Making glucose oxidase fit for biofuel cell applications by directed protein evolution

by

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A thesis submitted in partial fulfilment
of the requirements for the degree of
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School of Engineering and Science

Dedication
to my parents, my brother and my husband

致经纬
如果有来生，我愿与你结芦为舍，草色为帘，为你温一壶黄滕素酒，与你和一曲《高山流水》的缠绵 如果有来生，我愿与你站在一起，站成两棵树，抱崖而立，共餐风饮露，同耕云种月，相依相伴，岁岁年年.

Stay hungry, stay foolish…

Steve Jobs

ABSTRACT

Pioneered by Adam Heller, miniaturized enzymatic biofuel cells (mBCs) convert blood sugars into electrical energy by employing for example glucose oxidase (GOx) on the anode and bilirubin oxidase on the cathode. To match application demands it is crucial to increase life-time and power output of mBCs. The power output has been limited by the performance of GOx on the anode. However powerful directed protein evolution have been developed for tailoring proteins to our needs in industry applications. Inspired by nature Darwinian evolution, directed evolution involves mutation, recombination and screening or selection to accumulate the mutants required to achieve significant change in protein function.

The gene encoding *gox* harboring α-pheromone signal sequence from *Aspergillus niger* was expressed as secretary product in yeast *Saccharomyces cerevisiae*. Error prone PCR has been used to generate mutant libraries of GOx by adjusting the concentration of $MnCl_2$. 4000 mutants have been screened for catalytic activity and thermostability. One mutant (G4) showed two-fold higher activity than WT and an enhanced thermostability. For the screening of mutants, two novel and reliable assays were developed for medium-throughput screening. Glucose oxidase detection assay (GODA) and ferroecenemethanol based assay were developed for improving GOx properties by directed protein evolution. GODA is a reaction product detection assay based on coupled enzymatic reactions leading to NADPH formation which is recorded at 340 nm. The main advantage of the assay is that it detects the production of D-gluconolactone instead of the side-product hydrogen peroxide and enables to improve bioelectrochemical properties of GOx. Ferrocenemethanol based assay can directly screen electron transfer between oxidized form of ferrocenemethanol and reduced form of GOx by monitoring colorimetric development. It is a simple, less expensive and

rapid assay, more importantly; it is insensitive to nature electron acceptor dioxygen in pH 8.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABTS	2,2′-azino-bis(3-ethybenz-thiazoline-6-sulfonic acid
ANS	8-anilino-1-naphthalene sulfonic acid
Atm	Atmosphere
BCA	Bicinchoninic acid
BOD	Bilirubin oxidase
bp	Base pair
cm	Centimeter
CV	Coefficient of varient
DCPIP	2,6-dichloroindophenol
DMSO	Dimerthylsulfoxide
DNA	2'-Desoxynucleotide-5'-triphosphate
dNTP	Desoxyribonucleicacid
e	Electron
epPCR	Error-prone polymerase china reaction
ET	Electron transfer
FAD	Flavin adenine dinucleotide
g	Gram
GDH	Gluconate dehydrogenase
GK	Gluconate kinase
Glu	L-Glutamic acid
GODA	Glucose oxidase detection assay
GOx	Glucose oxidase
h	Hour
His	L-Histidine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxide
Ip	Isoelectric point
kDa	Kilo dalton
LB	Luria-Bertani
mBC	Miniaturized biofuel cell
ml	Milliter
min	Minute
mol	Molar
mM	Millimolar
Mw	Molecular weight
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein database bank
POx	Pyranose oxidase
PMS	Phenazine methosulfate
rpm	Rotation per minute
SC	Synthetic minimal defined medium for yeast

SD	Standard deviation
SDS	Sodium dodecyl sulfate
Sp.	Species
TMB	3,3′,5,5′-tetramethylbenzidine
U	Uracil
UV	Ultraviolet
YPD	Yeast Extract/Peptone/Dextrose
6-PGDH	6-PGDH 6-phosphogluconate dehydrogenase

1. INTRODUCTION

1.1 Glucose oxidase

1.1.1 Natural host

Glucose oxidase (EC.1.1.3.4) is found on the surface of fungi, where it helps protect against bacterial infection, and it is also found in honey, where it acts as a natural preservative (White et al. 1963). Glucose oxidase (GOx) was first isolated from *Aspergillus niger* (Müller 1928) and later it has been isolated and purified from several strains of *Aspergillus* and *Penicillium* species as well as from *Talaromyces flavus* (Fiedurek et al. 1986; Kim 1990). Furthermore, a nonglycosylated enzyme with a relative molecular mass of approximately 180000 has been described from the fungus *Phanerochaete chrysosporium* (Kelley and Reddy 1986). Genes encoding *gox* has been isolated from two different strains of *Aspergillus niger* (Fiedurek et al. 1986; Kriechbaum et al. 1989; Whittington 1990) and it has been shown that the DNA sequences of these two genes are identical, both in the coding and in the non-coding parts of the gene.

Table 1 contains nature strains list of GOx; interestingly most were found in fungi. GOx from *Aspergillus niger* is the most investigated GOx and more than 90 % of all publications on the topic report findings about GOx from *Aspergillus niger* and *Penicillium amagasakiense*.

Table 1 Nature host strains of GOx

Organism	Accession number	Localization	Expression	Remark	Literature
Aspergillus niger	P13006	Intracellular	*A. niger A.nidulans and S. cerevisiae*	Crystal structures available	(Kriechbaum et al. 1989; Park et al. 2000; Wohlfahrt et al. 1999)
Penicillium amagasakiense	P88156	Extracellular	*P. amagasakiense and S. cerevisiae*	Crystal structures available	(Kalisz et al. 1990; Kalisz et al. 1997; Wohlfahrt, and Hecht 1999)
Talaromyces flavus	Q92452	Extracellular	*Talaromyces species*	S. cerevisiae as host not investigated	(Murray et al. 1997)
Phanerochaete chrysosporium	Unknown	Periplasmic space	*Phanerochaete chrysosporium*	Broader substrate profile; C-1/C-2 specific glucose oxidase	(Kelley et al. 1986a; Kelley et al. 1988; Volc et al. 1996*)

GOx has in general an activity optimum at a pH between 4.5 and 6.0. At pH 7, the activity of GOxs is reduced by 20 % for GOx from *Aspergillus niger* (on a "wired" glucose sensor (Kenausis et al. 1997)); and by 40 % for GOx from *Penicillium amagasakiense* (not immobilized; (Kalisz et al. 1997)). Both GOxs are reversibly inhibited, especially at a low pH and in presence of halides such as Cl^-. Halides cause a reduced flavin reduction rate which, at low pH values determines the reaction rate (Bright and Appleby 1969; Rogers and Brandt 1971; Weibel and Bright 1971). The focus on GOx from *Aspergillus niger* is apart from the known gene sequence and the crystal structure probably motivated by its commercial availability and not by its superior properties. Indeed, the few data reported show that, for example, the GOx from *Penicillium amagasakiense* has a higher turnover number and lower K_m value for ß-D-glucose (K_m 6.2 ± 0.5 mM; (Witt et al. 1998). A comparison of the protein sequence of

the fungal GOx from *Aspergillus niger* to that from *Talaromyces flavus* and to that from *Penicillium amagasakiense* revealed only a 64 % and 79 % identity on the amino acid level respectively (Murray 1997; Witt et al. 2000). These findings indicate a huge diversity among fungal GOx for industry application. Enzymes need to function sufficiently well according to several application-specific performance parameters (Figure 1, (Lorenz and Eck 2005))

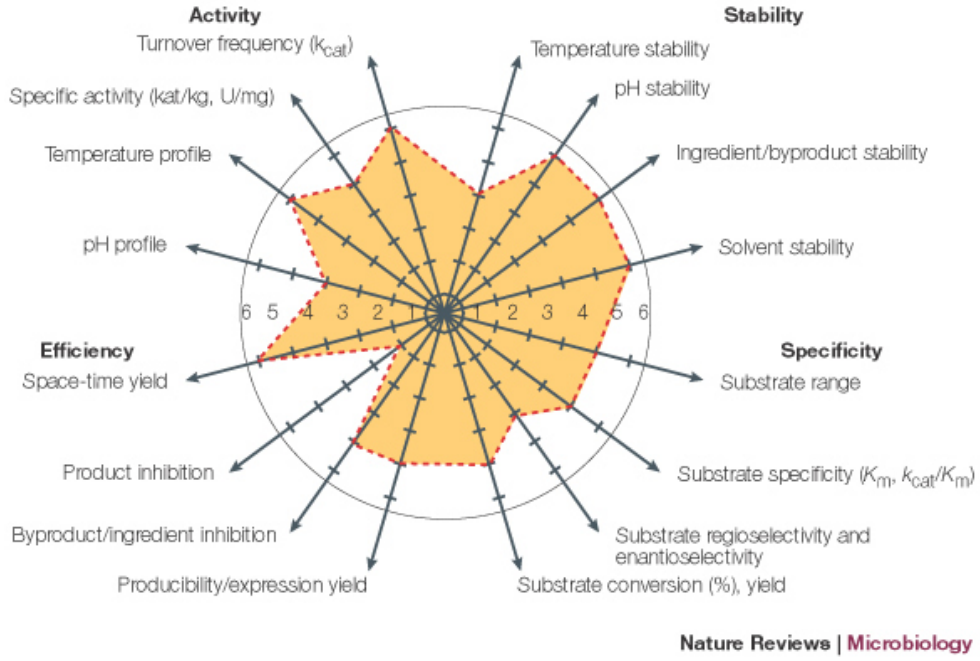
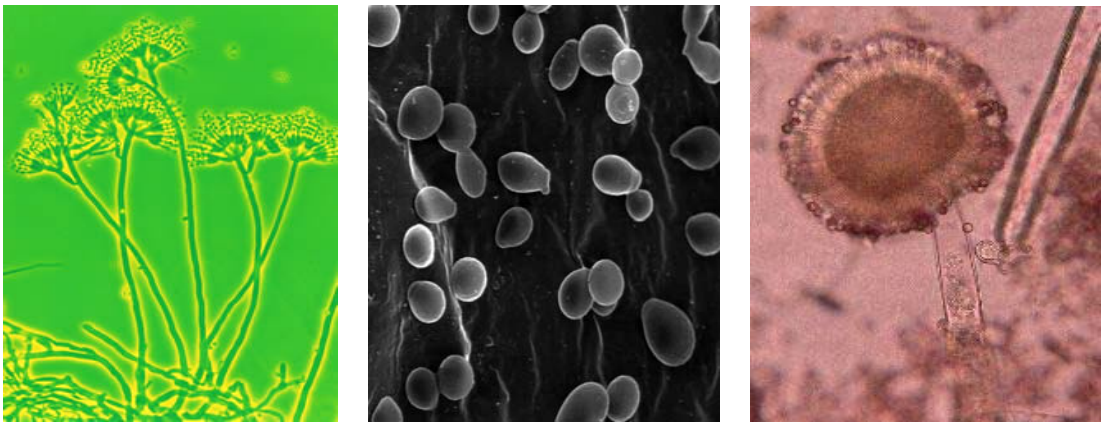


