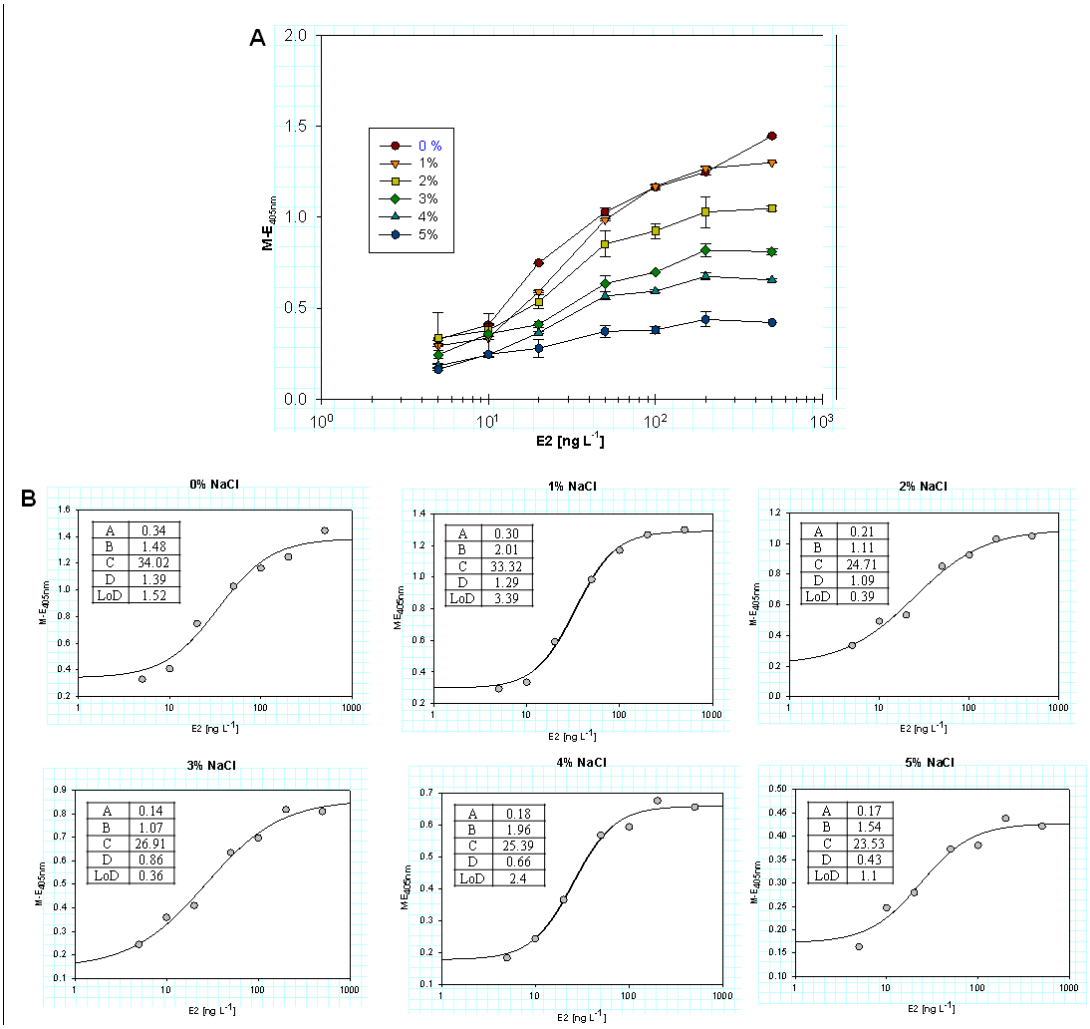

Fig. 3-23. Influence of salinity on phytase production by immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells. Immobilized cells were incubated with different E2 (0, 5, 10, 20, 50, 100, 200 and 500 ng L^{-1}) and NaCl (1, 2, 3, 4 and 5 %) concentrations in 48 deep-well plates in YMM-maltose at 37 °C, 220 x g for 4 h. Phytase activity was measured biochemically by $M\text{-}E_{405nm}$ in 0.1 M sodium citrate buffer (pH 3.9). (A) All curves combined. (B) Separated sigmoidal curves.

3.9. Analysis of estrogenic activities in wastewater with the EstraMonitor

Environmental samples may contain a multitude of unknown organisms and substances, each of which may cause interference of the matrix to the cells. It is, however, the purpose of the EstraMonitor to detect estrogenic activity directly in the effluent of the waste-

water. To prove this purpose, *A. adeninivorans* G1212/YRC102-hERα-phyK was used as microbial component to detect the estrogenic activities in respective number of wastewater samples obtained from different places in Magdeburg, Sachsen-Anhalt, Germany (Table 3-1).

Because of unknown and unanticipated estrogenic substances in the samples, E2 concentrations were spiked with variable amounts of wastewater in order to excite/amplify the measuring signals. Non immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (final cell density: OD_{600}=30) and immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (8 Lentikats) were incubated with different E2 concentrations and non-pretreated samples in YMM-maltose at 37 ºC for 4 h. The non-immobilized cells were cultivated at 330 x g in 96 deep-well plates and the immobilized cells were kept at 220 x g in 48 deep-well plates, respectively. The phytase activities in supernatants were biochemically and amperometrically measured using 0.1 M sodium citrate buffer (pH 3.9).

Table 3-1. Wastewater samples from various places in Magdeburg, Sachsen-Anhalt, Germany.

Wastewater sample no.	Source	pH
77	Zoo	7.6
80	Chemical industry	12.4
82	Mixed sample	8.7
83	Hospital	9.1
109	Hotel	5.03

3.9.1 Electrochemical behaviour of the oxidation in the sample

Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells produce phytase in response to estrogenic compounds. This phytase reacts with the substrate *p*-APP, generating the intermediate product *p*-AP. In order to investigate the behaviour of *p*-AP in wastewater samples, immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with 50 ng L^{-1} E2, in 20% (v/v) sample number 109 and YMM-maltose at 37 °C, 220 x g in 48 deep-well plate for 4 h. After *p*-APP was added, the oxidation of *p*-AP was investigated by voltammetry. Cyclic voltammograms (Fig. 3-24A) were obtained with a Pt-Ag/AgCl-Pt electrode and a scan rate of 50 mV s^{-1}. Scanning was from -200 to +200 mV. The voltammogram presented in Fig. 3-24A depicts a chemically reversible oxidation process under matrix condi-

tion. The anodic current peak corresponding to the oxidation of *p*-AP lies at about 150 mV. Oxidation of pure *p*-AP shows similar results (Fig. 3-1).

Five repetitive scans of *p*-AP by cyclic voltammetry illustrate electropolymerization, which results in fouling of the electrode surface (Fig. 3-24B). This may be avoided by using diluted samples.

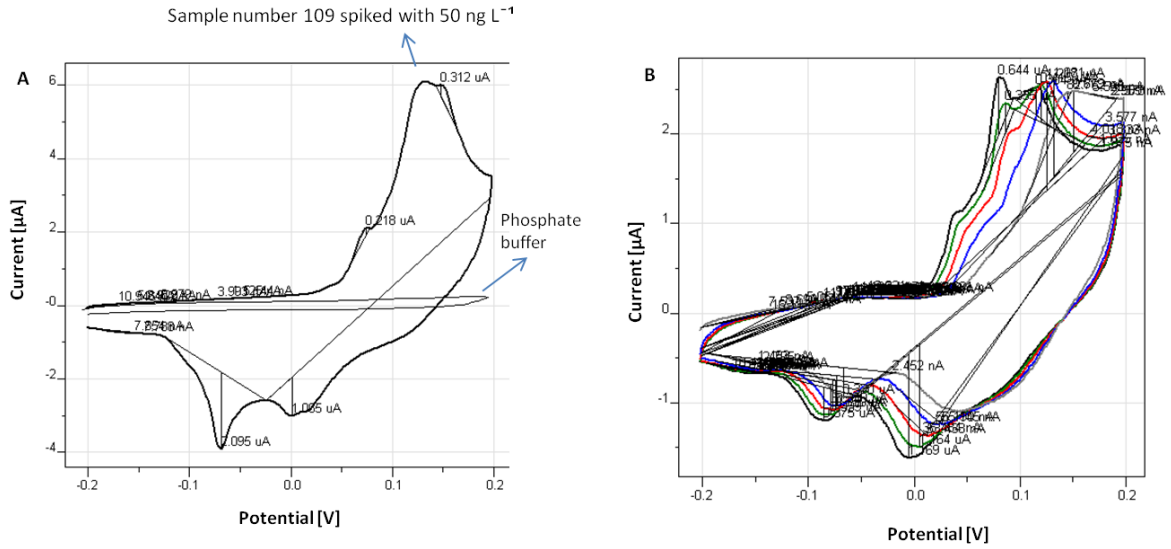

Fig. 3-24. Cyclic voltammogram of *p*-AP generated from wastewater sample 109. Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with 50 ng L^{-1} E2, 20% sample number 109 (v/v). Following the electrochemical substrate 1.5 mM *p*-APP (pH 3.9) was added, incubated for 10 min, after that the suspension was measured using cylic voltammetry at a scan rate of 50 mV s^{-1}, using a scanning range from -200 to +200 mV. Pt-Ag/AgCl-Pt electrodes were used. (A) Single scan. (B) Repetitive scans (5 lines in various colours depict 5 scans).

3.9.2 Biochemical and amperometric measurements with wastewater samples

3.9.2.1 Biochemical measurements using non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells

Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with 20% (v/v) or 80% (v/v) wastewater spiked with different E2 concentrations (0, 0.1, 0.5, 2, 10, 20, 50 and 200 ng L^{-1}) and YMM-maltose for 4 h at 37 °C. Phytase in the supernatants was measured biochemically using 0.1 M sodium citrate buffer (pH 3.9). In the presence of 20% (v/v) wastewater (Fig. 3-25A) *A. adeninivorans* G1212/YRC102-hERα-phyK cells responded very well to E2 under different wastewater matrices with a broad pH range from 5.03 to 12.4 (Table 3-1). The dose response curves in the presence of 80% (v/v) waste-

water (Fig. 3-25B) reveal that in the presence of sample numbers 77, 80, 82 the cells showed a dose dependent reaction whereas in sample 82 and 83 signals remained at a stable high plateau. This suggested that these last samples may contain large amount of estrogenic substances. The EC_{50} values of the curves obtained with 20% (v/v) wastewater samples were calculated using BioVal® software (Table 3-4).

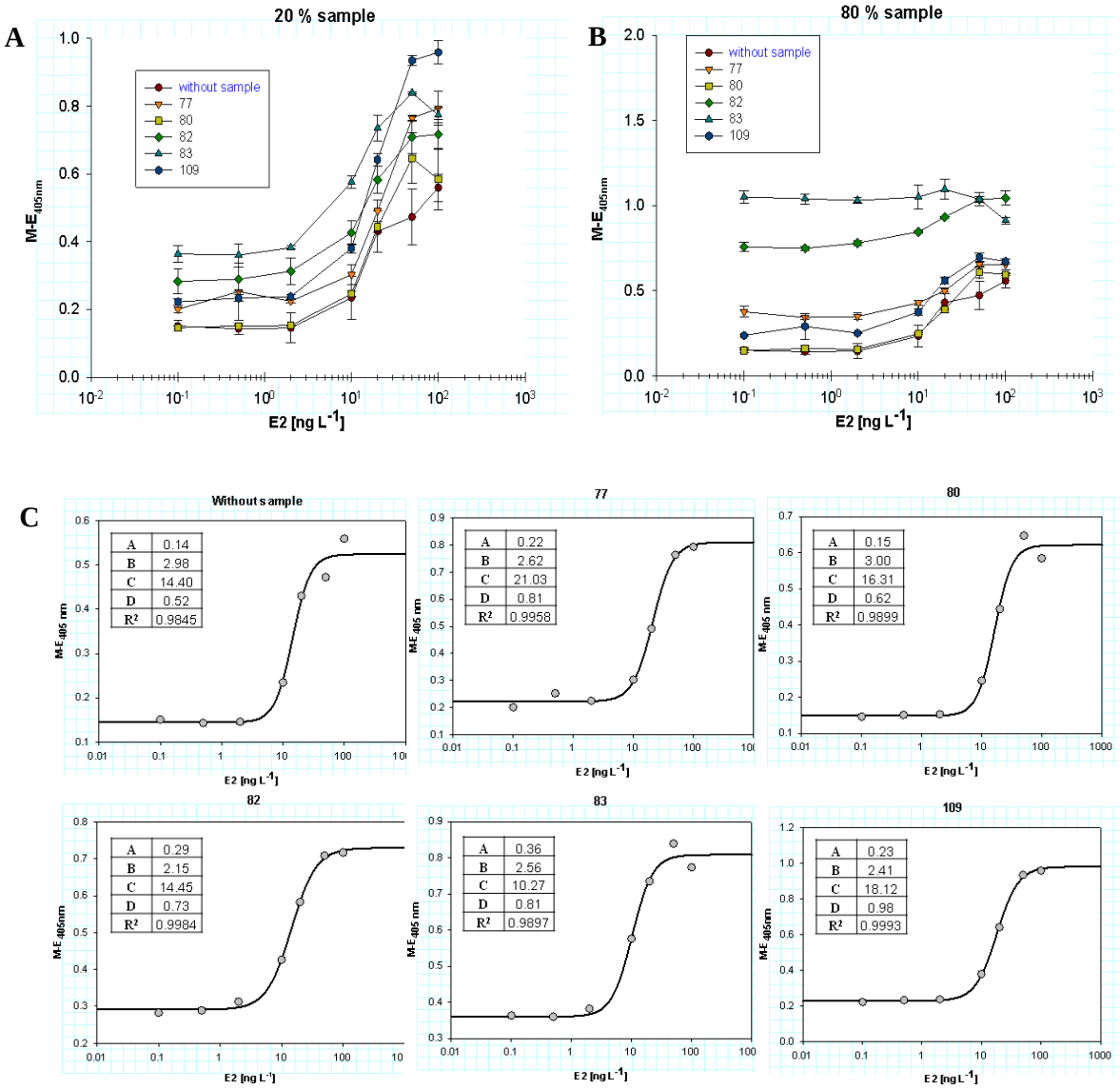

Fig. 3-25. Influence of the matrix on non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells. Non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with (A) 20% (v/v) or (B) 80% (v/v) wastewater under different E2 concentrations (0, 0.1, 0.5, 2, 10, 20, 50 and 200 ng L^{-1}). Phytase activity in the supernatant was measured biochemically using 0.1 M sodium citrate buffer (pH 3.9). (C) Sigmoidal curves of individual samples.