Fig.1.Multi-parameter footprint analysis. This figure illustrates the ideal biocatalyst concept. Each enzyme candidate from the metagenome is ranked, from low (rating of 1) to high (rating of 6) using a specific set of criteria, to produce a multi-parameter fingerprint (shown in yellow). Criteria include *in vitro* enzyme activity, efficiency, specificity and stability.

This decision matrix reveals the strengths and weaknesses of every candidate enzyme, so that the most promising candidate enzymes from diverse enzyme libraries can be selected for further process development by re-screening, protein engineering or directed evolution methods (Lorenz and Eck 2005).

Aspergillus niger is the main organism used for large-scale production of GOx (Kapat 1997). Much work has been done to optimize the process of GOx production through either genetic manipulation of the host strain or improvement of cultivation conditions. Mutageneses by UV-irradiation and classical screening have been used successfully to increase either the intracellular production of this enzyme (Markwell et al. 1989) or its extracellular secretion (Fiedurek and Szczodrak 1995). In order to keep a optimizing of cultivation conditions, the enzyme is induced in media containing trace elements, high concentration of β-D-glucose and low concentration of nitrogen and phosphate. High aeration rates are required and the pH of the medium must be maintained in the range of 4.5 to 5.5 (Whittington 1990).



a b c

Fig.2. Host strains of GOx: a: *Penicillium sp* b: *Saccharomyces cerevisiae* c: *Aspergillus niger*

1.1.2 Recombinant glucose oxidase

The nature host organism did not prove to be ideal for GOx production. The major difficulty of using *Aspergillus niger* as a production organism is the problem of concomitant secretion of catalase, amylase and cellulase, which acts as important contaminants impairing the application of GOx (Witteveen et al. 1992). To circumvent

this problem, *Saccharomyces cerevisiae* has been selected as a recombinant host for production of GOx (Frederick et al. 1990; Whittington 1990). Several studies have shown that GOx was one of the largest foreign proteins able to be expressed heterologously in yeasts such as *Saccharomyces cerevisiae*. The yeast *Saccharomyces cerevisiae* has a well-defined secretory system (Schekman 1982). Expression of foreign proteins in yeast is known to have advantages over other expression systems in that secretion of foreign proteins can be achieved by fusing the leader peptide or the yeast α-factor prepro-sequence to the foreign gene (Brake 1984). Moreover it was found to be successfully secreted into culture media at the concentration of 3.0 and 2.25 g l^{-1}, respectively (Debaetselier et al. 1991; Debaetselier et al. 1992; Hodgkins et al. 1993). GOx secreted in *Saccharomyces cerevisiae* shows extensive N-linked hyperglycosylation in comparison to *Aspergillus niger* GOx (Debaetselier et al. 1991).Witt has reported the possibility to express GOx from *Penicillium amagasakiense* in bacteria *Escherichia coli* as non-glycosylated enzyme, which comes to inclusion bodies, consequently it has to be purified from cell homogenates and reactivated by means of 8 M urea denaturation with following renaturation (Witt et al. 1998). Another group found that additional carbohydrate moiety of GOx has minimal effect on the catalytic efficiency of the enzyme (Ko et al. 2002). Figure 3 shows the most important factors which will have influence on the production of GOx from *Saccharomyces cerevisiae* (Debaetselier et al. 1992; Kapat 1997; Kapat et al. 2000; Park et al. 2000; Kapat et al. 2001).

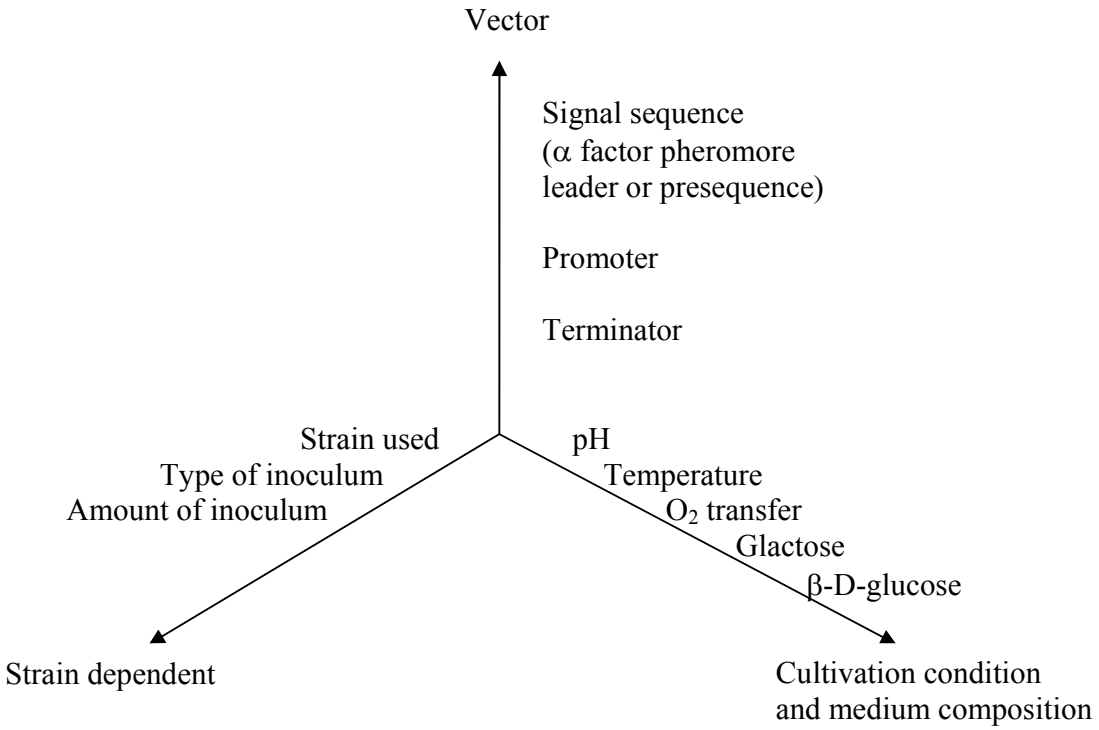


Fig.3. Factors affecting the expression of GOx in *Saccharomyces cerevisiae*

Debaetselier (Debaetselier et al. 1992) reported a marked difference in production was observed with respect to the signal sequence used: a 9-fold increase was noticed when the homologous GOx signal sequence was used instead of the α factor. The level of GOx synthesis was found to be highly dependent on the genetic background of host strain, cultivation condition and medium composition, resulting in a 100-fold difference between the lowest and highest producing strain.

1.1.3 Structure and catalytic metabolism of glucose oxidase

1.1.3.1 Structure of GOx

GOx (β-D-glucose:oxygen-1-oxidoreductase) is a homodimeric enzyme (Swoboda 1969a; 1969b; Kriechbaum et al. 1989). The molecular weight varies from 110-325 kDa

depending on the degree of glycosylation. Each subunit of this protein contains one coenzyme molecule of flavin adenine dinucleotide (FAD) which is acting as a redox carrier in catalysis. The monomer folds into two distinct domains: one that binds noncovalently but very tightly the FAD moiety and another that binds the β-D-glucose substrate (Pazur and Kleppe 1964; Swoboda and Massey 1965). The first area consists mainly of β-sheets and the second one, 4 α-helices supporting antiparallel β-sheets (Hecht et al. 1993). The dimer contains two disulfide bonds and two free sulfhydryl group (Tsuge et al. 1975). GOx from *Aspergillus niger* is a glycoprotein with a high-mannose type carbohydrate content of 10–16% of its molecular weight (Hayashi and Nakamura 1981). The carbohydrate moieties are *N* or *O*-glycosidically linked to the protein (Takegawa et al. 1991a; Takegawa et al. 1991b).

The primary structure of GOx from *Aspergillus niger* has been determined; a single polypeptide chain of one subunit has 583 amino acid residues (Kriechbaum et al. 1989; Frederick et al. 1990). The crystal structure of enzyme was solved at 2.3 Å resolution (Hecht et al. 1993).

The corresponding dimensions of the dimer are 70 Å x 55 Å x 80 Å. The minimum distance between the flavin and the surface of the monomer is 13 Å. The two isoalloxazine moieties are separated by a distance of about 40 Å, a distance which excludes any electrical communication between them.