Because EC_{50} values differed between tests with E2 only and tests where E2 was spiked with sample, and because signals were stronger in the 80% samples, it can be concluded that these samples contained estrogenic substances. EEQ values were calculated and listed in the Table 3-2. Relevant EEQ values correlated to the measuring signals shown in the curves in Fig. 3-25.

Table 3-2. Estrogenic activities in wastewater samples using non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells.

Sample number	**77**	**80**	**82**	**83**	**109**
EEQ [ng L^{-1}]	12.93	10.47	20.14	27	16.81

3.9.2.2 Measurements using immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells

Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with 20% (v/v) wastewater spiked with different E2 concentrations (0, 0.1, 0.5, 2, 10, 20, 50 and 200 ng L^{-1}) and YMM-maltose in 48 deep-well plate, 220 x g for 4 h at 37 °C. Phytase activity was measured biochemically and amperometrically using 0.1 M sodium citrate buffer (pH 3.9).

Biochemical measurements

The results (Fig. 3-26) obtained in the biochemical method reveal that the same behaviours of the immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells to the estrogenic substances were received when compared with non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells. However, the level of absorbance was lower than that when using non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells. This was caused by the difference in the cell number between non-immobilized and immobilized cells. The EEQ values are shown in the Table 3-3.

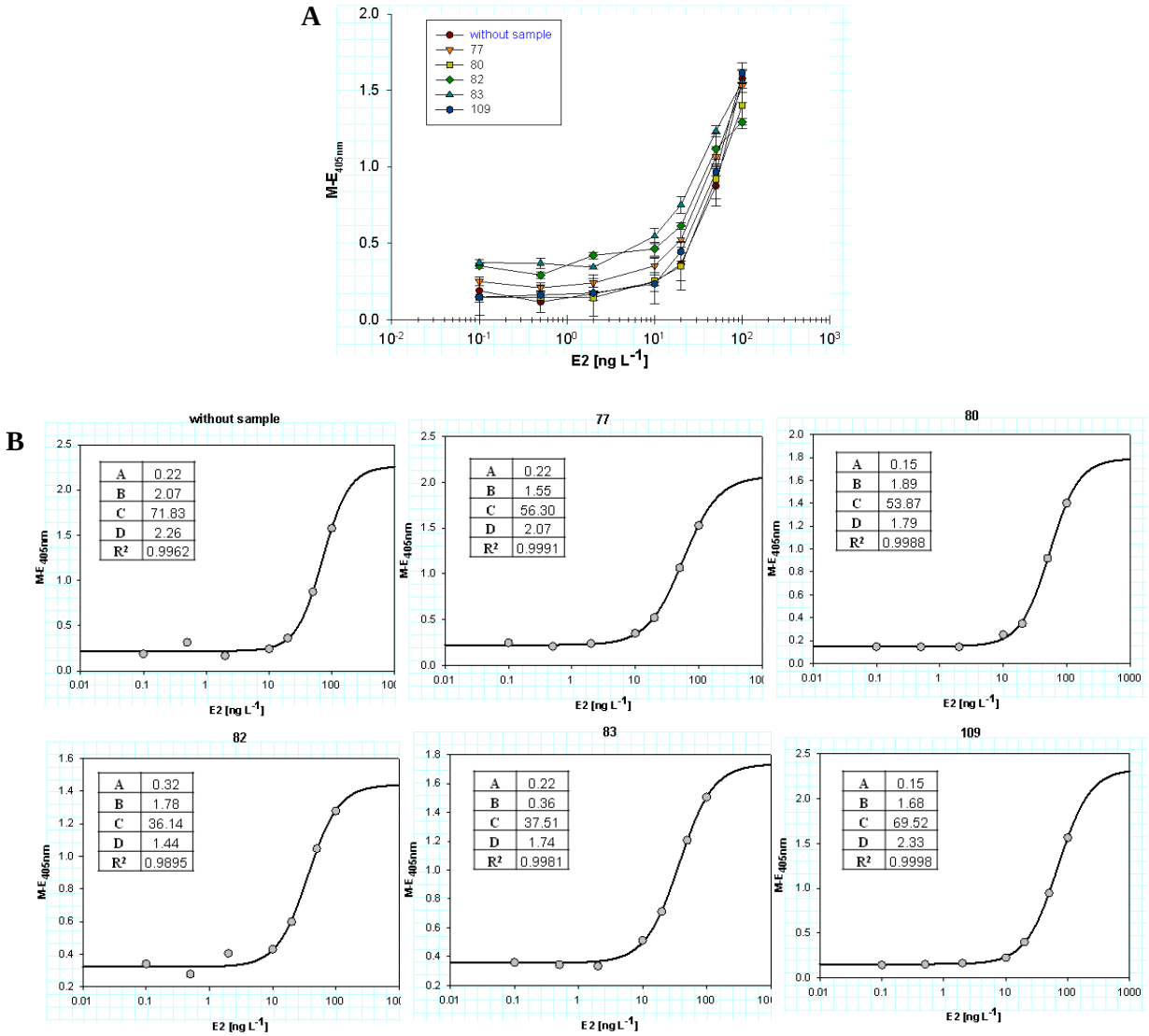

Fig. 3-26. Influence of matrix on immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells. Immobilized cells were spiked with E2 in variable concentrations (0, 0.1, 0.5, 2, 10, 20, 5 and, 200 ng L^{-1}) and incubated with 20% (v/v) wastewater sample. Phytase activities in the supernatants were measured biochemically using 0.1 M sodium citrate buffer (pH 3.9). (A) All curves combined (B) Curves were presented separately.

Table 3-3. Estrogenic activities in wastewater samples measured by immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells and biochemical detection method.

Sample number	77	80	82	83	109
EEQ [ng L^{-1}]	19.66	9.56	25.15	30.17	23.15

Amperometric measurements

In this trial, the immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells and amperometric method were used for estrogenic detection in the wastewater samples (Fig.

3-27). The separated sigmoidal curves in the Fig. 3-27A show that the amperometric measurements gave the good measuring signals. 20% of the samples did not cause the adverse affect to the amperometry. The logistic regression results of those dose curves were given in the Fig. 3-27B.

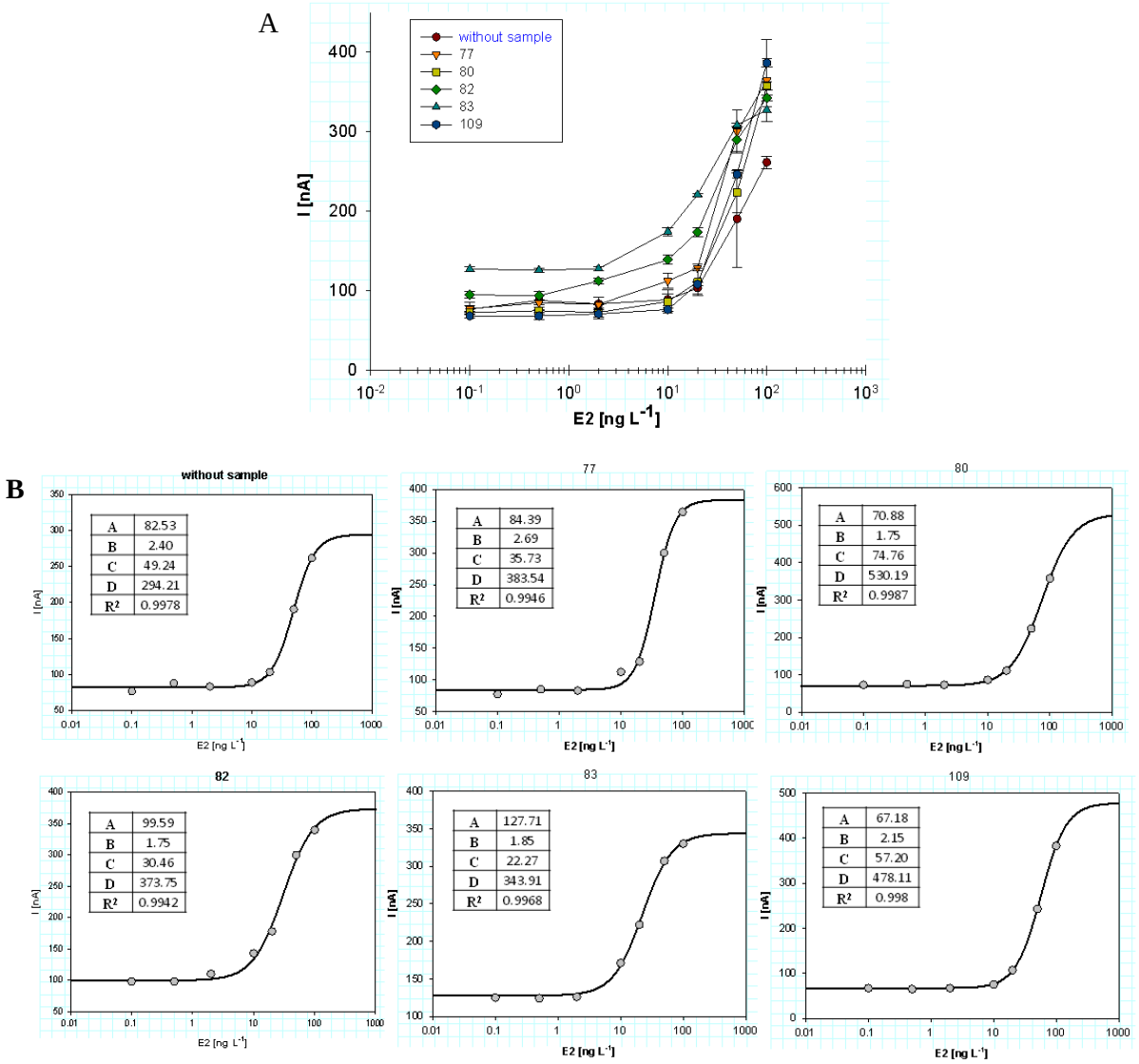

Fig. 3-27. **Influence of the matrix on immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells.** Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were spiked with E2 (0, 0.1, 0.5, 2, 10, 20, 50 and 200 ng L^{-1}) and incubated with different wastewater samples (20%, v/v) at 37 °C, 220 x g for 4 h. Phytase activities in the supernatants were measured electrochemically using 0.1 M sodium citrate buffer (pH 3.9). (A) All curves combined. (B) Individual curves.

The interpolated EEQ values (Table 3-4) based on the amperometric method were a bit higher than those of the biochemical assay.

Table 3-4. Estrogenic activities in wastewater samples measured by using immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells and amperometric detection method.

Sample number	77	80	82	83	109
EEQ [ng L^{-1}]	23.25	9.47	22.29	28.03	21.1

3.9.3 Comparison of EEQ values obtained by amperometric and biochemical detection methods

The EEQ values in the wastewater samples determined by biochemical and amperometric methods using non-immmobilized and immobilized cells are summarized in Fig. 3-28. These responses are the sum of all estrogenic or estrogenic-like compounds capable of binding to the receptor. The EEQ values obtained by the biochemical method using non-immobilized cells were lower than the values obtained from the biochemical and electrochemical methods using immobilized cells. The reason could be, because of the entrapment of cells inside the supportive and stable PVA, the cell's integrity was protected and the effect of the matrix containing wastewater sample on the cells was therefore reduced. The results with amperometric and biochemical methods utilizing immobilized cells were somehow similar.

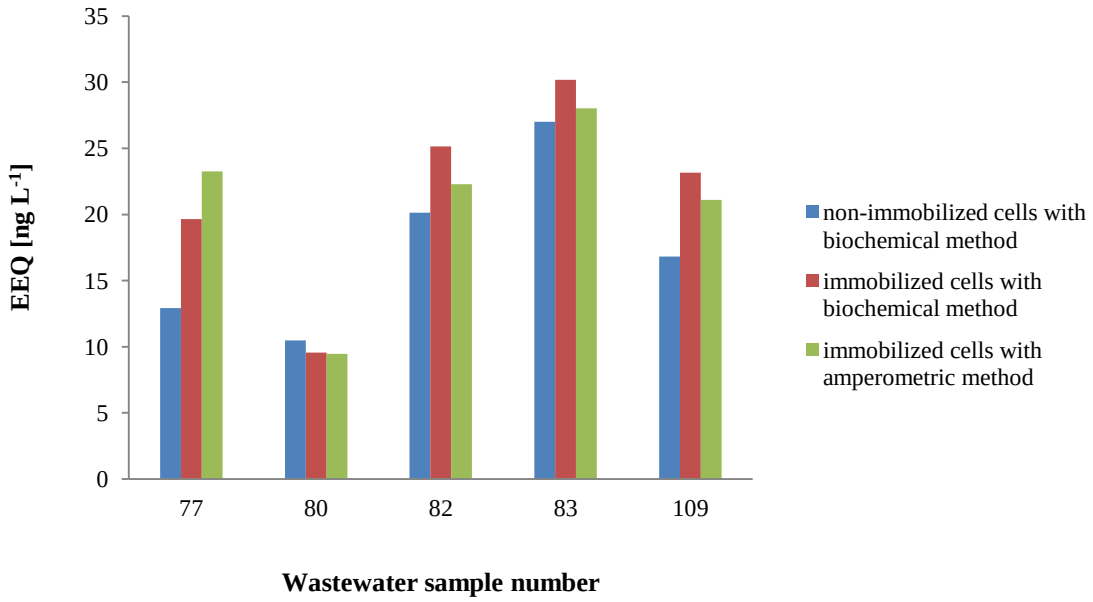

Fig. 3-28. Comparison of EEQ values obtained with biochemical and amperometric methods. Immobilized and non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells were incubated with 20% (v/v) wastewater samples spiked with different E2 concentrations (0, 0.1, 0.5, 2, 10, 20, 50 and 200 ng L^{-1}) in YMM-maltose for 4 h at 37 °C. 48/96 deep-well plate and 220/330 x g was used for cultivating immobilized and non-immobilized cells, respectively. The phytase activities in the

supernatants were measured with both biochemical and amperometric methods using 0.1 M sodium citrate buffer (pH 3.9).

3.10. Development of the alternative substrate AA2P (ascorbic acid 2-phosphate) for amperometric detection method

AA2P is an alternative substrate for the alkaline phosphate (ALP) assay and has been compared with the widely employed *p*-APP substrate for use in the amperometric detection method. Since AA2P renders great sensitivity to the assays (Kokado *et al.*, 2000; Sun and Jiao, 2005) and is relatively inexpensive, its possible application in the EstraMonitor system in order to enhance economic efficiency has been studied. The procedure of enzymatic hydrolysis reaction is shown in Fig. 3-29.

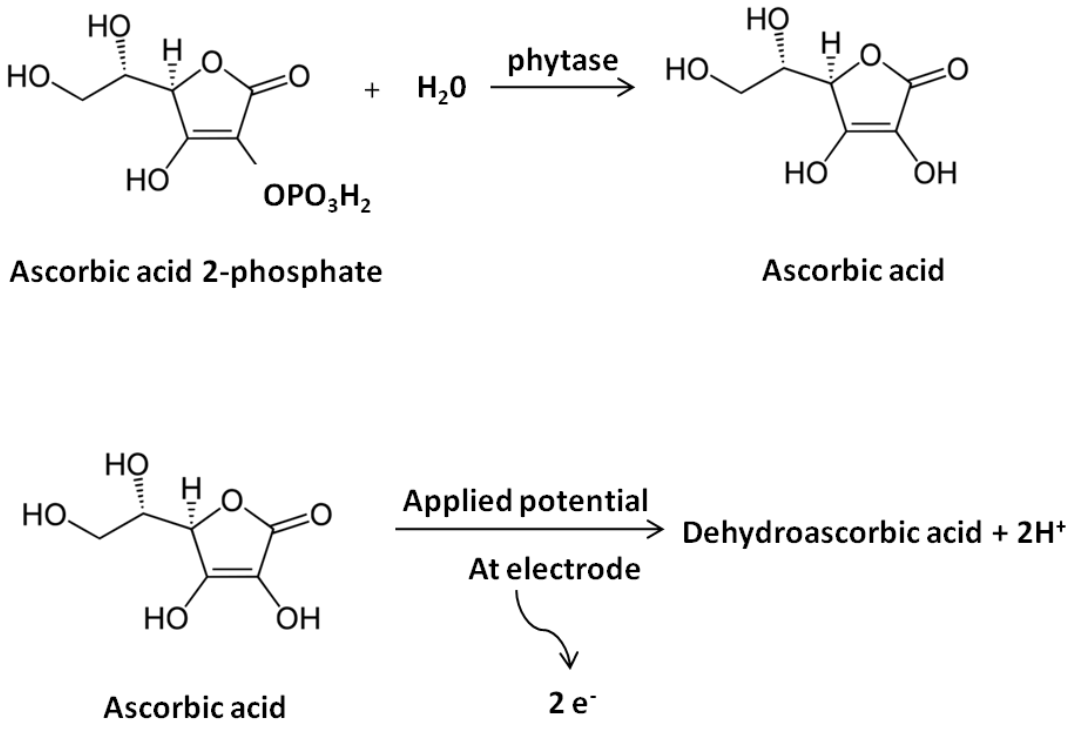

Fig. 3-29. Scheme of enzymatic hydrolysis and electrochemical reaction. Phytase transforms ascorbic acid 2-phosphate (AA2P) into the intermediate product ascorbic acid (AA) which is electroactive and can be oxidized at an electrode by applied potential. Electrons released from the AA oxidation can be detected as an electric current.

In the first step, to investigate the suitability of AAP2 for the EstraMonitor, the applied potential of the amperometric detection had to be determined.

3.10.1 Electrochemical behaviour of the enzyme substrate product ascorbic acid (AA)

The oxidation of 5 mM AA product was investigated by voltammetry. Typical cyclic voltammogram was achieved with a Pt-Ag/AgCl-Pt electrode and a scan rate of 50 mV s^{-1} (Fig. 3-30). Scanning was from -200 to 600 mV and solutions were stirred before the scan. The voltammogram given in the Fig. 3-30 exhibited a single chemically irreversible peak. The anodic current peak corresponding to the oxidation of AA was observed at about 400 mV versus Ag/AgCl.

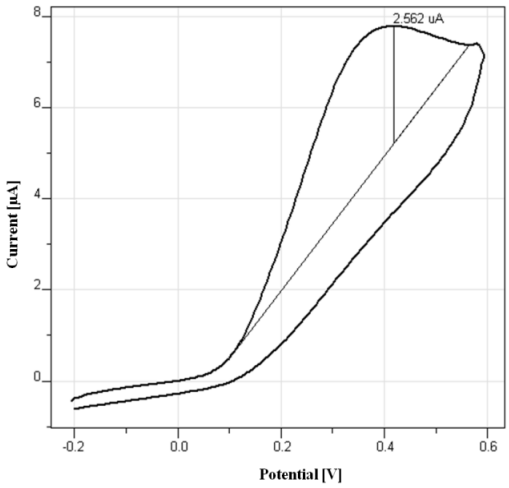

Fig. 3-30. Cyclic voltammogram of 5 mM AA in 0.1 M sodium citrate buffer (pH 3.9). Scan rate of 50 mV s^{-1} and Pt-Ag/AgCl-Pt electrode were used.

3.10.2 Establishing the optimal electrode potential for the new substrate

Hydrodynamic voltammetry was used to determine the optimal applied potential for the detection of the intermediate product AA with the Pt-Ag/AgCl-Pt electrode. The hydrodynamic voltammograms of the oxidation of the new electrochemical substrate AA2P (10 mM) and that of the intermediate product AA (10 mM) as shown in Fig. 3-31A were performed between 0 to 500 mV. The electro-oxidation current response (ΔI) of the intermediate product AA increases as a function of the applied potential and reached a stable maximum from 300 mV onwards. Up until a potential of 500 mV AA2P was not oxidized and ΔI thus results only from the oxidation of 10 mM AA. A potential of 300 mV was used in all subsequent studies.

The electro-oxidation current density (ΔI) correlated with the AA concentration in a range from 1 to 10 mM. Regression analysis shows a linear relationship between both param-

eters with a high correlation coefficient (R2 = 0.9811). The equation (y = 1.0531x - 0.4395) can be used to calculate the phytase activity, and thus the E2 concentration (Fig. 3-31B).

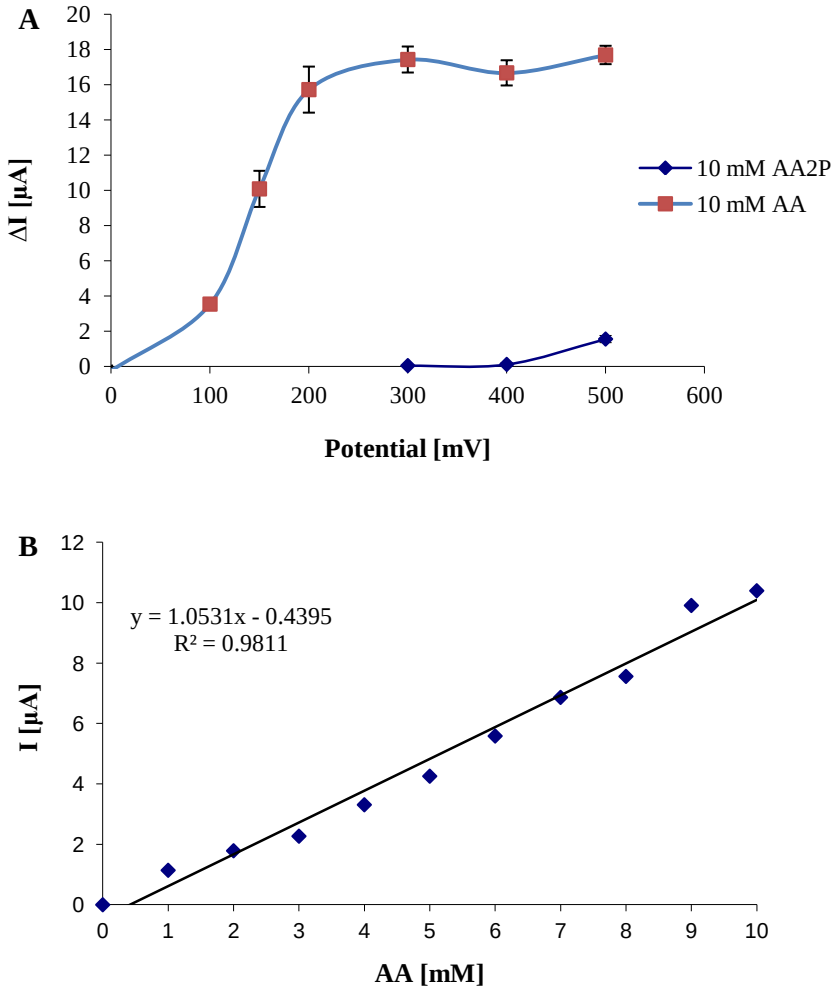

Fig. 3-31. (A) Values for hydrodynamic voltammograms at various potentials of 10 mM AA2P and 10 mM AA in 0.1 M sodium citrate buffer (pH 3.9) at 37 °C. ΔI values were calculated as $\Delta I = I_P - I_B$ (I_P = value for 10 mM AA2P or 10 mM AA; I_B = value for citrate buffer). **(B) Correlation of AA concentration to the current (I)**. The equation was obtained by regression analysis.

3.10.3 Stability of substrate AA2P

In order to use AA2P as a substrate for the amperometric reaction to detect estrogenic activities, its stability under storage was investigated. To do this, 10 mM of AA2P was dissolved in 0.1 mM sodium citrate buffer pH 3.9 and stored at 4 °C or 25 °C. Aliquots of AA2P solution was taken at 1, 2, 3, 4, 5 and 6 days of storage and treated with phytase. Substrate solution and the mixture of the enzyme substrate reaction were amperometrically measured. Fig. 3-32A demonstrates that as expected the signals obtained from the samples after

enzyme substrate reaction are much higher than those from samples with substrate only. After six days of incubation at 25 °C, the signal obtained from the only substrate solution increased dramatically (see Fig. 3-32A). Subtracting the signals of the control samples (substrate only) from those of the enzyme substrate reaction, showed that signals were stable for the first 5 days of incubation and reduced substantially afterwards (Fig. 3-32B). Although the substrate seemed to remain more when stored at 4 °C, for reliable measurements it was decided that substrates should not be used for more than 5 days.