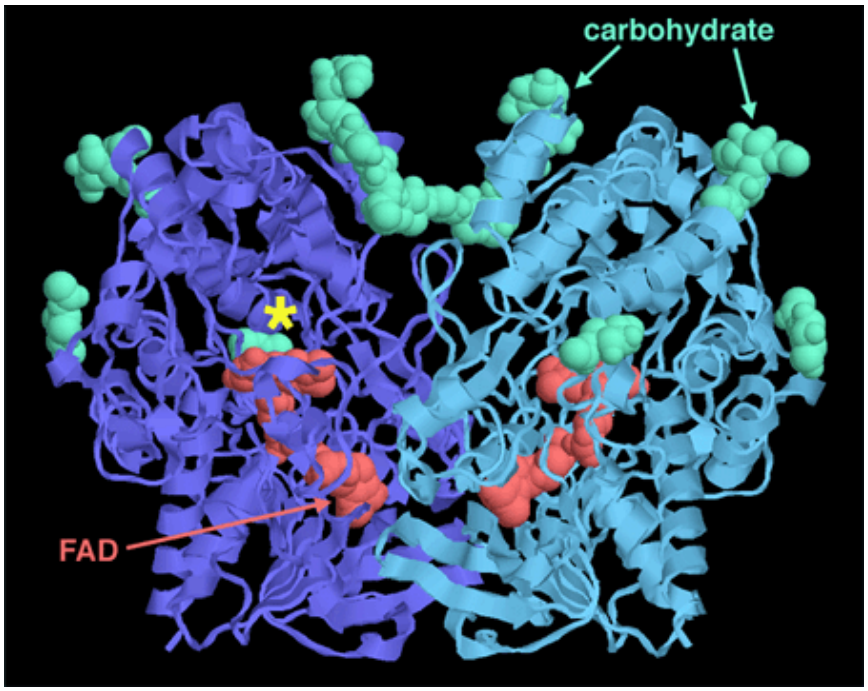


Fig.4. GOx from a *Penicillium* mold in PDB entry 1gpe.

The oxidation reaction is performed by the FAD cofactors bound deep inside the enzyme, shown in red. The active site where β-D-glucose binds is just above the FAD, in a deep pocket shown with a star. Notice that the enzyme, like many proteins that act outside of cells, is covered with carbohydrate chains, shown in green.

1.1.3.2 FAD as cofactor

FAD functions as a coenzyme because of its ability to undergo reversible redox reactions (Gibson et al. 1964). The redox active centre of the FAD coenzyme is the isoalloxazine ring system. On reduction, the yellow colour associated with the coenzyme disappears since reduced flavin is colorless. On exposure to air, the yellow colour of the oxidised form reappears. The FAD is not covalently bound and can be released from the holoprotein following a partial unfolding of the protein. The apo-enzyme missing the

FAD cofactor is not biocatalytically active, but it still binds β-D-glucose as observed from a decrease in its intrinsic tryptophan fluorescence (Eftink and Ghiron 1975b; 1975a). Sodium sulfite can be bind to FAD and form FAD-bisulfite complex. The decrease in absorbance at 450 nm was measured (Swododa and Massey 1966). It can be also reconstituted with native or artificially modified FAD cofactor for biofuel cell applications (Xiao et al. 2003).

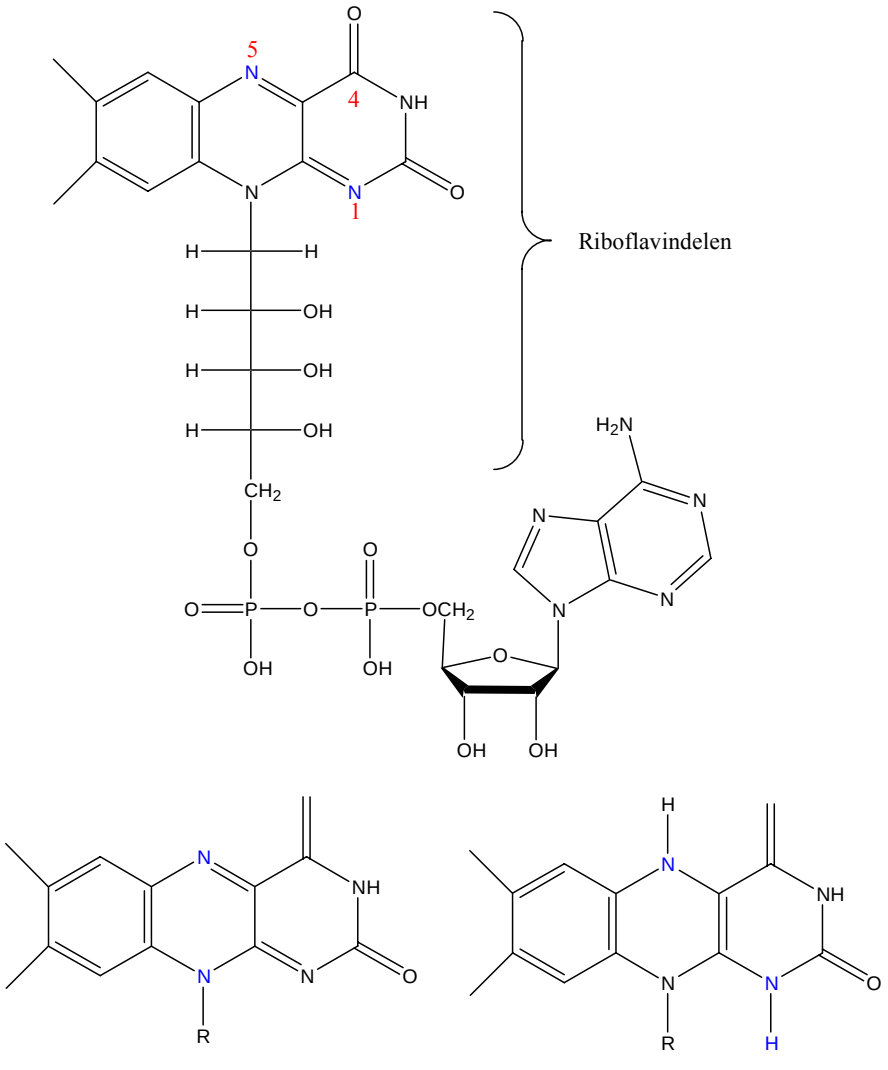


Fig.5. Structure of cofactor FAD and its redox state.

1.1.3.3 Active-site residues

There are only three amino acid side chains within close proximity of this reaction center, the side chain of glutamic acid, Glu412, and the side chains of two histidine residues, His516 and His559. His559 is fixed by a strong hydrogen bond between its Nε and Oε of Glu412. Glu412 is partly buried inside the protein molecule while the side chain of His516 is more flexible and more exposed to the solvent (Leskovac et al. 2005).

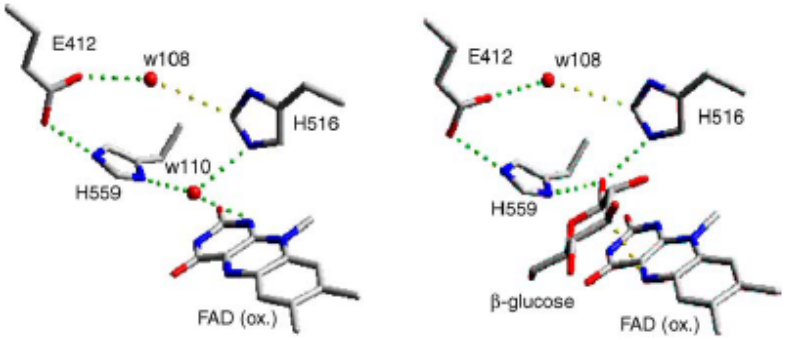


Fig.6.Active site of the substrate-free, oxidized enzyme and the enzyme·FAD_{ox} β-D-glucose complex (Wohlfahrt et al. 1999).

The structural features of the active site of enzyme clearly show that one of the two histidines, His516 or His559, must be the base catalyst in the overall catalytic cycle. There are several lines of evidence that point out to His516 as the general base catalyst in the reductive and the oxidative half-reaction. The genetic mutant of the *Aspergillus* enzyme, with His516 replaced by alanine, is almost inactive. His516 is strictly conserved in the primary structure of many oxygen-consuming oxidases (glucose oxidases, alcohol oxidases, cholesterol oxidases), while His559 is not; this clearly suggests that His516 serves a crucial, genetically mandated, role in catalysis (Roth and Klinman 2003).

The kinetic and structural data indicate that the p*K*a value of Glu412 (bound to His559), in the free reduced enzyme, is approximately 3.4. On the other hand, the p*K*a value of His516, in the free reduced enzyme, is approximately 6.9. The other histidine in the active site, His559, is bound to Glu412 by a hydrogen bond; the p*K*a of this histidine is probably elevated by one or two pH units, particularly in the reduced enzyme, which is beyond the pH range covered in this work.

1.1.3.4 Catalytic metabolism of glucose oxidase

GOx catalyzes the oxidation of β-D-glucose in a highly specific way. Other monosaccharides are oxidized at the much lower rates (Pazur and Kleppe 1964).

The catalytic cycle can be divided into two half-reactions, referred to as the reductive and the oxidative half-reactions (Figure 7). In the reductive half-reaction, GOx catalyzes a two-electron oxidation of β-D-glucose to D-gluconolactone, which is hydrolysed non-enzymically to gluconic acid. The flavin ring of GOx thereby becomes reduced to $FADH_2$. Thus two electrons and two protons are transferred to the flavin ($2e^-$ and $1H^+$) and to a histidine residue ($1H^+$) of the enzyme.