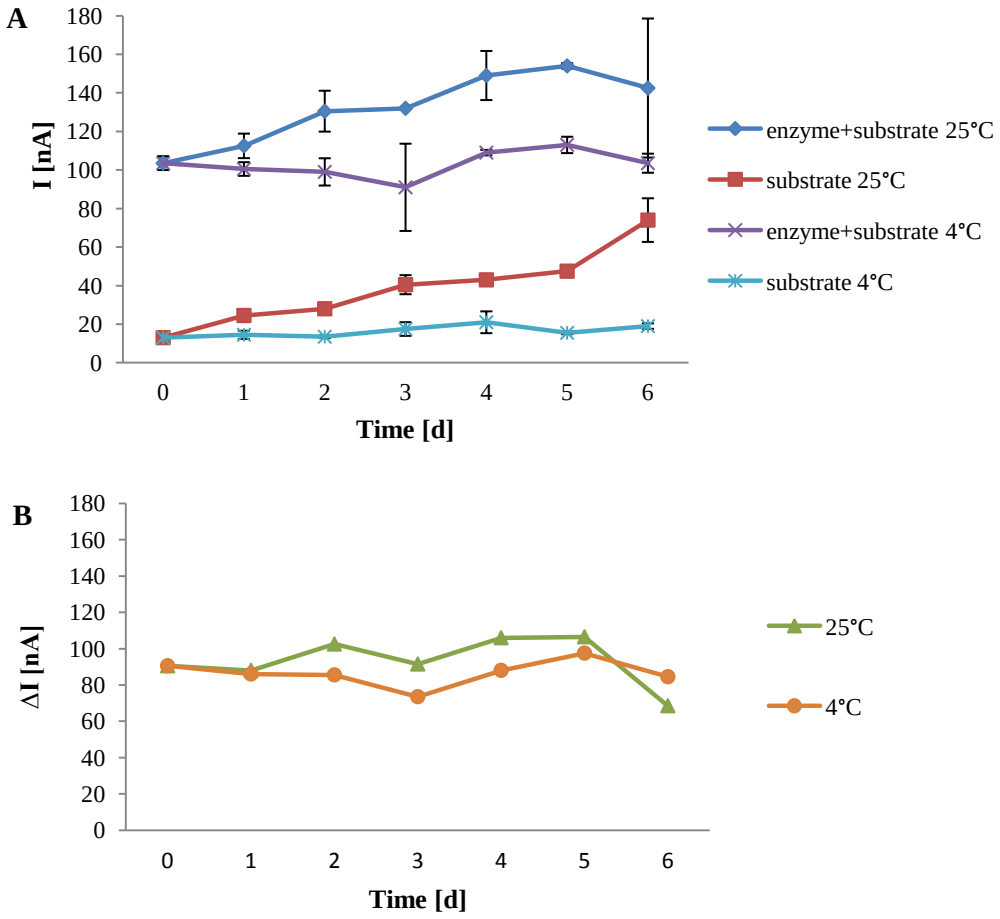

Fig. 3-32. Stability of substrate AA2P. (A) Real values. (B) Subtracted values (ΔI=value $_{enzyme+substrate}$ – value $_{substrate}$). 10 mM AA2P was dissolved in 0.1 M sodium citrate buffer (pH 3.9) and stored at 4° C or 25 °C. Enzyme reactions with phytase were carried on a daily basis. Electrochemical measurements were performed 5 min after the start of the reaction.

3.10.4 Amperometric measurements with AA2P

The calibration curve for E2 using immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (8 Lentikats) and 10 mM AA2P is shown in Fig. 3-33. Phytase activities

were amperometrically measured for 10 min. The results reveal a highly reproducible and typical sigmoidal curve. This indicates that phytase synthesized by the induction of immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells interacts well with the alternative substrate AA2P, resulting in the intermediate product (AA), which is oxidized at the electrode and thus generates an electric current. The EC_{50} and LoD values calculated by BioVal® software were 15.69 ng L^{-1} and 0.92 ng L^{-1} respectively.

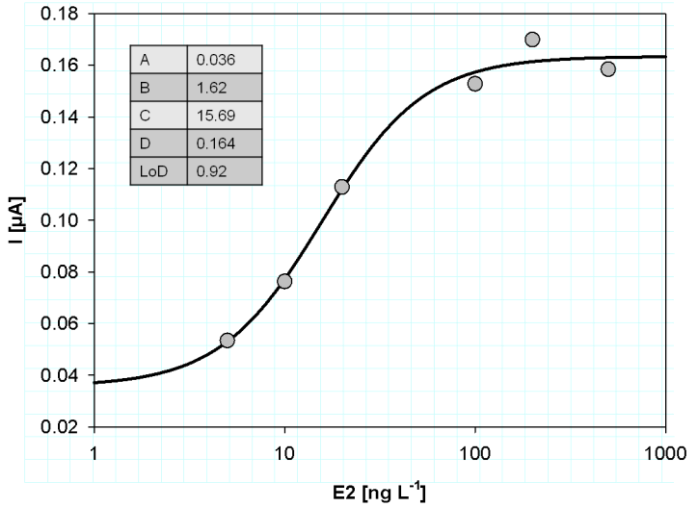

Fig. 3-33. Standard calibration curve for E2 in amperometric measurements with substrate AA2P. Immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (8 Lentikats) were incubated with different E2 concentrations (0, 5, 10, 50, 100, 200 and 500 ng L^{-1}) at 37 °C, 190 x g in 48 deep-well plate for 4 h. After 4 h incubation, AA2P (10 mM, prepared in 0.1 M sodium citrate buffer, pH 3.9) was added and phytase activity measured amperometrically for 10 min.

3.11. Development of alternative *A. adeninivorans* G1214/YRC103-hERα-phyK for the microbial component of the EstraMonitor

To enhance the sensitivity and to reduce measuring time of the EstraMonitor, an alternative microbial component, *A. adeninivorans* G1214/YRC103-hERα-phyK, was developed. The construction of this microbial component and its suitability for the system were investigated.

3.11.1 Generation of *AURA3mm* selection marker gene

The auxotrophic selection marker, *AURA3mm*, was excised from plasmid pCR4-AURA3mm-13 using *Sal*I and *Afe*I restriction enzymes (Fig. 3-34). After agarose separation, two bands were obtained of 1339 bp and 4287 bp, corresponding to *AURA3mm* and plasmid

fragments, respectively. The *AURA3mm* gene, after digestion, was extracted from the agarose gel and precipitated for use in subsequent experiments.

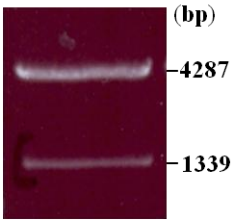

Fig. 3-34. Pattern of pCR4-AURA3mm-13 restriction with endonuclease *Sal*I and *Afe*I. The 1339 bp band represents *AURA3mm*, the 4287 bp represents plasmid pCR4 fragments.

3.11.2 Generation of backbone plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK/*Afe*I-*Sal*I

The backbone plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK was created by restriction of the plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK with *Afe*I and *Sal*I enzymes. Since there are 2 sites for *Afe*I enzymes in this plasmid (one near the promoter of *ALEU2* and the second in the *phyK* gene region, see Fig. 3-35), three fragments were obtained after enzyme digestion.

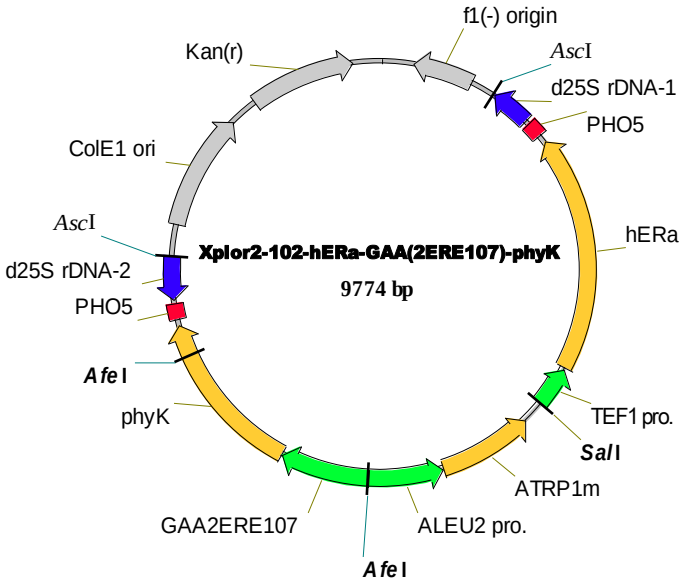

Fig. 3-35. Physical map of plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK. The plasmid contains: *E. coli* elements (ColE1 ori; Kan(r); f1(-)origin; module for selection (*ALEU2* promoter-*ATRP1m*); *phyK* expression module (*A. adeninivorans*-derived *TEF1* promoter-*phyK* reporter gene); *hERα* expression module (*A. adeninivorans*-derived *GAA2(ERE^{107})* promoter-*hERα* receptor gene);

two d25S rDNA target sequences disrupted by the insertion of modules; *Asc*I restriction sites between *E. coli* part and two d25S rDNA.

The results shown in Fig. 3-36 reveal three (expected) bands with approx. size of 6624, 1704 and 1446 bp. The 6624 bp band denotes the backbone fragment, the 1704 bp band represents the *phyK-GAA*2(*ERE*^{107}) fragment and the 1446 bp band implies the *ATRP1m-ALEU*2 fragment.

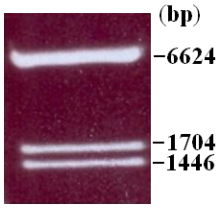

Fig. 3-36. Patterns of restriction endonuclease *Sal*I and *Afe*I of the plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK. The 6624, 1704 and 1446 bp bands represent backbone plasmid, *phyK-GAA*2(*ERE* ^{107}) and *ALEU*2-*ATRP1m* fragments, respectively.

3.11.3 Construction of plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK

After digestion and separation by electrophoresis in agarose gel, *AURA*3*mm*, *GAA(2ERE^{107})-phyK* and backbone plasmid Xplor2-102-hERα-GAA(2ERE^{107})-phyK fragments were extracted from the gel and ligated using T4-DNA ligase in one reaction. The ligation product was transformed into *E. coli* and the proper orientation of the *phyK* gene was confirmed by digestion with *Eco*RI. According to the restriction patterns, 9 positive clones (fragment of 2324 bp) were identified (1st-2nd, 4th-7th, 9th, 12th-13th), whereas 6 negative clones (fragment of 3349 bp) were obtained (3rd, 8th, 10th-11th, 14th-15th 17th). The negative clones had the phytase fragment insertion in a wrong orientation.

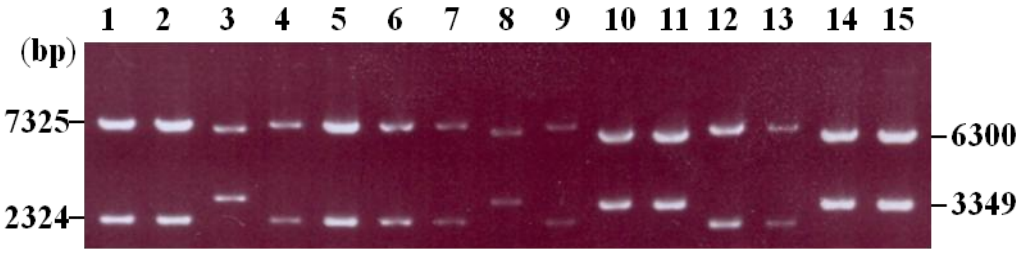

Fig. 3-37. Digestion patterns of recombinant plasmid Xplor2-103-hERα-GAA(2ERE^{107})-phyK with *Eco*RI. Lane 1-15*:* Xplor2-103-hERα-GAA(2ERE^{107})-phyK 1st-15th. The 2324 and 7325 bp bands represent positive clones, 3349 and 6300 bp bands denoted negative clones.

3.11.4 Integration of *AURA3mm* selection marker containing cassette in the auxotrophic uracil mutant *A. adeninivorans* G1214

Plasmid Xplor2-103-hERα-GAA(2ERE[107])-phyK is based on the Xplor 2 vector system and has *Asc*I restriction sites between two d25S rDNA segments and *E. coli* portion. To remove the *E. coli* part, the plasmid was digested with *Asc*I enzyme. The resulting linearized fragment, YRC103-hERα-phyK (shown in Fig. 3-38) is targeted to rDNA and integrated in *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*] by transformation (see section 2.2.4.2). Transgenic *A. adeninivorans* G1214/YRC103-hERα-phyK strain can grow in YMM without uracil supplementation.

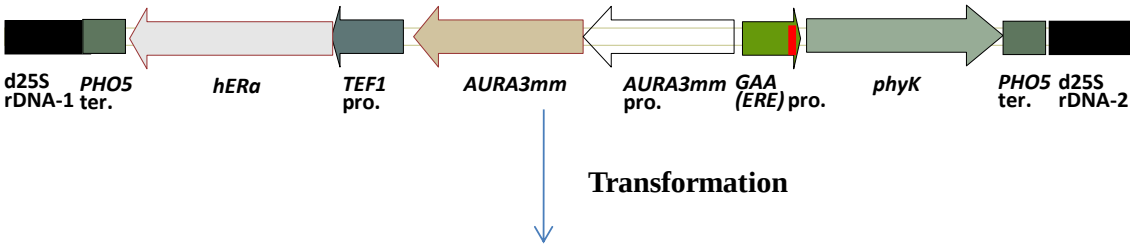

Fig. 3-38. Schematic illustration of transformation of *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*] with YRC103-hERα-phyK. YRC103-hERα-phyK, containing the *hERα* receptor, *phyK* reporter gene expression modules and the *AURA3mm* selection module, was integrated into auxotrophic *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*].

3.11.5 Screening of transgenic *A. adeninivorans* G1214/YRC103-hERα–phyK transformants for phytase activity

After transformation, 10 selected *A. adeninivorans* G1214/YRC103-hERα-phyK strains were chosen for genetic stabilization by cultivation in selective and non-selective medium for 3 weeks. Afterward, the phytase activity of these strains was tested biochemically (Fig. 3-39).

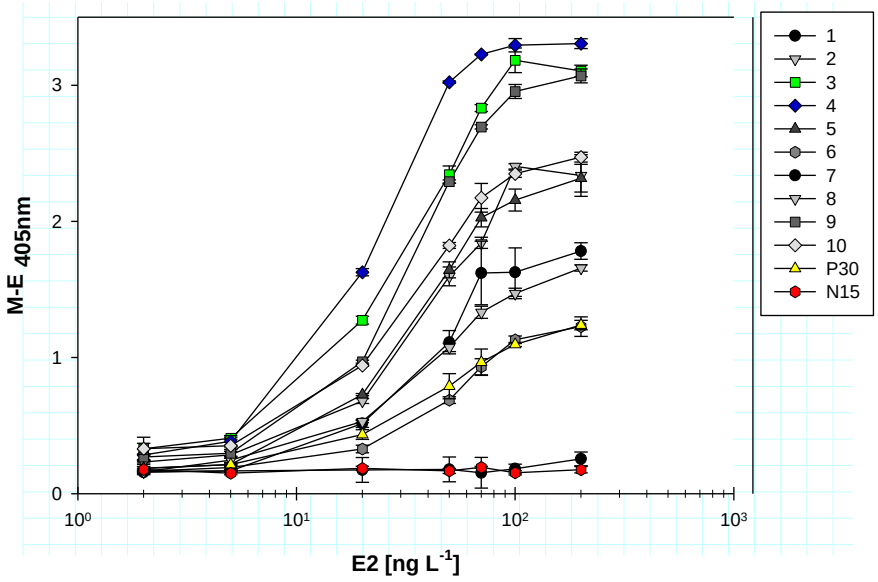

Fig. 3-39. Screening of *A. adeninivorans* G1214/YRC103-hERα-phyK transformants. Transformants were incubated with different E2 concentrations at 37 °C in 96 deep-well plate in YMM-maltose at 330 x g for 4 h. Phytase activity was measured biochemically using 0.1 M sodium citrate buffer (pH 3.9) by M-E_{405nm}. P30 = current *A. adeninivorans* G1212/YRC102-hERα-phyK strain; N15 = *A. adeninivorans* G1212/YRC103-phyK strain.

The combined test results are depicted in Fig. 3-39. All strains show a clear response in the presence of E2. However, the phytase productions of most new transformants were clearly higher than that of the current *A. adeninivorans* G1212/YRC102-hERα-phyK (P30) strain.

3.11.6 Selection of optimal transgenic *A. adeninivorans* G1214/YRC103-hERα-phyK strains

To increase the time efficiency of the system, two transgenic *A. adeninivorans* G1214/YRC103-hERα-phyK strains with the highest signal response (numbers 3 and 4 in Fig. 3-39) were chosen for further studies. The two strains were incubated for 1, 2, 3 or 4 h with different E2 concentrations in YMM-maltose at 37 °C. Afterwards, the phytase activities were biochemically measured using 0.1 M sodium citrate buffer (pH 3.9) and the results were summarized in Fig. 3-40. It was interesting to observe that in all strains tested for 2 h incubation appeared sufficient to generate a signal. When longer incubation times of 2 to 4 h were applied, (Fig. 3-40B-D) it was found that signals resulting from *A. adeninivorans* G1214/YRC103-hERα-phyK strains were distinctly higher than those from *A. adeninivorans* G1212/YRC102-hERα-phyK (P30) strain (Fig. 3-40A). Unfortunately, the obtained measur-

ing signals after 1 h incubation showed to be flat (Fig. 3-40A). It seemed that it was impossible to detect the signal within 1 h incubation.

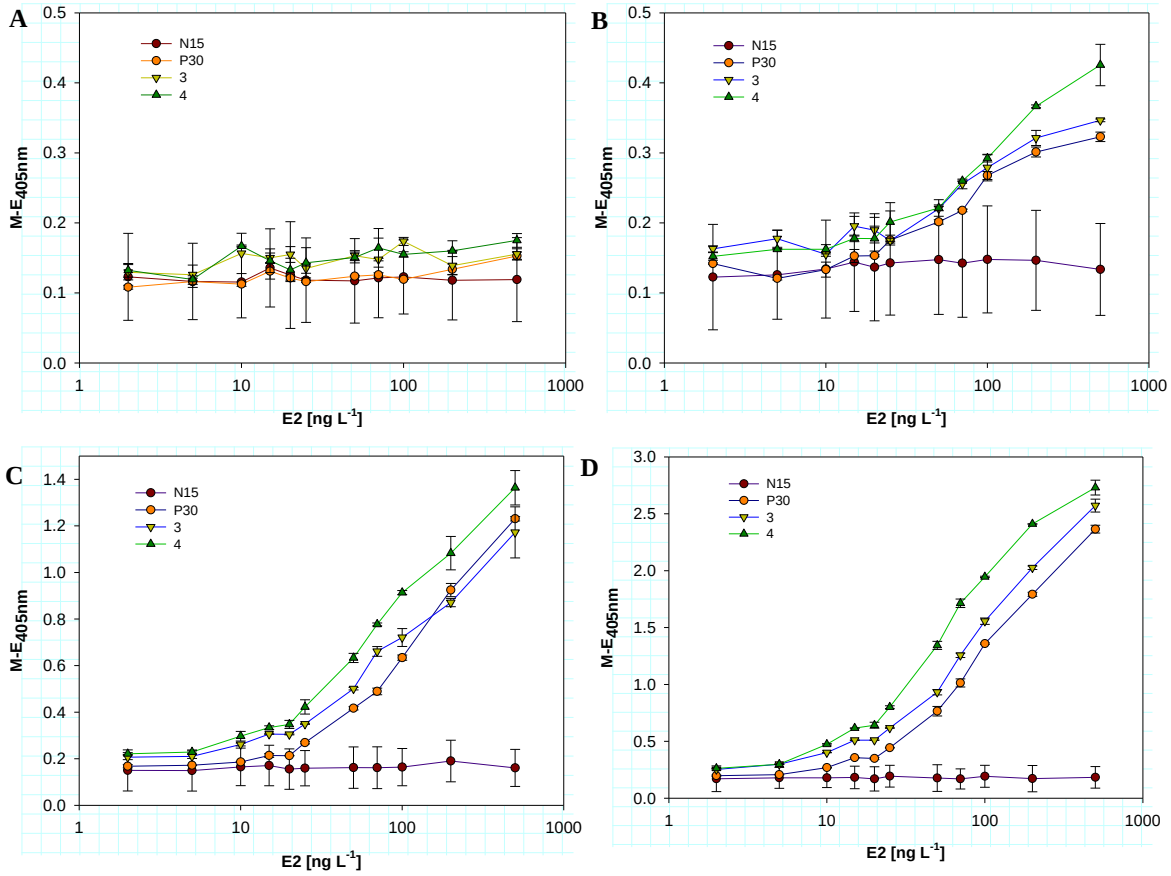

Fig. 3-40. Comparing the phytase production of different transgenic *A. adeninivorans* G1214/YRC103-hERα-phyK strains. *A. adeninivorans* G1214/YRC103-hERα-phyK strains number 3, 4; *A. adeninivorans* G1212/YRC102-hERα-phyK (P30) and *A. adeninivorans* G1212/YRC102-phyK (N15) were incubated with different E2 concentrations at 37 °C in 96 deep-well plate in YMM-maltose, at 330 x g for 1 h (A), 2 h (B), 3 h (C) and 4 h (D). Phytase activity was biochemically measured using 0.1 M sodium citrate buffer (pH 3.9) by M-E_{405nm}.

The EC_{50} and LoD values were calculated using BioVal® software and are presented in Table 3-5. The data show very low LoD and EC_{50} values of 1.34 and 45.86 ng L^{-1} for strain number 4 when compared to strain number 3 and P30 (EC_{50} values of 77.83 and 72.96 ng L^{-1}, LoD values of 5.38 and 3.69 ng L^{-1}, respectively).These results therefore open a new trend in the search for alternative microbial components with higher sensitivities and stronger signal responses. Further investigations on *A. adeninivorans* G1214/YRC103-hERα-phyK strains are in progress.

Table 3-5. EC_{50} and LoD values of the *A. adeninivorans* G1214/YRC103-hERα-phyK strains number 3 and 4, and *A. adeninivorans* G1212/YRC103-hERα-phyK (P30) after 2 h incubation with E2.

Strains	Min	Max	EC_{50} [ng L^{-1}]	Hillslope	LoD
P30	0.13	0.33	72.96	1.54	3.69
3	0.16	0.35	77.83	1.72	5.38
4	0.15	0.47	45.86	1.3	1.34

4 Discussion

In recent years, there has been an increased awareness regarding the occurrence of estrogenic active substances in the environment and their hazardous effects on aquatic organisms. Monitoring estrogenic contaminations is therefore urgently needed. To meet the increasing demands, numerous estrogenic detection methods have been developed such as E-screen-assays, yeast-based assays and receptor binding assays. In the present study, the EstraMonitor is introduced, a novel system based on robust transgenic *A. adeninivorans* yeast strains as a microbial biosensor in combination with an amperometric detection method. The EstraMonitor is designed as a complete apparatus, adapted for estrogenic detection in aquatic samples with automated, continuous and *semi-online* manners. The microbial, biochemical and electrochemical components have been optimized to achieve a highly sensitive and time-saving system. The EstraMonitor, therefore offers a practical and effective application for monitoring estrogenic activities in sewage treatment plants.

4.1. Advantages of *A. adeninivorans* cells as microbial components

For a long time microbial biosensors suffered from a poor selectivity because of non-specific cellular responses to a substrate. However, through the introduction of biotechnology and the availability of complete genome sequence for microorganisms, it has become possible to genetically engineered microbes with specific metabolic pathways tailored to a particular target (Su *et al.*, 2011).

The transgenic yeast strain *A. adeninivorans* G1212/YRC102-hERα-phyK was engineered to co-express the *hERα* and the *Klebsiella* derived phytase (*phyK*) reporter gene under control of an *Arxula* derived *glucoamylase* (*GAA*) promoter modified by insertion of *EREs* (Hahn *et al.*, 2006; Kaiser *et al.*, 2010). The physical maps of the cassettes used for constructing *A. adeninivorans* G1212/YRC102-hERα-phyK and *A. adeninivorans* G1212/YRC102-phyK are shown in Fig. 4-1A.

For the genetic modification of *A. adeninivorans*, the Xplor 2 vector system was employed. Using this system, Kaiser *et al.* (2010) reported a highly efficient expression of target genes (*hERα* receptor and *phyK* reporter genes) in *A. adeninivorans* G1212/YRC102-hERα-phyK cells. As a consequence, Kaiser *et al.* (2010) were able to successfully apply *A. adeninivorans* G1212/YRC102-hERα-phyK cells for estrogenic detection in a biochemical nAES-assay. The present study has proven that *A. adeninivorans* G1212/YRC102-hERα-phyK cells can also be effectively employed in a microbial biosensor system in combination with an am-

perometric detection method (see Fig. 3-4A). The results show that the measured currents correlate with the E2 concentrations. This means that there is a linear correlation between the phytase levels produced by the *A. adeninivorans* cells and the E2 concentrations in a range from 0 to 200 ng L^{-1}. The recombinant phytase reacted well with the electrochemical substrate *p*-APP and generated the electrochemical active intermediate product *p*-AP which was oxidized at the electrodes. The clear electric signals detected by the EstraMonitor accredit the *A. adeninivorans* G1212/YRC102-hERα-phyK strain high detection sensitivity for estrogenic contaminations. Interestingly, also after 2 h incubation of the samples, reliable measurements were still possible. This demonstrates the robustness of *A. adeninivorans* G1212/YRC102-hERα-phyK to E2.

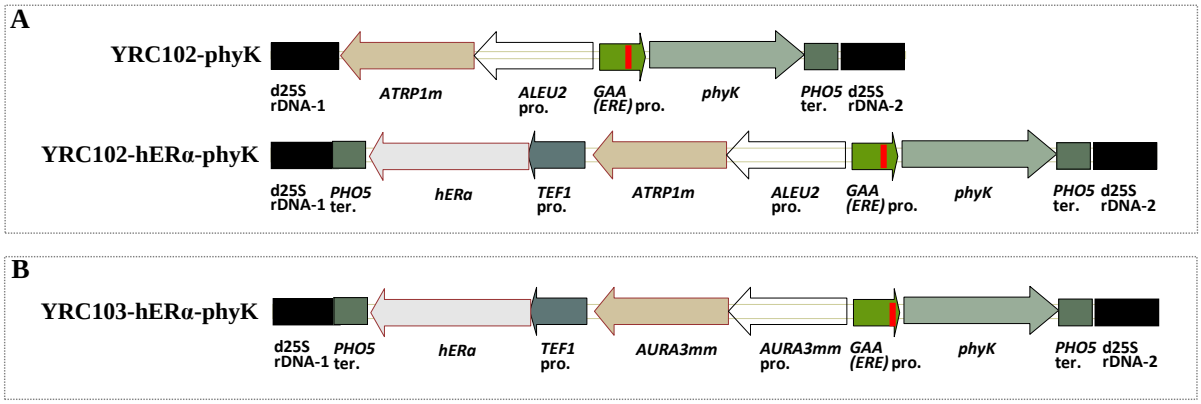

Fig. 4-1. Physical maps of cassettes used for transformation in *A. adeninivorans* G1212 (A) (Kaiser *et al.*, 2010) and *A. adeninivorans* G1214 (B). YRC102-phyK contains a selection marker module (*ATRP1m* fused to the *ALEU2* promoter), and reporter gene expression module (*GAA2xERE*$^{-107}$ promoter-*phyK* gene-*PHO5* terminator). This cassette was used to design the control strain *A. adeninivorans* G1212/YRC102-phyK. Cassette YRC102-hERα-phyK was based on YRC102-phyK with an insertion of the receptor gene expression module (*TEF1* promoter-*hERα-PHO5* terminator). The YRC102-hERα-phyK cassette was used to design the measuring strain *A. adeninivorans* G1212/YRC102-hERα-phyK. YRC103-hERα-phyK contains the *AURA3mm* gene selection marker module, the receptor and reporter gene expression modules. All cassettes were flanked by d25S rDNA sequences for targeting.