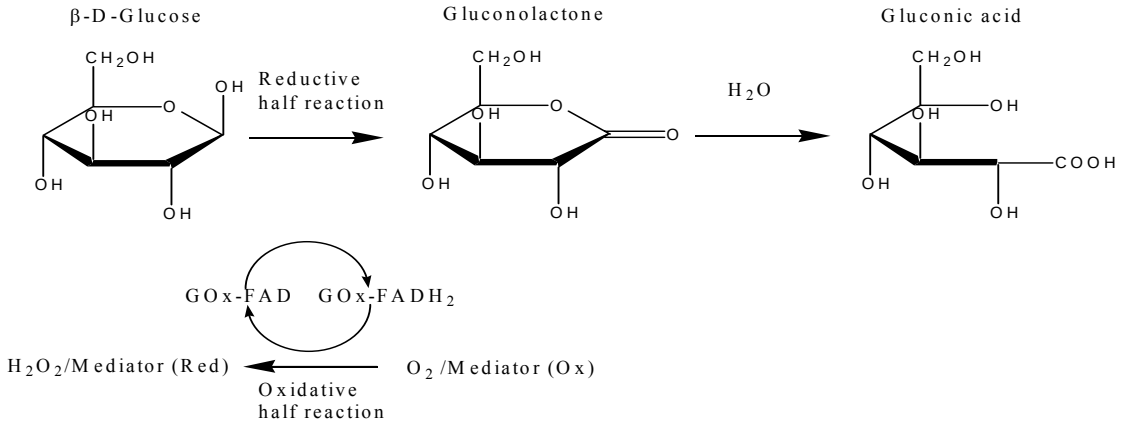


Fig.7. Scheme of representation of GOx reaction

In the oxidative half-reaction, $FADH_2$ acts as a transducer molecule and provides electrons and protons to dioxygen/Mediator (ox) to yield H_2O_2/ Mediator (Red) and complete the catalytic cycle.

The natural electron acceptor in GOx-catalyzed reactions is molecular oxygen (Gibson et al. 1964). However, many other electron acceptors, natural and synthetic ones, are also able to function in GOx reactions (Wilson and Turner 1992; Bourdillon et al. 1993). The electron acceptors were divided into three main groups: molecular oxygen, quinones, and one-electron acceptors. The kinetic and chemical mechanism of action for each group of substrates was examined in turn with a wide variety of kinetic methods and by means of molecular modeling of enzyme-substrate complexes. The steady-state kinetics of the oxidation of glucose by molecular oxygen, as well as by all other substrates strictly obeys the Ping Pong Bi Bi mechanism (Bright 1975). The pH dependence of individual steps in GOx reaction has been studied in detail and a reaction mechanism has been proposed.

1.2 GOx electrodes for biofuel cell applications

GOx is of considerable commercial importance (Crueger and Cruger 1984). The enzyme is used in food processing, immunoassay, and the production of gluconic acid (Röhr et al. 1983). One of its most important applications is in biosensors or biofuel cell made from the enzyme-electrode system to measure blood glucose in diabetic patients (Wilson and Turner 1992).

Biofuel cells use enzymes as biocatalysts (Katz et al. 1999) or whole cells (Wingard 1982b; 1982a) to convert chemical energy into electrical energy. A biofuel cell requires an anode, a cathode, a supporting electrolyte medium to connect the two electrodes, and an external circuit to use the extractable power. Since Clark & Lyons (1962) described the first specific β-D-glucose electrode based on GOx, GOx has become the prime enzyme for development of biosensor and biofuel cell due to its high specificity and efficiency. However, one obstacle in applying redox enzymes on electrodes is that electrochemical communication between redox enzymes and electrodes is usually inhibited due to the buried electroactive sites of the enzymes (Pishko et al. 1990; Moser et al. 1992). Crystallographic investigations of the tertiary structure of glucose oxidase indicate that the access to the flavin system in the active site is provided by a deep pocket; the coenzyme, FAD, is located at the bottom of this pocket (Hecht et al. 1993). This problem was not solved till 1984 when Cass (Cass et al. 1984) first reported a glucose biosensor using ferrocene to mediate the electron transfer between the redox site of GOx and an electrode surface. Adam Heller's group reported that his miniature membrane-less biofuel cell consists of two carbon fibres, 2 cm long and 7 thousandths of a millimetre wide, each coated with a catalyst that assists the chemical reactions of glucose burning. This reaction takes place in two halves, one at each electrode. One electrode is coated with a polymer and GOx, which strips β-D-glucose of electrons. The polymer makes an electrical connection between the enzyme and the carbon fiber. At the other electrode, another polymer-wired enzyme adds electrons to dissolved oxygen. As the reaction proceeds, electrons are fed in a current around an electrical circuit. The device works at a temperature and alkalinity close to those of normal blood: at 37 ºC, and at pH 7.2. It

produces about the same amount of power as a wristwatch battery: about 1.9 microwatts. It could drive a miniature glucose sensor for monitoring diabetics (Kim 1990). Electron-transfer theory (Siders et al. 1984) defines the rate of electron transfer, k_{et}, between a donor-acceptor pair by equation (1):

$$k_{et} \propto e^{-\beta(d-d_0)} * e^{\frac{(-\Delta G^o + \lambda)^2}{4RT\lambda}} \quad (1)$$

Where ΔG^0 is the free energy associated with electron transfer and generation of redox products
λ is the reorganization energy
d_0 is the Van-der Waals distance
d actually distance between donner and acceptor
β is the electronic couple coefficient

This equation shows that the separation distance, d, between electron donor and acceptor is a major factor that limits/controls electron transfer rates.

In order to reduce the distance between active site of GOx and electrode, a lot of effort has been done by cleavage of GOx' carbohydrate moiety. Moreover, deglycosylated glucose oxidase immobilized to a sensor has been demonstrated to communicate more rapidly than the glycosylated enzyme with electrode (Kalisz and Kuennecke 1989).

Various strategies have been developed to achieve efficient electron transfer (ET) between the redox enzymes and electrodes. These strategies can be divided into two types: Chemical modification and genetic modification

1.2.1 Chemical modification

1.2.1.1 Wiring of glucose oxidase

Adam Heller (Mano and Heller 2005) reported the biofuel cell based on a stationary glassy carbon disk, which can detect glucose at 2 fM concentration in a physiological buffer solution at 1 atm O_2 pressure. Stationary glassy carbon disk is surrounded by an

also stationary platinum ring. The disk was coated with a film of GOx, electrically "wired" with polymer I, having a redox potential of -0.19 V versus Ag/AgCl. The ring was coated with bilirubin oxidase (BOD) "wired" with polymer II, having a redox potential of + 0.36 V versus Ag/AgCl, The wired BOD disk scavenged the O_2 so effectively that the β-D-glucose-reduced $FADH_2$ of GOx was not oxidized by O_2, the natural cosubstrate of enzyme.

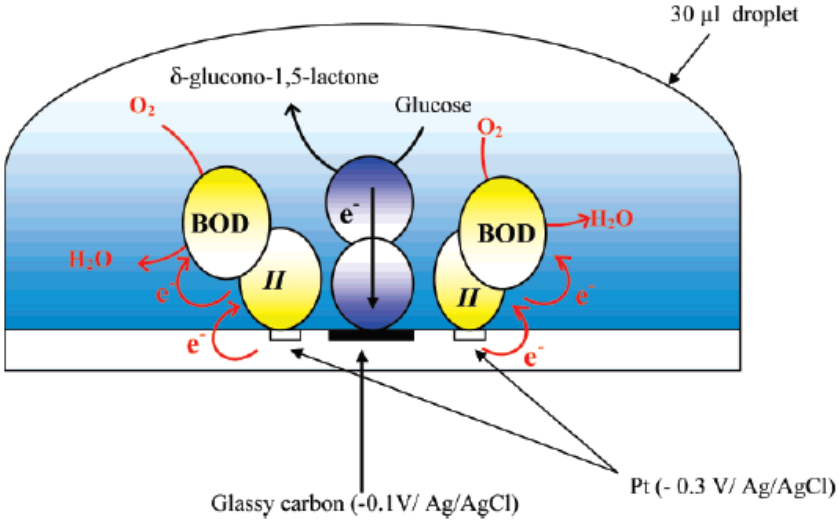


Fig.8. Schematic diagram of the coated rotated ring-disk electrode (Mano and Heller 2005)

At the anode, electrons are transferred from β-D-glucose to GOx, from GOx to polymer **I**, and from polymer **I** to the electrode. At the cathode, electrons are transferred from the cathode to redox polymer **II**, from redox polymer **II** to BOD, and from BOD to O_2.

1.2.1.2 Electrical nanoplug

Prof. Willner group have devised a way to use gold nanoparticles as tiny electrical wires to plug GOx into electrodes (Xiao et al. 2003). The gold "nanoplugs" help align the molecules for optimal binding and provide a conductive pathway for the flow of electrons.

The reconstitution of apo–GOx, on a 1.4-nanometer gold nanocrystal functionalized with the cofactor FAD and integrated into a conductive film yields a bioelectrocatalytic system with exceptional electrical contact with the electrode support. The electron transfer turnover rate of the reconstituted bioelectrocatalyst is 5000 per second, compared with the rate at which molecular oxygen, the natural cosubstrate of the enzyme, accepts electrons (700 per second).