Additionally, an auxotrophic mutant *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*] has recently been selected by gene disruption of the auxotrophic mutant *A. adeninivorans* G1211 [*aleu2*] (Giersberg *et al.*, 2012). *A. adeninivorans* G1212 [*aleu2 atrp1::ALEU2*] (Steinborn *et al.*, 2007b) was also based on the *aleu2* mutant strain *A. adeninivorans* G1211, which was selected after chemical mutagenesis of the wild-type strain LS3 (Samsonova *et al.*, 1996). It would be interesting to investigate the phytase production capacity of *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*] and G1212 [*aleu2 atrp1::ALEU2*], which have the

same genetic background, to establish a potential application in the EstraMonitor system. Therefore, using the Xplor 2 vector system, we constructed a new cassette, YRC103-hERα-phyK, which is similar to YRC102-hERα-phyK except for the selection marker modules (see Fig. 4-1B). The results in Fig. 3-40 and Table 3-10 demonstrate that the phytase activity of the non-immobilized *A. adeninivorans* G1214/YRC103-hERα-phyK cells (strain 4) was significantly higher than that of *A. adeninivorans* G1212/YRC102-hERα-phyK (P30 strain in Fig. 3-40). The EC_{50} and LoD values for E2 obtained with *A. adeninivorans* G1214/YRC103-hERα-phyK strain 4 were 45.86 and 1.34 ng L^{-1}, respectively, whereas they were 72.96 and 3.69 ng L^{-1} in *A. adeninivorans* G1212/YRC102-hERα-phyK. In particular, the *A. adeninivorans* G1214/YRC103-hERα-phyK strain was capable to accumulate sufficient target protein which is considered as an estrogenic response signal after induction with E2. It further seemed that the *aura3* deficient yeast mutant has a faster response to estrogenic compounds than the *atrp1* mutant. An explanation might be that *A. adeninivorans* is a dimorphic yeast (Wartmann *et al.*, 1995), and that the mutant *A. adeninivorans* G1212 forms more budding cells, whereas the mutant *A. adeninivorans* G1214 prefers the mycelia form (data unpublished). Wartmann *et al.* (2000) reported that budding cells and mycelia exhibit different gene expression levels and that mycelia secrete two times more protein than budding cells. It is conceivable that mutagenesis can also cause alteration in cell wall composition and morphology. This may lead to different cell wall permeabilities to protons and metabolites including estrogenic compounds between mutants. The target protein in this process is synthesized and secreted as the estrogenic response and it is possible that an altered cell wall composition can influence the retransport of E2. Overal it was concluded that the alternative *A. adeninivorans* G1214/YRC103-hERα-phyK strain represents an improved microbial component in terms of sensitivity and time-effectiveness for the EstraMonitor.

4.1.1 Advantages of using immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells

The advantage of immobilization is that cells are retained within a matrix that permits their recovery and subsequent reuse. In our case, *A. adeninivorans* G1212/YRC102-hERα-phyK cells were successfully immobilized in PVA (Lentikat®) and were analyzed under confocal laser scanning microscopy (CLSM) (Fig. 3-12). Trapping cells within a porous and stable network prevents the cells from diffusing into the surrounding medium while still allowing mass transfer of nutrients and metabolites.

The interval activation step (see 3.5.2) is an essential aspect of the immobilization process. This step enables the cells to adapt to and proliferate within the new environment inside the Lentikats. Number of cells per Lentikats and inclusion of an activation time proved equally important. Fig. 3-13 shows that signals reach maximum values with 7.5 x 10^7 cells per Lentikat in combination with an activation time of 30 h. Longer activation times should not be applied since they raise the possibility of accumulating dead cells in the Lentikats thus reducing their efficiency.

An effort was made to establish the optical number of Lentikats to be used in the EstraMonitor. The results showed a linear correlation between Lentikat number and signal increase (Fig. 3-14). Due to the size limitations of the measuring chamber, the maximal number of Lentikats tested was ten. Although obviously saturation was not achieved, using eight or more Lentikats give sufficient signal for an analysis. It was therefore decided to use eight Lentikats in all subsequent experiments. The result presented in Fig. 3-15 show that *A. adeninivorans* G1212/YRC102-hERα-phyK cells immobilized in Lentikat® liquid (PVA) maintain their activity over thirty days before deteriorating. This shows that the PVA matrix provides a suitable environment that prolongs cell activity. The long term stability of the immobilized yeast cells should increase the field of application.

The effective property of the Lentikat is its reusability. In the presence of E2 at a concentration of 50 ng L^{-1}, immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells could be reused for up to 15 times before the enzyme activity dropped below the 50% level. At E2 concentrations of 100 and 200 ng L^{-1}, the same immobilized cells could only be reused 2 times. At these high concentrations the E2 probably has a toxic effect on the yeast cells (see Fig. 3-16). Reusability of immobilized *Alcaligenes faecalis* cells was reported by Cheng *et al.* (2006). These authors entrapped *A. faecalis* in PVA crosslinked with boric acid. The penicillin G acylase (PGA) activity of the immobilized *A. faecalis* cells was assayed using substrate potassium penicillin G (PGK). No activity loss was measured after 15 cycles and even at the 31st cycle enzyme activity was still an impressive 65% (Cheng *et al.,* 2006). In 2007, Wang *et al.* reported on an immobilized *Acinetobacter* sp. strain PD12 for phenol degradation functions. These cells retained their stability after 50 cycles and, when stored at 4 °C, for 50 days (Wang *et al.*, 2007).

Standard calibration curves with immobilized cells using amperometric and biochemical detection methods are shown for E2 (Fig. 3-17). These results confirm the functionality of immobilized cells in response to E2. Interestingly, the EC_{50} and LoD values obtaining when

measuring with immobilized cells were 20.9 ng L^{-1} and 8.3 ng L^{-1} (Fig. 3-17A), while they were 68.9 ng L^{-1} and 44.5 ng L^{-1} when measuring with suspended cells (Fig. 3-5B).

Kaiser *et al.* (2010) previously reported that more than 97% of the total phytase enzyme activity detected resided in the extracellular phase. In case of the nAES assay (Kaiser *et al.*, 2010) and other YES-assays, the cell suspension, after induction with E2 or estrogenic compound, must be removed by centrifugation to avoid that cell pellet or other substances may disrupt the enzyme substrate reaction. The latter is analyzed by supplementing the clear supernatant after centrifugation with the substrate for the enzyme reaction. With immobilized *A. adeninivorans* cells there is no need for cell lysis and further centrifugation steps to determine phytase activity. However, it is possible that cells entrapped within a porous matrix could be released from the inclusion particles. In such a case, a system would contain both immobilized and free cells. In the EstraMonitor, this problem has been solved by the inclusion of a decontamination system. All waste, before discharging into the reservoir, has to pass through a heat-based decontamination system. The results in Fig. 3-10 show that all cells released from measuring chambers were dead, hence, this decontamination system ensures no genetically modified yeast cells released into the environment.

4.1.2 Effect of temperature and air sparging on phytase production of *A. adeninivorans* G1212/YRC102-hERα-phyK

A. adeninivorans is thermo-tolerant and grows at temperatures of up to 48 °C. The morphology of this yeast changes according to temperature. For instance, it exists as budding cells at 30 °C and 37 °C, as pseudomycelia at 42 °C and as mycelia at 45 °C (Wartmann *et al.*, 2002) (Fig. 4-2). It is well known that temperatures affect the accumulation of recombinant proteins. In our study, the highest phytase production occurred during 4 h incubation at 37 °C. This productivity was 2 times higher than that at 30 °C, the conventional temperature for yeast cells cultivation (see also Fig. 3-3).

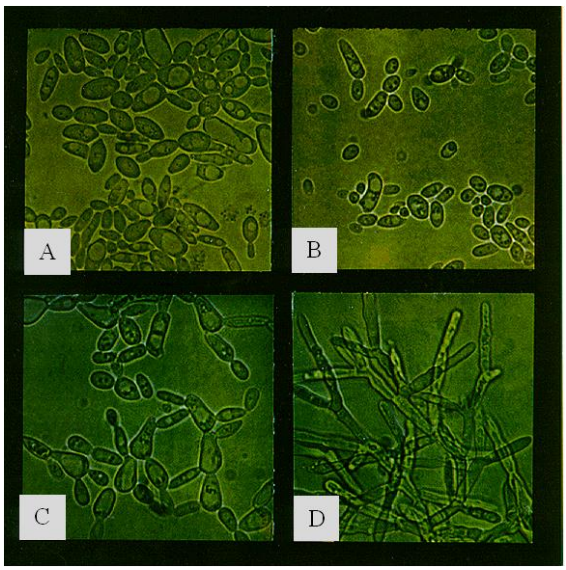

Fig. 4-2. Influence of temperature on morphology of *A. adeninivorans.* (A) 30 °C: budding cells. (B) 37 °C: budding cells. (C) 42 °C: pseudomycelia. (D) 45 °C: mycelia (Wartmann *et al.*, 1995).

Oxygen is essential for *A. adeninivorans*, experiments with non-immobilized cells showed that the signals obtained with oxygen sparging were three times higher than those without oxygen sparging (Fig. 3-9). This effect of air sparging on immobilized cells was also observed in Fig. 3-1 where signals from immobilized cells with air sparging were two times stronger than without air sparging. Therefore, it was emphasised that an air sparging system should be included in the EstraMonitor system.

4.1.3 Osmo-tolerance of *A. adeninivorans* G1212/YRC102-hERα-phyK

Salinity may cause proliferation inhibition, toxicity, or death of the tested organisms (Calmano, 2001). Furthermore, it may interfere with bioavailability of environmental pollutions. Salinity may thus influence bioassays in many ways (Kase *et al.*, 2008). In enzyme link receptor assays (ELRA), salinity may also act with components of the ELRA, such as the buffering system (Kase *et al.*, 2008). The ionic strength may adversely influent the conformation and solubility of proteins, altering the binding capacities of receptors (Kase *et al.*, 2008). In E-screen and YES assay, salinity causes strong inhibition in growth rates. *A. Adeninivorans*, however, is an osmo-resistant yeast species which can tolerate high levels of osmolytes like NaCl (Böer *et al.*, 2004). The results in Fig. 3-22 and 3-23 confirm the robustness of transgenic *A. adeninivorans* (see also section 1.7). This makes *A. adeninivorans* G1212/YRC102-hERα-phyK well adapted for the analysis of the extreme samples containing up to 5% NaCl (Kaiser *et al.* 2010). In contrast to this, YES assay only works in less than 1% NaCl (Kase *et al.*, 2008). Kase *et al* (2008) also reported that, for E-screen-assay the growth

rate of yeast was reduced by 20% in the presence of 0.5% NaCl and by more than 70% at 1.5% NaCl. Interestingly, *A. adeninivorans* has a high salinity tolerance, and dramatically decreases in growth rate only occur at NaCl concentrations higher than 10% (Wartmann and Kunze, 2000). The osmo-tolerant character of *A. adeninivorans* was also used by Riedel *et al.* (1998) in developing a rapid Biochemical Oxygen Demand (BOD) method for the application in wastewater and brackish water. This character has been also exploited in producing an estrogen assay which can be used in high salty environments (Riedel *et al.*, 1998). Additionally, Fig. 3-22 and 3-23 show typical sigmoidal curves with low standard deviation for samples containing up to 5% NaCl.

This characteristic is found in both non-immobilized and immobilized cells. Interestingly, the LoD values obtained with immobilized cells, were 1.52, 3.39, 0.39, 0.36, 2.4 and 1.1 ng L^{-1} (corresponded to 1, 2, 3, 4 and 5% NaCl), and lower than those obtained with non-immobilized cells (4.25, 2.73, 1.67, 1.54, 1.18 and 1.66 ng L^{-1} respective with 1, 2, 3, 4 and 5% NaCl).

4.1.4 Functionality of *A. adeninivorans* in the matrix of the real wastewater samples

Currently, in most bioassays, the environmental samples must be pre-treated by solid phase extraction (SPE) before the estrogenic activity can be determined. This is because estrogens normally occur in a bound form which reduces the sensitivity of an assay (Kaiser *et al.*, 2010; Fine *et al.*, 2006). Still, it is possible to measure estrogenic activity in un-treated sample with adequate sensitivity as shown by Balsiger *et. al.* (2010), who used a four – hour yeast bioassay for the direct measure of estrogenic activity in the environmental samples without extraction, concentration or sterilization.

In trials with environmental samples we also analyzed the function of immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells under different conditions of the complex matrix. Both amperometric and biochemical detection methods (modified nAES assay) were used for this purpose. Because the concentrations of estrogenic compounds in environmental samples may be lower than the sensitivity of detection methods, environmental samples, collected from different sources in Germany, were spiked with E2 to stimulate and amplify the signals. The signal obtained after subtracting the value of the un-spiked sample from that of the spiked sample was used for calculation of the EEQ (E2 equivalent). Based on the sigmoidal standard curve for E2 and the 4-parameter logistic equation (see section 2.2.9), the EEQ was interpolated. It is likely that E2 concentration used for spiking should be optimized for

each detection system in order to estimate the correct E2-equivalent (EEQ). Based on the standard curve for E2, the E2 concentration used for spiking should be situated in the curve point or curve range with a high hill slope level. In order to understand this effect, further studies need to be investigated. In our first trials, wastewater was used at concentrations of 20 and 80% (v/v) spiked with different E2 concentrations. The results with non-immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells (Fig. 3-25) confirmed the robustness and functionality of the cells. They were able to grow in 80% (v/v) wastewater form different environmental sources. The functionality of the immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells was also proven in Fig. 3-26 and 3-27.

4.2. The use of an amperometric detection method in the EstraMonitor

Amperometric detection method has been extensively exploited in environmental microbial biosensors (D'Souza, 2001). It provides rapid and sensitive tools for the detection of pollutants. Combining an electrochemical transducer, particularly an amperometric one, to a microbial biosensor, increases the sensitivity to estrogens and reduces the incubation time below that of other methods such as fluorescent, colorimetric or bioluminescent assays. Thomson *et al* (1991) reported that amperometric detection should provide a five times greater sensitivity than the spectrophotometric method. The amperometric microbial biosensor is a rapid and sensitivity tool in health and fermentation applications (Su *et al.,* 2011). Taking advantage of a short response time (normally about 5 min), numerous applications using amperometric detection have been introduced since 2005. For a list see Table 4-1.

However, according to our knowledge, there are only a few applications of amperometric detection to monitoring estrogens contaminations in the environment. The EstraMonitor is a novel system based on an amperometric microbial biosensor which is sensitive, selective and rapid in the determination of estrogenic activity.

Typical dose-response curves (Fig. 3-17A) together with EC_{50} and LoD values of 20.9 ng L^{-1} and 8.3 ng L^{-1}, respectively were achieved using immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells and the amperometric detection method. These results were slight higher than those achieved by biochemical detection (modified nAES assay) (EC_{50} and LoD were 13.9 and 3.4 ng L^{-1}, respectively, see also Fig. 3-17A, B). The LoD value of 2.8 ng L^{-1} for the nAES assay as previously determined (Kaiser *et al.,* 2010), demonstrates the higher sensitivity of nAES assay compared to our trial.

Table 4-1. Amperometric microbial biosensors.

Target	Microorganism	Working electrode	Range of detection	Reference
Nitrophenol	*Moraxella sp.*	Modified Carbon paste	20 nM	Mulchandani *et al.*, 2005
Paraxon	*P. putida* JS444	Clark oxygen	0.2-50 µM	Lei *et al.*, 2005
Paraxon	*M. sp*	Dissolved oxygen	Up to 50 mM	Mulchandani *et al.*, 2006
Phenol	*P. putida*	Graphite-epoxy composite	8-40 µM	Kirgoz *et al.*, 2006
Phenol	*E. coli*	Carbon ink	1.6-16 ppm	Neufeld *et al.*, 2006
Cu^{2+}	*S. cerevisiae*	Oxygen	1.6-6.35 mg L^{-1}	Tag *et al.*, 2007
Fe^{2+}	*A.ferrooxidans*	Oxygen	Up to 2.5 mM	Zlatev *et al.*, 2006
BOD	*P. syringae*	Dissolved oxygen	5-100 mg L^{-1}	Kara *et al.*, 2009
Glucose	*P. fluorescens*	Carbon paste	60-750 µM	Yeni *et al.*, 2008
Glucose	*G. oxydans*	Graphite	0.1-2.5 mM	Tuncagil *et al.*, 2009
Ethanol	*P. angusta*	Clark oxygen	-	Voronova *et al.*, 2008
Caffeine	*P. alcaligenes*	Clark oxygen	0.1-1 mg L^{-1}	Babu *et al.*, 2007

Selectivity

Selectivity is an essential criterion for any analytical tool. The cyclic and hydrodynamic voltammograms, which are presented in Fig. 3-1 and 3-2, respectively, show the optimal applied potential for *p*-AP oxidation of 150 mV (versus Ag/ AgCl reference). This low potential results in a relatively low interference and provides a low background signal for the system. It is a fact that the higher potential applies, the more substances are able to be reduced at high potential. In other words, a high potential would mean more non-specific electrochemical oxidation of non-target compounds. Repetitive scans of the cyclic voltammogram with pure *p*-AP (figure 3-1) demonstrated that the *p*-AP did not foul the electrode surface used for the EstraMonitor.

The amperometric investigations were also evaluated for matrix effect of naturally occurring compounds in true environmental samples (see Fig. 3-27). The typical sigmoidal curves achieved imply the functionality of the amperometric detection under matrix effects.

Response time

For field monitoring, especially *semi-online* or *online*, the biosensor should be simple to operate and have a short response time. To accomplish this, the EstraMonitor was designed with a simple operating system. An analytical procedure with the EstraMonitor, including incubation of immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells with E2, and the subsequently incubation with electrochemical substrate (*p*-APP) followed by signal recording, are completed within only 4 h and 10 min. Amperometric detection containing substrate incubation and signals recording accounts for only 10 min out of the total time. The speed of the amperometric detection is one of the most significant advantages over other methods like e.g. colorimetric, bioluminescence and photometric assays. Photometric detection takes about 1 h in the AYES and nAES-P assay (Hahn *et al.*, 2006; Kaiser *et al.*, 2010), and it takes 2 h for a chemiluminescent detection in the modified YES assay (Balsiger *et al.*, 2010), and 4 h for fluorescent measurement in the recombinant yEGFP yeast assay (Xu *et al.*, 2010). Quantifiable bioluminescence using a BYES assay can be obtained after 1 h though maximum bioluminescence is not reached until after 3-4 h (Sanseverino *et al.*, 2005). Therefore, the speed of the amperometric detection is almost an order of magnitude below that of other commercial systems.

Precision and accuracy

The parameters involved in sigmoidal curves have been calculated with the BioVal® software (quo data GmbH, Dresden, Germany). Relative standard deviation (RSDs) for all calibration curves are given in the range of values from 4.4 to 29.1%. These RSDs express the precision and repeatability of the detection.

The results carried out by the EstraMonitor were compared with those obtained with other well-known commercial handheld potentiostat (Palmsens) (see Fig. 3-5). Also in these experiments Pt-Ag/AgCl-Pt electrodes were used. The EstraMonitor and Palmsens analyses showed similar values for E2, e.g. EC_{50} values of 69.9 ng L^{-1} versus 78.1 ng L^{-1} and LoD values of 44.5 ng L^{-1}versus 43.9 ng L^{-1}, respectively. This demonstrates the flawless performance of the electric part of the EstraMonitor.

Similarly, importance were the results (obtained from the experiments with environmental samples) of the dose-response curves which gave regression coefficient values (R^2) ranging from 0.9845 to 0.9998 (see Fig. 3-25C, 3-26B, 3-27B). These R^2 values demonstrate the accuracy and reliability of the amperometric detection method used for the EstraMonitor system.

An additional advantage of combining the amperometric detection method with *A. adeninivorans* G1212/YRC102-hERα-phyK is the ability of using a broad range of electrochemical compounds. This benefit results from the capacity of phytase to catalyze the hydrolysis of multiple substrates including not only *p*-APP, but also the much cheaper AA2P. AA2P is a stable form of vitamin C. Its usefullness has been already shown in several studies (Kokado *et al.*, 2000; Sun and Jiao, 2005). AA2P is inexpensive and widely available on the industrial market. Its application in the EstraMonitor system was investigated. The simple procedure of the enzymatic hydrolysis reaction is shown in Fig. 3-29. Like *p*-APP, the AA2P is hydrolyzed by phytase and converted into AA. Through oxidation at the electrodes, the concentration of AA, and thus indirectly that of phytase, can be measured as an electric current. AA2P has already been employed as an alternative substrate in the EstraMonitor system. The dose response curve with E2 is shown in Fig. 3-33. The interpolated EC_{50} value for E2 was 15.69 ng L^{-1} with a LoD of 0.92 ng L^{-1}. This indicates that the use of AA2P renders the system a higher analytical sensitivity than when *p*-APP is used (EC_{50} and LoD values of 15.69 and 0.92 ng L^{-1} versus 20.09 and 8.3 ng L^{-1}, respectively). Therefore, by increasing the sensitivity at a lower price, AA2P should be the substrate of choice for estrogenic detection with the EstraMonitor.

4.3. Efficiency of the EstraMonitor in comparison to other assays

To compare different assay platforms, the EC_{50} and LoD values for E2, as well as the incubation and working time are summarized and tabulated in the Table 4-3.

The highest sensitivity (LoD of 0.326 ng L^{-1}) was obtained by the E-Screen assay. This assay is based on MCF-7 cells proliferation and compares the number of cells present after 6 days incubation (Körner *et al.*, 1999). However, the E-screen is the most expensive of all assays and also the 4-6 day long incubation time that it requires is the longest of all systems. Some assays, like the receptor binding assays with hERα (RB-assay) based on fluorescence polarization are at 3.5 h analysis time, slightly faster than the EstraMonitor, but more expensive and not as sensitive, having an LoD of 3.5 µg L^{-1} (Schultis and Metz, 2004).

Table 4-3. EC_{50}, LoD and time of various estrogen assays.

Estrogen assay	EC_{50} for E2 (ng L^{-1})	LoD for E2 (ng L^{-1})	Incubation and working time (h)	Reference
E-creen-assay	1.4	0.326 ± 0.054	148	Schultis and Metzger, 2004; Körner *et al.*, 1999
RB assay (hERα)	ND	2040 ± 136	3.5	Schultis and Metzger, 2004; Schultis *et al.*, 2002
RB assay (hERβ)	ND	1060.8 ± 136	3.5	Schultis and Metzger, 2004; Schultis *et al.*, 2002
LYES-assay	15	11.152 ± 0.816	9	Schultis and Metzger, 2004
YES-assay (hERα/ERE/lacZ)	65	38 ± 8.16	73.5	Schultis and Metzger, 2004; Routledge and Sumpter, 1996
YES assay (modified)	30.74-153.408	ND	45	De Boever *et al.*, 2001
A-YES- assay	ND	5	30	Hahn *et al.*, 2006
yEGFP-yeast assay	31.28	ND	4	Xu *et al.*, 2010
nAES –assay	33.2	2.8	5-24	Kaiser *et al.*, 2010
4 h YES-assay	7.27×10^{5}	ND	4	Balsiger *et al.*, 2010
Bateria assay	ND	27.2	2	Liang *et al.*, 2011
YTH system (*hERα, lac Z*)	ND	0.29	18	Schwartz-Mittelman *et al.*, 2005
Electrochemical detection	ND	$2.72 \times 10^{-3} - 2.72 \times 10^{3}$	5	Baronian and Gurazada, 2007
Electrochemical detection	0.403	11.1×10^{-3}	9	Gurazada PhD thesis, 2008
Electrochemical detection	ND	2.72×10^{5}	ND	Im *et al.*, 2010
EstraMonitor	33.6	5.3	4	Current study

ND: not determined

YES optical detection systems are popular because of their simple handling. These assays are based on a transgenic strain of the yeast *S. cerevisiae*. This strain contains e.g. the receptor gene *hERα* and the reporter gene *lac*-Z encoding the enzyme β-galactosidase. The enzyme is produced in response to the presence of estrogenic compounds and releases chlorophenol red from the chromogenic substrate chlorophenol red- β-D-galactopyranoside (CPRG). In general, these YES assays are both sensitive and cost effective. However, they are also time-consuming as they often require 72 h of incubation (Routledge and Sumpter, 1996). The most time-consuming step of these assays is the release of the intracellular β-galactosidase.

The modified LYES assay was developed to include a degradation of the cell wall with the enzyme lyticase and a permeabilization of the yeast cell membrane with triton X-100 (Schultis and Metzger, 2004). This brings about a faster conversion of CPRG to chlorophenol red and reduces the detection time to 7 h. Furthermore, Schultis and Metzger (2004) reported that the sensitivity of LYES assay is an order of magnitude better than that of the original YES assay.

The A-YES and nAES assays employ *A. adeninivorans* instead of *S. cerevisiae* (Hahn *et al.*, 2006; Kaiser *et al.*, 2010) and a photometric detection system to determine estrogenic activities in wastewater, seawater, brackish water and urine. The genetically modified *A. adeninivorans* cells were engineered to co-express the *hERα* receptor and phytase (*phyK* derived from *Klebsiella*) reporter gene under control of the *GAA* promoter (glucoamylase promoter derived from *A. adeninivorans*) which has been inserted by EREs (Hahn *et al.*, 2006). Because *A. adeninivorans* produces extracellular phytase, there is no need for cell lysis. Hahn *et al* (2006) and Kaiser *et al* (2010) have successfully established and evaluated A-YES and nAYES assay with a very high sensitivity of 5 and 2.8 ng L^{-1} E2 respectively. Interestingly, the nAES assay is even less less time-consuming than the modified LYES assay (5 h versus 7 h, respectively).