1.2.1.3 The fluorescence emission of the apo-GOx

Joseph R. Lakowicz group found a new method of β-D-glucose sensing using an inactive form of GOx from *Aspergillus niger*. They removed cofactor FAD to make apo enzymes by the acid ammonium sulfate precipitation method. The apo-GOx bound β-D-glucose with an affinity comparably to the holo enzyme. Importantly, the intrinsic tryptophan fluorescence of apo-GOx was sensitive to β-D-glucose binding. Additionally, apo-GOx non-covalently bound 8-anilino-1-naphthalene sulfonic acid (ANS). The ANS bound to GOx displayed decreases in both intensity and lifetime upon addition of β-D-glucose. These results suggested a new approach to obtaining protein sensors for β-D-glucose, this being β-D-glucose converting enzymes rendered inactive by removal of necessary cofactors (D'Auria et al. 1999).

1.2.2 Genetic modification

Chen reported a novel method for improving glucose biosensors by genetically modifying GOx. GOx was genetically modified by adding a poly-lysine chain at the C-terminal with a peptide linker inserted between the enzyme and poly-lysine chain. The

poly-lysine chain was added in order to anchor more electron transfer mediator, ferrocenecarboxylic acid, to GOx for the purpose of improving sensitivity and stability of glucose biosensors. The modified GOx had similar K_m and k_{cat} to those of the wild type enzyme. After interacting with the electron transfer mediator, the modified enzyme retained 90.01% of its native activity, while the commercial GOx and the wild type GOx (*Aspergillus niger*) retained only 22.43 and 22.17%, respectively (Chen et al. 2002).

All biofuel cells still face considerable challenges, however. Nature didn't evolve GOx to work with circuitry, especially in physiological condition. So chemical and genetic modifications have to be combined together to solve the problems. Most important, blood and other complex bodily fluids contain numerous compounds that can deactivate or block the enzymes essential to fuel-cell function, causing them to stop working within hours or days. But if researchers can improve their stamina, biofuel cells could pave the way to a new generation of implanted devices powered by the body itself.

1.3 Directed protein evolution

Powerful directed evolution mimics natural evolution by combining mutation with selection or screening to identify improved variants (Kuchner and Arnold 1997). It allows us to tailor enzymes with improved specificity, activity, stability and solubility that operate under industrial process conditions and finally to elucidate structure-function relationship (Tao and Cornish 2002).

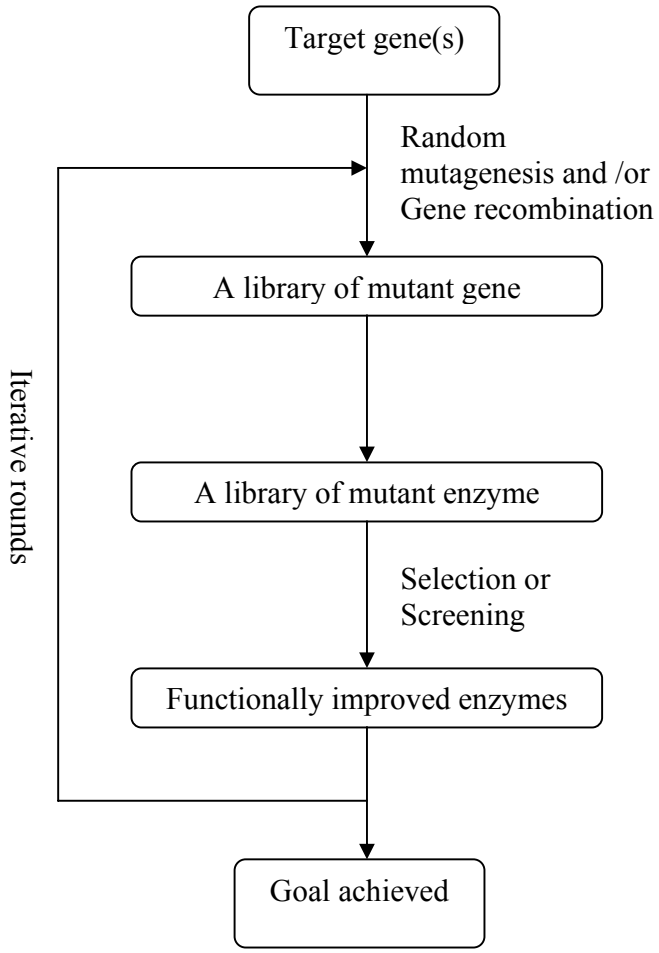


Fig.9. Flowchart of major steps of directed protein evolution

The major steps in a typical directed enzyme evolution experiment are outlined in Figure 9. The genetic diversity for evolution is created by mutagenesis and/or recombination of one or more parent sequences. These altered genes are cloned back into a plasmid for expression in a suitable host organism (bacteria or yeast). Clones expressing improved enzymes are identified in a high-throughput screen, or in some cases, by selection, and the gene(s) encoding those improved enzymes are isolated and recycled to the next round of directed evolution.

There are four requirements for successful directed evolution: first, the desired function must be physically possible; second, the function must be biologically, or evolutionarily,

feasible – this means that there exists a mutational pathway to get from here to there through ever-improving variants; third, the researches must be able to make libraries of mutants complex enough to contain rare beneficial mutations. This usually means functional expression in a suitable microorganism such as *Escherichia coli* or *Saccharomyces cerevisiae;* fourth, there must be a rapid screen or selection that reflects the desired function. Just how rapid the screen must be depends on how rare mutations leading to the desired property are and how many must be accumulated to achieve the desired result. A reliable high throughput assay system is the key to success (Kuchner and Arnold 1997).

Directed evolution consists of two major steps (Kuchner and Arnold 1997):

a) The creation of genetic diversity in the target gene
b) An effective screening system based on catalytic functions

1.3.1 Creation of genetic diversity

An ideal mutagenesis method replaces any amino acid of a polypeptide chain by 19 different amino acids in a statistical manner without limiting protein expression in the host organism (Wong 2006). The common strategies used for the creation of genetic diversity are as followed.

1.3.1.1 Error prone polymerase chain reaction (epPCR)

Error-prone PCR introduces random copying errors by imposing imperfect, and thus mutagenic, or 'sloppy', reaction conditions (e.g. by adding Mn^{2+} or Mg^{2+} to the reaction mixture (Cadewell 1991). This method has proven useful both for generation of

randomised libraries of nucleotide sequences, and also for the introduction of mutations during the expression and screening process in a mutagenesis step. All known epPCR methods are significantly limited in their ability to create diversity on the gene level. Instead of changing one amino acid to all other 19 possible amino acids, an average of less than five other amino acids can be obtained with current epPCR methods. The reasons lie mainly in the redundant genetic code and the biased mutational spectra of DNA polymerases. Additionally, most directed evolution experiments are performed at low to moderate mutation frequencies in order to avoid a large portion of inactive clones (Wong et al. 2004).

1.3.1.2 Mutator strain

The XL1-Red strain (endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10 (Tetr)) introduces random mutations into a cloned gene of interest. *E. coli* XL1-Red (Strategene) is a mutator strain that is deficient in three of the primary DNA repair pathways (*mutS*, *mutD* and *mutT*). During cell cultivation, mutations are accumulated in the genomic DNA of the mutator strain, the vector, and the gene of interest. Thus the random mutation rate in this strain is increased 5000-fold compared to the wild type.
Mutagenized vectors are subsequently isolated, re-transformed into a standard expression host with low mutation frequency and screened for beneficial variants (Wong 2006).

1.3.1.3 Saturation mutagenesis

Saturation mutagenesis is a method implemented to generate a library of mutations at a targeted DNA sequence. It can be used to introduce all possible mutations at key sites or

adjacent sites to explore a larger fraction of the protein sequence space (Sakamoto et al. 2001). It also can provide much more comprehensive information than can be gained by single amino acid substitutions as well as overcome the drawbacks of random mutagenesis, in that a single mutation randomly placed in codons generates on average only 5.6 out of 19 possible substitutions (Bylina et al. 2000; Rui et al. 2004).

1.3.1.4 Site directed mutagenesis

The principle of site-directed mutagenesis is that a mismatched oligonucleotide is extended, incorporating the "mutation" into a strand of DNA that can be cloned. Using site-directed mutagenesis the information in the genetic material can be changed. A synthetic DNA fragment is used as a tool for changing one particular code word in the DNA molecule. This reprogrammed DNA molecule can direct the synthesis of a protein with an exchanged amino acid. *In vitro* site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships, gene expression and vector modification (Cedrone et al. 2000; Bolon et al. 2002).

1.3.2 Screening mutant libraries of GOx

Although the production of an extensive and unbiased library is very important, the bottleneck for most directed protein evolution is the availability of a genuinely high-throughput screen for the target activity (Wong et al. 2005).

Screening and selection methodologies should meet the two demands:

a) ‘you get what you select for’ is the first rule of directed evolution (Arnold and Volkov 1999)

b) The assay should be sensitive over the desired dynamic range

Since the enzyme activity was first reported by Muller (Müller 1928) in extracts of *Aspergillus niger*. The four types of screening method were developed and summarized in figure 10 and following sections.

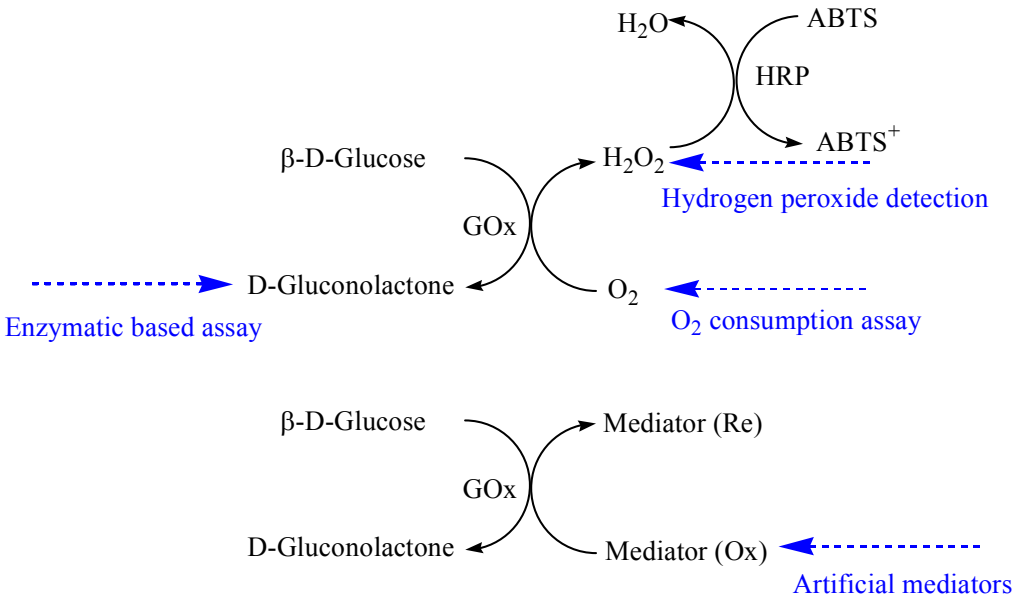


Fig.10.Reaction schemes of the screening method of GOx

1.3.2.1 Based on electron acceptors

1) Dioxygen as natural electron acceptor

In the presence of molecular oxygen, β-D-glucose is oxidised by the enzyme GOx to gluconic acid and hydrogen peroxide: Dioxygen generally undergoes reactions in a stepwise manner via formation of free radical intermediates with one unpaired electron (Klinman 2001).

$$O_2 + e^- \rightarrow O_2^- \quad (2)$$

$$O_2^- + H^+ \rightarrow HO_2 \quad (3)$$

$$HO_2 + H^+ + e^- \rightarrow H_2O_2 \quad (4)$$

Although dioxygen is a strong oxidant at pH 7 (when it is a concerted four-electron-transfer agent), it is a weak one-electron oxidant. In terms of the reduction potential, the limiting step is the first electron transfer to O_2.

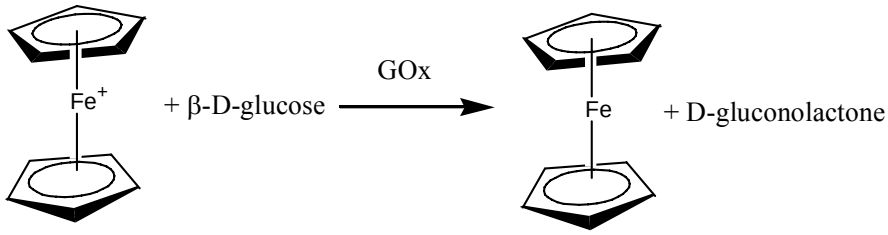
Under the conditions of the assay, oxygen consumption is directly proportional to activity of GOx. A single detection of GOx can be assayed at 30 °C with an oxygen electrode (Clark electrode) by recording oxygen consumption during the reaction. Oxoplate (PreSens GmbH, Regensburg, Germany) can be used to detect O_2 consumption in 96-format, but the plate is not reusable.

2) Artificial electron acceptor

Artificial mediator could be an alternative to place O_2 as electron acceptor, The reaction of GOx has been extensively studied with a number of artificial electron acceptors including organic dyes such as phenazine methosulfate, 2,6-dichlorophenolindophenol, and *N*,*N*,*N'*,*N'*-tetramethyl-4- phenylenediamine (Aleksandrovskii et al. 1981) . However, these mediators have a number of limitations such as poor stability and the pH dependence of their redox potentials. Other simple inorganic redox species such as hexacyanoferrate, hexacyanoruthenate and pentaamine pyridine ruthenium do not suffer from these problems, however they also do not show obvious signal development (color, fluorescence) during redox reaction. It is difficult to build up an efficient screening system. Since 1984, Cass first reported a glucose biosensor using ferrocene to mediate the electron transfer between the redox site of GOx and an electrode surface (Cass et al. 1984). Organometallic redox compounds, such as ferrocene detrivative, ruthenium

complexes and osmium complex are playing more important role as electron transfer mediator.

One of most popular classes of mediators has been ferrocene and its derivatives. These molecular are well behaved electrochemically and exhibit rapid kinetics for enzyme oxidation (Luong et al. 1994). Since the β-D-glucose/D-gluconolactone and $FADH_2$/FAD couples are $2e^-+2H^+$ systems, and ferrocenes are one-electron oxidizing systems, two equivalents of ferrocene and ferrocenium are involved in reaction; thus, the oxidative half-reaction and reductive half reaction can be represented by Equation (5)



(5)

Reaction (5) is a multistep process, in oxidative half reaction, two electrons and two protons are exchanged between the reduced form of the enzyme ($FADH_2$) and two molecules of substrates and two molecules of the base contained in buffer. Here, one has to draw a clear distinction between the substrates that can accept only one electron but no proton (such a ferrocenium salts), and oxidizing systems that are the $1e^- +H^+$ couples (such as stable nitroxide radicals). Bourdinllon reported that the oxidation rate constant at pH 8 is indeed larger then that observed with dioxygen. So it is possible to understand why glucose oxidase using positively charge ferrocenium as electron acceptor is insensitive to dioxygen (Bourdillon et al. 1993).

Artificial electron acceptor based assay can be used for high throughput screening due to their convenient and cheap preparation. The oxygen competition and selection of redox potential is however the main concern with such assays system.

1.3.2.2 Based on detection hydrogen peroxide

GOx detection based on hydrogen peroxide production is a very successful screening method in term of widespread use. GOx catalyses the oxidation of β-D-glucose to D-gluconolactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. The amount of hydrogen peroxide produced can be detected by a colorimetric method or a florescence method using a combination of peroxidase and its substrates (chromogens) such as o-dianisidine, 2,2′-azino-bis(3-ethybenz-thiazoline-6-sulfonic acid (ABTS) and 3,3′,5,5′-tetramethylbenzidine (TMB)(Yamaguchi et al. 2001); or fluorescent substrate such as amplex red and resazurin. Hydrogen peroxide based assays are very sensitive and specific assays due to combination with horseradish peroxide (HRP), however, the drawback of this assay is to detect byproduct H_2O_2 which can also be formed in cells metabolism and the inherent variability of dissolved air concentration. It has been reported that ABTS and o-dianisidine are mutagenic and carcinogenic (Voogd et al. 1980), and TMB has the weak point of being labile in aqueous buffers.

1.3.2.3 Enzymatic based assay

Enzymatic based detection of GOx is based on pentose phosphate pathway showed from Figure 10. GOx is marked in red. It employs other enzymes, which specifically catalyze the main product of glucose oxidase reaction, such as D-gluconolactone, to screen for

activity of GOx. It is a very specific and direct method for GOx detection compared to other assay systems.

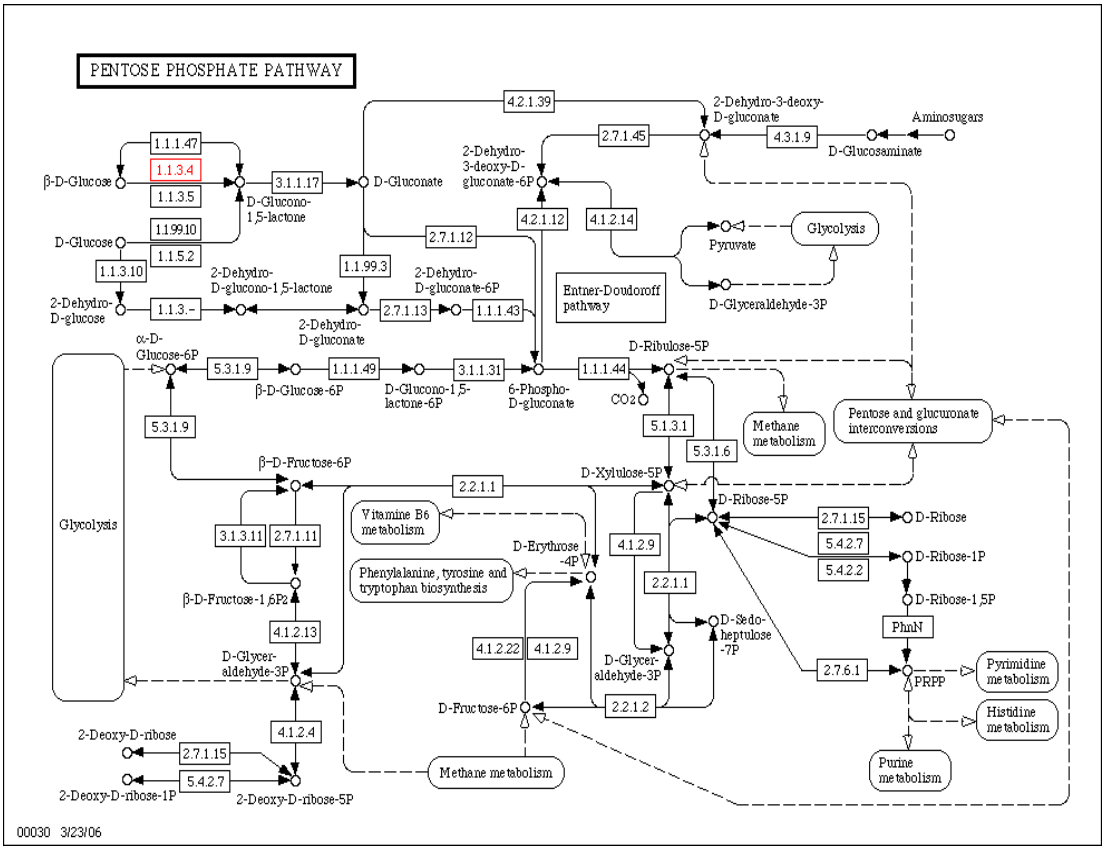


Fig.11. Pentose phosphate pathway

Glucose oxidase detection assay (GODA) is developed as medium-throughput screening system for improving GOx properties by directed protein evolution. GODA is a reaction product detection assay based on coupled enzymatic reactions leading to NADPH formation which is recorded at 340 nm. The main advantage of the assay is that it detects the production of D-gluconolactone instead of the side-product hydrogen peroxide thereby enabling specific improvement of bioelectrochemical properties of GOx (Zhu et al. 2006).