In 2010, Balsiger *et al* reported on a recombinant yeast bioassay with a measuring time of just 4 h. Their assay used a commercially available chemiluminescent substrate for the detection of estrogenic activity. This allowed the detection of signals within 30 min and out of a total assay time from start to finish of 4 h. The assay is relatively cheap and wastewater samples can be assayed without the need for sample extraction, concentration and sterilization. However, at about 190 ng $L^{-1}$the EC_{50} value for E2 was substantially higher than in the EstraMonitor.

Recently, bacterial assays for estrogenic detection have been introduced, which are based on an estrogen sensitive intein (a segment of protein that excises itseft and rejoin the remaining portion) (Liang *et al.*, 2011). The particular *E. coli* strain, DIER (detection strain with intein VMA^{ER}) was designed on an artificial estrogen –sensitive VMA^{ER} intein and ß-galactosidase colorimetric assay. The *E. coli gapA* promoter controls the expression of *lacZ* gene which is interrupted with the VMA^{ER}. The VMA^{ER} contains the intein splicing region of *S. cerevisiae* VMA and the estrogen–binding domain of hERα. In the presence of estrogenic substances, VMA^{ER} is spliced and able to excise itself out of the LacZ protein. This activates *lacZ* to synthesis ß-galactosidase used for a colorimetric assay. This new assay accomplishes the whole detection procedure within 2 h at a LoD of 27.2 ng L^{-1}. This assay offers a short time for estrogenic detection but it still not that sensitive than the EstraMonitor.

Nowadays, electrochemical detection methods are widely employed. For example, the assay, based on a recombinant *S. cerevisiae* harbouring a yeast two-hybrid-E2 system (YTH), was used to investigate the effect of estradiol induced dimerization of hERα (Schwartz-Mittelman *et al.*, 2005). The YTH system is a powerful technique to identify protein interactions such as dimerization (Wang *et al.*, 1995). This assay involves a redox product of β-galactosidase activity via protein-protein interaction. Eukaryotic transcriptional factors like Gal4p consist of two domains, the DNA-binding domain and the activation domain. An ERα monomer is fused to the DNA-binding domain derived from a Gal4p transcription factor and a second ERα monomer is fused to the activation domain of the same transcription factor (Schwartz-Mittelman *et al.*, 2005). ER dimerization induced by estradiol leads to the reconstitution of a functional Gal4p transcription factor which can be measured as β-D-galactosidase activity. An additional benefit of this assay is the use of sensitive electrochemical method to determine β-galactosidase activity. The substrate, *p*-aminophenyl β-D-galactopyranoside, is transformed into *p*-aminophenyl which is oxidized at the electrode and can be detected electrochemically by β-galactosidase. The LoD value of this assay is given as low as 0.29 ng L^{-1}, however, a complete analysis takes up to 18 h.

An electrochemical assay using wild-type yeast to quantify estrogens was firstly reported by Gurazada (2008). This study described the development and optimization of a simple, cost effective *in vivo* estrogen bioassay for the detection of estrogens using either genetically modified or a wild-type *S. cerevisiae* strains. The assay was based on catabolic repression by glucose to achieve specificity to estrogens in complex environmental samples without the requirement of sample preparation. The other attractive features of this bioassay are the use of a wild-type *S. cerevisiae* strain, its time-efficiency with only 9 h to complete the meas-

urement, its high specificity and sensitivity with a LoD of 11.1 x 10^{-3} ng L^{-1}. In addition, the electrochemical detection method makes this easy to interface with a variety of electronic devices.

Another system employing electrochemical impedance spectroscopy (EIS) was reported by Im *et al* (2010). Here the estrogen receptor was immobilized on the modified surface of a gold electrode through amide bonding. The detection of estrogenic activity was accomplished by the relative impedance changes in the EIS measurements. The apparatus contains a frequency response detector model 1025 (Oak Ridge, TN, USA) coupled with an EG&G 263A potentiostat. Though the LoD value of 2.72 x 10^{5} ng L^{-1} is quite high, it is expected that it can be applied to the fabrication of a portable estrogen sensor.

In this study, we have demonstrated that the EstraMonitor offers significant competitive advantages over other commercial estrogenic detection assays. These include short time processing and high sensitivity. Our novel system allows estrogenic detection within 4 h and 10 min, and over the same range as with a modified YES-assay (Balsiger *et al.*, 2010), and RB assays (3.5 h). The only system faster than the Estramonitor is the bacterial assays (2 h) which, however, is far less sensitive (LoD value of Estramonitor 8.3 ng L^{-1} versus 27.2 ng L^{-1} for the bacterial assay (Liang *et al.*, 2011)).

The immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells have eliminated the need to remove cells by centrifugation, or to pre-sterilize samples, since immobilized cells can work under non-sterile conditions. This helps to reduce time and lab requirements for estrogenic detection. The long-term stability and the possibility to reuse the immobilized cells also diminish cell preparation time. This allows measurements without continuous or repeated cultivation. Moreover, the reliable functioning of the immobilized *A. adeninivorans* G1212/YRC102-hERα-phyK cells under a wide variety of wastewater samples abolishes the need of sample pretreatment.

The EC_{50} and LoD results (20.9 and 8.3 ng L^{-1}, respectively) of the EstraMonitor are quite similar to those of the LYES assay (EC_{50} and LoD values of 15 and 11 ng L^{-1}), and of the nAES assay (EC_{50} and LoD values of 33.2 and 2.8 ng L^{-1}). The YTH system (Schwartz-Mittelman *et al.*, 2005) may be slightly more sensitive (LoD value of 0.29 ng L^{-1}), but requires substantially more time (18 h versus 4 h).

Despite all these positive features, there is still room for improving the current model of the EstraMonitor. The liquid controlling system for fluid exchange should be optimized and an air sparging module should be included for the incubation step. Furthermore,

the prototype of the EstraMonitor contains only 2 measuring chambers, which limit a scale-up. An improved high-throughput model of the EstraMonitor is in the pipeline. Additional chambers for the enzyme substrate reaction and detecting signals (generally called as measuring chambers) are planned. A longer cell viability may be achieved if the incubation and measuring chambers are separated since the substrate and products of the enzyme-substrate reaction can be toxic for the cells.

Finally, the EstraMonitor is a complete automated system with sample cycling, measuring and calibration processes and an alarm function. It can be applied for *semi-online* and continuous monitoring without additional instrumentation. The ability in *semi-online* and continuous monitoring is a distinct benefit which other estrogenic detection systems do not have.

Taking sample preparation time, assay time, sensitivity, capacity in *semi-online* and continuous monitoring all into consideration, then the EstraMonitor has the potential to become an important and feasible *semi-online* device for monitoring estrogenic activity in sewage treatment plants.

Future works

So far the EstraMonitor has been developed as a built-in estrogen detection system with advantage in time, cost, sensitivity and compatible to various environmental matrices. In order to enhance the sensitivity as well as to reduce the time (from 4 h to 2 h) for sample determination, the quest for an alternative *A. adeninivorans* strain, G1214/YRC103-hERα-phyK need further investigations. To reduce the operational costs, the alternative substrate (AA2P), which is both cheap and widely available, should be considered. Furthermore, to improve the automatically determination of estrogenic activity, imperfections regarding the liquid handling system for fluid exchange should be handled and an air sparging device for the incubation step should be included. Additionally, it is worth considering a system including various electrodes as well as designing separate measuring and incubation chambers. Furthermore, it is necessary to confirm the broad validation of the *semi-online* EstraMonitor system in order to guarantee acceptance on the market.

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6 Subscription

6.1. List of figures

6.2. List of tables

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8 Curriculum vitae

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Title of the thesis: Study on cloning of Cry4A mosquitocidal toxin coding gene from 01-17 *Bacillus thuringiensis* var *israelensis* strain isolated in Vietnam.

List of publication related to the current study

1. **Ha Thi Minh Pham**, Martin Giersberg, Steffen Uhlig, Gerold Hanke, Kirsten Simon, Kristin Kunath, Keith Baronian, Gotthard Kunze (2012). EstraMonitor-A monitor for amperometric detection of estrogenic activity with *Arxula adeninivorans* yeast cells as the microbial component. Sensor and Actuators B Chemical. 161: 137-145

List of publications un-related to the current study

1. **Pham Thi Minh Ha**, Vo Thi Thu, Raj Bhatnagar, Siva Ready (2007). Construction of expression vector for *cry4Ba2* gene encoding mosquitocidal toxic protein in green algae. The National Conference on Life Sciences. Vietnamese Science and Technics Publishing House.
2. **Pham Thi Minh Ha**, La Thi Nga, Vo Thi Thu, Raj Bhatnagar (2005). Expression of cry4Ba2 gene encoding mosquitocidal toxic protein from *Bacillus thuringiensis* var. *israelensis* 01-2 in *Escherichia coli*. The National Conference on Life Sciences. Vietnamese Science and Technics Publishing House.
3. Vo Thi Thu, Truong Ba Hung, Nguyen Minh Duong, La Thi Nga, Le Thi Thu Hien, **Pham Thi Minh Ha**, Le Danh Toai, Nguyen Truong Son, Le Tran Vinh, Dao Thi Thanh Xuan (2005). Bioproduct BioF fermentation and application in aquaculture. Proceedings of national workshop on research and technologies applied in aquaculture. Vietnamese Agricultural Publishing House.
4. Vo Thi Thu, Truong Ba Hung, Nguyen Minh Duong, La Thi Nga, Le Thi Thu Hien, **Pham Thi Minh Ha**, Le Danh Toai, Nguyen Truong Son, Dao Thi Thanh Xuan (2005). Manufacturing water treatment bioproduc Biochie by using *Bacillus subtilis, Bacillus megaterium, Bacillus licheniformis* and *Lactobacilus acidophilus*. Proceedings of national workshop on research and technologies applied in aquaculture. Vietnamese Agricultural Publishing House.
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6. Dang Minh Hieu, **Pham Thi Minh Ha**, La Thi Nga, Vo Thi Thu, (2003) Cloning of cry4A gene from *Bacillus thuringiensis* var. *israelensis* strain 01-17. The National Conference on Life Sciences. Vietnamese Science and Technics Publishing House.

Oral presentations

1. **Ha Thi Minh Pham** (2011). EstraMonitor: Studies on Microbial, biochemical and electrochemical components. Meeting in the frame of the "PA_EPASGO" project. Duisburg, 21st November, 2011.
2. **Ha Thi Minh Pham** (2011). Improvement of the substrate and mixing system for the estrogenic detection. Meeting in the frame of the "PA_EPASGO" project. IPK Gatersleben, 10th May, 2011.
3. **Ha Thi Minh Pham** (2011). EstraMonitor-feasibility and difficulties Meeting in the frame of the "PA_EPASGO" project. Duisburg, 03rd March, 2011.
4. **Ha Thi Minh Pham** (2010). Adaptation of *A. adeninivorans* cells to automatic measurements. Presentation in the "PA_EPASGO" project meeting: Monitoring Committee for IGF Research Project No. 16145, Gatersleben, 25th October, 2010.
5. **Ha Thi Minh Pham** (2010). Progress on Estramonitor. Meeting in the frame of the "PA_EPASGO" project. Dresden, 19th August, 2010.
6. **Ha Thi Minh Pham** (2010). Microbial biosensor for the detection of estrogenic activities. Presentation in the frame of Physiology and Cell Biology department progress seminar – IPK, Gatersleben, 20th October, 2010.
7. **Ha Thi Minh Pham** (2010). Amperometric detection of estrogenic activities by yeast cell biosensor. Presentation in the frame of PhD seminar – IPK, Gatersleben, 15th July, 2010.
8. **Ha Thi Minh Pham** (2009). Amperometric detection of estrogenic activities by yeast cell biosensor. Presentation in the frame of Physiology and Cell Biology department progress seminar – IPK, Gatersleben, 11th October, 2009.

Poster presentations

1. **Ha Thi Minh Pham** , M. Körner, C. Kaiser, S. Uhlig, K. Simon, T. Gerlach, K. Kunath, K. Florschütz, G. Kunze (2009) . Amperometric detection of estrogenic activities in tap, waste water and milk by an *Arxula adeninivorans* yeast cell biosensor. 5th Plant Science Student Conference, June 23rd-26th, University of Halle, Halle, Germany.
2. **Ha Thi Minh Pham,** M. Giersberg, C. Kaiser, M. Körner, S. Uhlig, K. Florschütz, G. Kunze (2010). Immobilized *Arxula adeninivorans* yeast cells as microbial compound of an electrochemical biosensor for the detection of estrogenic activities in waste water. 6th Plant Science Student Conference June 15th-18th, IPK, Gatersleben, Germany.
3. Giersberg M., Worch S., Hähnel U., Riechen J., **Pham T.M.H.,** Trautwein A., Jankowska D., Schwarz M., Kunze M. & Kunze G (2011). *Arxula adeninivorans* – a valuable tool for basic research and biotechnological applications. Institute's day, IPK Gatersleben, Germany.

Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Gatersleben, 08. März 2012

Ha Thi Minh Pham