1.3.2.4 Solid phase screening (Colony screening)

Solid phase screening is a method to screen libraries of variant enzyme directly on agar plate which is greatly simplified by establishing a direct physical link between gene, protein expression, and desired function (Wahler and Reymond 2001). It is an attractive option because of its relative ease and high throughput comparing to liquid phase screening. The assay mix will be applied to top of colony using filter, membrane or overlay detection method. The activity of enzyme will be screened by formation of halo, color, and florescence signal. With development of digital image analysis, high throughput (10,000 clones/day) screen for dioxygenase activity was designed by Professor Frances H. Arnold group for dioxygenase activity (Joern et al. 2001). Another system, called Kcat Technology, combined digital imaging spectroscopy and a new solid-phase screening method to screen enzyme variants. It can be used to distinguish small differences in both the kinetic and spectral properties of enzyme variants expressed in microcolonies, which grown at a nearly confluent density of 500 colonies per cm^2 on an assay disk (Delagrave et al. 2001).

1.4 Objectives of PhD study

The objective of the PhD is to improve properties of GOx for its putative use in the anodic compartment of miniaturized biofuel cells (mBC). Specific objectives are derived from in-body-applications (implantation) of mBC as power sources.

The specific objectives are ranked in descending order of importance:

1. Developing directed evolution protocol of glucose oxidase
2. Developing glucose oxidase screening methods
    1) Enzymatic detection assay

2) Ferrocenemethanol mediated assay

3. Improving activity and thermostability

Prerequisite for directed evolution of GOx are high-throughput screening systems for identifying improved GOx mutants

Besides increasing the power output of GOx in mBC, we hope on the long run to discover fundamental structure-electron transfer relationships for understanding and tuning electron transfer in GOx.

2 MATERIALS AND METHODS

All chemicals used were of analytical reagent grade or higher quality and purchased from Sigma–Aldrich Chemie (Taufkirchen, Germany) and Applichem (Darmstadt, Germany). Toyopearl Butyl-650S chromatographic resin was purchased from TOSOH BioScience GmbH (Stuttgart, Germany). T7Gene6 Exonuclease was purchased from Amersham Bio sciences Europe GmbH (Freiburg, Germany). pYES2 shuttle vector, *E. coli* strain DH5α, XL1-Red mutator strain, and *S. cerevisiae* strain INVScI were purchased from Invitrogen (Karlsruhe, Germany). Nucleotides and all other enzymes were purchased from Fermentas (St.Leon-Rot, Germany) D-glucose detection kit and D-gluconolactone detection kit were purchased from Roche (Darmstadt, Germany), if not stated otherwise.

2.1 Microorganism methods

2.1.1 Strains and plasmids

E. coli DH5α strain was used as the host for the cloning and propagation of plasmid. *E. coli* mutator strain XL1-Red was used for diversity of generation. *S. cerevisiae* strain INVScI was used throughout as the host for the expression of GOx.

2.1.2 Media and culture conditions

Luria-Bertani (LB) medium (1% petone, 1% NaCl, 0.5% yeast extract) and 100 µg/ml Ampicillin) were used to cultivate the host and *E. coli* transformant. YPD (Yeast Extract/Peptone/Dextrose) medium (1% yeast extract, 2% peptone, and 2% D-glucose) was used to cultivate the host and yeast transformants. A SC-U (Synthetic minimal defined medium for yeast-Uracil) growth medium (Table 2) was used for the selection of

the yeast transformants. A SC-U induction medium (Table 2) was used for expression of GOx. Complex synthetic media was used for scale-up fermentation of rGOx in *S. cerevisiae* (Table 3).

Table 2 Compositions of SC-U growth and induction medium

		Nitrogen	Amino acid (0.5 %)	Amino acid (1 %)
SC-U Growth Medium	D-glucose 2 %	Yeast nitrogen base 0.67 %	Aspartic acid, histdine, isoleucine, serine, proline, tryptophan, methionine, phenylalanine.	Adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan
SC-U Induction medium	D-glactose 2 %	Yeast nitrogen base 0.67 %	Aspartic acid, histdine, isoleucine, serine, proline, tryptophan, methionine, phenylalanine.	Adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan

Table 3 Compositions of synthetic medium

Synthetic medium	Per liter
D-glucose/Galactose	20 g
$(NH_4)HPO_4$	2 g
$(NH_4)_2SO_4$	17.5 g
KCl	2.2 g
$MgSO_4$ x $7H_2O$	2 g
$CaCl_2$ x 2 H_2O	0.3 g
$FeCl_3$	144 mg
$MnSO_4$ x H_2O	102 m
$ZnSO_4$ x $7H_2O$	86.4 mg
$CuSO_4$ x 5 H_2O	23 mg
Myoinositol	150 mg
Ca-Panthothenat	75 mg
Thiamine HCl	15 mg
Pyridoxine HCl	3.75 mg
Biotin (H)	0.075 mg

L-Histidine	125 mg
L-Leucine	300 mg
L-Tryptophan	200 mg

Cultivation of *E. coli* cells was carried out in a shaker (Multitron II, Infors GmbH, Einsbach, Germany; 37 °C, 250 rpm). Cultivation of yeast cells and protein expression were carried out in a shaker ((Multitron II, Infors GmbH, Einsbach, Germany; 30 °C, 250 rpm). The mutant libraries of GOx in 96-format deep-well plate were cultivated in a microplate shaker (Multitron II, Infors GmbH, Einsbach, Germany; 30 °C, 990 rpm)

2.2 Cloning of recombinant glucose oxidase

A thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) and thin-wall PCR tubes (Multi-Ultra tubes; 0.2 ml; Carl Roth, Germany) were used for all PCRs and thermostability measurements. The PCR volume was always 50 µl; larger volumes were prepared in multiple 50 µl PCRs. The amount of DNA was quantified using a NanoDrop photometer (NanoDrop Technologies, Germany).

2.2.1 Source of *gox* gene

A *gox* gene from *A. niger* has been obtained from Professor Van Rensburg (Stellenbosch University, South Africa). Sequence analysis of South African's gene showed 100% identity to *gox* sequence of *A. niger* available on pdb database (PDB code 1gal). *A. niger* structural *gox* gene from Professor Van Rensburg was cloned into an integration vector (YIp5) containing the yeast mating pheromone α factor secretion signal (MFα1S).

2.2.2 PCR amplification of *gox* gene

YIp5 plasmid was extracted using the QIA prep Miniprep Kit (Qiagen, Hilden, Germany) and used as template for generating wild type (WT) plasmid by PCR. PCR was performed using a thermal cycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany) with the following conditions: 94°C for 4 min, 1 cycle; 94°C for 1 min/55°C for 1 min/72°C for 2.25 min, 29 cycles; 72°C for 10 min, 1 cycle. PCR mix in thin-wall PCR tubes (Multi-Ultra tubes 0.2 ml; Carl Roth, Germany) had a total volume of 50 µl and consisted of 0.2 mM dNTP mix, 40 ng YIp5 template, and 10 pmol of each primer (forward primer: 5'-CATCGGGGTACCATGAGATTTTCCTTC-3' and reverse primer: 5'-GAAGCTCTAGAGCTCACTGCATGG-3'). PCR was hot started when the reaction mix reached 94°C by adding 2.5 U *Taq* polymerase. PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The 2 kb PCR product, containing the *gox* gene fused in frame to MFα1s was analyzed by standard agarose gel electrophoresis. PCR product was purified with QIAquick Gel Extraction Kit (Qiagen).

2.2.3 DNA manipulation and plasmid construction

1 µg pYES2 plasmid and purified PCR product were digested by two restriction enzymes KpnI and XbaI using double digestion conditions (5 U/µg DNA, KpnI buffer, 0.2 U XbaI, 0.1 U KpnI, 6 h, 37°C) and purified with QIAquick Gel Extraction Kit (Qiagen).

2.2.4 Ligation and transformation in *E. coli*

Purified digested PCR product was subcloned into the KpnI and XbaI restriction sites of pYES2 using a classic ligation method. A typical ligation reaction (10 µl final volume)

contained 1x T4 DNA ligase buffer, ~500 ng inserts, ~50 ng plasmid and 0.5 U T4 DNA ligase and was incubated at room temperature for 1 hour and kept at 4°C overnight. 2 µl ligation mix was transformed into DH5α *E. coli* cell with DMSO heat shock method.

2.2.5 Re-ligation removal

Plasmids from all *E. coli* cells were extracted with QIAprep Miniprep Kit (Qiagen). Re-ligation band was analyzed by standard agarose gel electrophoresis and removed using QIAquick Gel Extraction Kit (Qiagen). Plasmids containing gene were purified with QIAquick Gel Extraction Kit (Qiagen), transformed into DH5α *E. coli* cell with DMSO heat shock method, and finally extracted using QIAquick Gel Extraction Kit (Qiagen).

2.2.6 Yeast transformation

1 µg plasmid pYES2-GOx, which was extracted from DH5α *E. coli* cell, was transformed into competent INVScI *S. cerevisiae* cells using *S.c.* EasyComp transformation kit (Invitrogen).

2.3 Purification of recombinant GOx

2.3.1 Scale-up GOx expression

S. cerevisiae clones carrying wild-type and mutant variant were grown to an OD_{600nm} of 3 in 300 ml shaking flasks containing 50 ml of SC-U growth media (48 h, 30°C, 250 rpm) using a Multitron II incubator shaker (Infos GmbH). *S. cerevisiae* cells were centrifuged (Eppendorf 5810R; 10 min, 4°C, 3220 g) in 50 ml Falcon tubes (Greiner Bio-One), resuspended and diluted to OD_{600nm} 0.4 in 2 l shaking flasks containing 500 ml of SC-U

induction media (SC-U containing 2 % D-galactose) and grown in a shaking incubator. Cells were centrifuged (Eppendorf 5810R; 10 min, 4°C, 3220 g) and the supernatant containing recombinant GOx was collected after 11 hours induction (Multitron II; Infors GmbH; 30°C, 250 rpm). rGOx had been purified with three different methods.

2.3.2 Ion exchange chromatography

Recombinant GOx from *S. cerevisiae* was purified by anion-exchange chromatography DEAE 650S column (15 cm^3) using an ÄKTA purifier (Amersham Pharmacia Biotech, Freiburg, Germany). The column was equilibrated with a starting buffer $NaxPO_4$ (Buffer A, 50 mM, pH 6) that allows GOx bind to the oppositely charged beads. Adsorbed GOx was finally eluted with elution buffer B (Buffer A + 1 M NaCl) with a flow speed 5 ml/min and a linear gradient, detection wavelengths of GOx are 280 nm (protein), 375 nm (FAD), 450 nm (FAD).

2.3.3 Hydrophobic interaction chromatography

Another alternative purification method was hydrophobic interaction chromatograph. After 11 hours of induction, the yeast cells were spun down at 4×10^3 g for 10 min and supernatant collected. Conductivity in supernatant containing rGOx was 20 mS cm^{-1}, pH was 3.6. The final conductivity of supernatant had been adjusted up to 183 mS cm^{-1} by adding $(NH_4)_2SO_4$ (Laboratory conductivity meter 703; Knick GmbH, Berlin, Germany), which corresponds to 1.5 M solution of ammonium sulphate. The pH of the supernatant had been adjusted up to 5.5. The supernatant containing rGOx was first filtered using a 0.45 μM hydrophilic polypropylene membrane filter (VWR International GmbH,

Darmstadt, Germany). Followed by purification with hydrophobic interaction chromatography (Sasaki et al., 1982) using an ÄKTA purifier (Amersham Pharmacia Biotech) and Toyopearl Butyl-650S resin (TOSOH BioScience GmbH) in a 15/125 mm column (KronLab GmbH, Sinsheim, Germany). It was pre-equilibrated at pH 5.5 with 50 mM sodium acetate buffer containing 1.5 M $(NH_4)_2SO_4$. The unabsorbed proteins were washed out of the column with the same buffer (10 column volumes; flow rate 5 ml/min) and GOx was finally eluted by a step gradient ($(NH_4)_2SO_4$ 1.5 M to 0 M; 50 mM acetate buffer pH 5.5; 3 column volumes; flow rate 5 ml/min). GOx elution was monitored at 280 nm (protein), and 382/452 nm (FAD). Collected GOx fractions were subsequently desalted and concentrated using centrifugal filters with 50 kDa cut-off membranes (Centricon YM-50; Millipore, Schwalbach, Germany).

2.3.4 Size exclusion chromatography

500 ml supernatant containing rGOx from induction was first concentrated to 1 ml by ultrafiltration stirred cell with 30 kDa cut-off membranes (AMICON, Millipore, Schwalbach, Germany) before loading to the size exclusion chromatography column. After flushing twice with five times excess loop volume buffer (50 mM, ammonium acetate buffer pH 5.5), 1 ml concentrated sample was injected to injection loop. Size exclusion chromatograph was carried out on Superdex 200 HR 16/60 column calibrated by molecular mass standards (Amersham Pharmacia, excl. lim = 200 kDa). The Column was equilibrated and run in ammonium acetate buffer (50 mM, pH 5.5) at a flow rate of 1 ml/min, 0.3 MPa, at room temperature. rGOx elution was monitored at 280 nm (protein), and 382/452 nm (FAD). The purified sample was concentrated with 50 kDa cut-off

membranes (Centricon YM-50; Millipore). The concentration of rGOx was determined by BCA™ assay kit (Pierce, Bonn, Germany).

2.3.5 Separation of protein using PAGE

SDS-polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (1970) using 6% acrylamide separating gel and 5% stacking gel containing 1% SDS. Purified rGOx from *S. cerevisiae* and cGOx from *A. niger* samples were heated at 95°C for 5 min in tris–glycine buffer containing 2% SDS, 0.1 % bromophenol blue and 100 mM dithiothreitol. Electrophoresis was carried out at a constant current of 10 mA for 2 h using a running buffer of tris–glycine containing 0.1% SDS. After electrophoresis, the gel was stained with 0.025 % Coomassie Brilliant Blue R-250 solution (4 hours, room temperature), and was finally destined in methanol: acetic acid solution without the dye (8 hours, room temperature) with changing the destining solution three or four times.

2.4 Fermentation (10 l) in batch

The batch fermentation experiment was carried out in 16 l stirred-tank bioreactor (Bioengineering AG, Switzerland). The fermentor was controlled by Intelligent Front Module (IFM), which was equipped with controls for temperature, pH, oxygen concentration, antifoam, stirring speed, airflow and feed. The operating parameters were a temperature of 30°C, pH 5, agitation from 400 to 700 rpm to maintain dissolved oxygen in the fermentor broth at 75-90% saturation, and pressure of 1 bar. Media contains: Yeast Nitrogen Base, essential amino acids and β-D-glucose. The pH was controlled and adjusted by 5 M NaOH and 2 M HCl. Foam was suppressed, when necessary, by the addition of antifoam reagent SP1.

A *S cerevisiae* strain (INVScI) bearing a 2 μm plasmid, pYES2 (containing a GAL1 promoter, *gox* gene, and URA3 selection) was grown in 200 ml SC-U growth medium of 1 l flask for two days. The optical density of the inoculum measured at absorbance of 600 nm was in a range 2.5-3 before the 200 ml inoculum was added to 16 l fermentor which contained 10 l SC-U growth medium. D-glucose was regularly check by glucose detection kit (Roche). After 37 hours growing, D-glucose was consumed from 20 g/l to 0.16 g/l, the sterilized inducer D-galactose (2%) dissolved in 200 ml SC medium was added. Samples were taken every hour; activity of GOx was monitored by ABTS assay. After 29 hours induction, the supernatant which was containing expressed rGOx was collected.

2.5 Construction of Mutant library in 96 well format

2.5.1 Expression of GOx in 96-well format

Colonies grown on SC-U selective agar plates containing 2 % D-glucose were picked with toothpicks and transferred into 96-deep well plates (BRAND, Wertheim, Germany) containing 1 ml SC-U growth media (containing 2 % D-glucose) in each well and grown to saturation (48 h, 30°C, 700 rpm) using a Multitron II incubator shaker (Infors GmbH, Einsbach, Germany). GOx production was induced by removing SC-U growth media through centrifugation (Eppendorf 5810R, 10 min, 4°C, 3220 g) followed by resuspension of cell pellets in 1 ml induction media (SC-U medium containing 2 % D-galactose) using a Liquidator (Steinbrenner GmbH, Wiesenbach, Germany). Recombinant GOx expression was performed using Multitron II incubator shaker (24 h, 30°C, 700 rpm). Cells were centrifuged (Eppendorf 5810R, 10 min, 4°C, 3220 g) and 100

µl of the clear supernatant which contain the secreted GOx from each well was used for activity determination.

2.5.2 Standard deviation in 96-well format

Apparent standard deviation was calculated based on absorbance values obtained. The whole procedure was repeated using supernatant without GOx gene insert. Absorbance values obtained were considered as background absorbance. "True" standard deviation was calculated after subtracting the background absorbance.

2.5.3 Generation of mutant library by epPCR using ligase free method

YIp5 plasmid was used as template for mutant library generation by error-prone PCR (epPCR). epPCR was performed using a thermal cycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany) and had the following conditions: 94°C for 4 min, 1 cycle; 94°C for 1 min/56°C for 1 min/72°C for 2.25 min, 29 cycles; 72°C for 10 min, 1 cycle. PCR mix in thin-wall PCR tubes (Multi-Ultra tubes 0.2 ml; Carl Roth, Germany) had a total volume of 50 µl and consisted of 0.2 mM dNTP mix, 40 ng pYIP5 template, 0.05 mM $MnCl_2$ (Cadwell and Joyce, 1992) for adjusting the mutation frequency and 10 pmol of each primer:

Forward primer: 5'-GGGAATATTAAGCTTGG TAC*C*AGGATGAGATT-TCCTTC-3'

Reverse primer: 5'-TAGTGGATCCGAGCTCGGTA*A*-ATCACTGCATGGAAGC-3'.

Nucleotides marked with a (*) contain a phosphothioate bond which terminates T7 Gene 6 Exonuclease digestion (Zhou and Hatahet, 1995). PCR was hot started when the

reaction mix reached 94°C by adding 2.5 U *Taq* polymerase. PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

Ligation of mutated *MFα1s*-GOx gene into pYES2 shuttle vector was performed according to a ligase free cloning method (Zhou and Hatahet, 1995). Firstly, 1 µg pYES2 plasmid was linearized by KpnI digestion (5 U/µg DNA, 6 h, 37°C) and purified with QIAquick Gel Extraction Kit (Qiagen). Secondly, 3 µg PCR product and 250 ng linearized pYES2 plasmid were treated with T7 Gene 6 Exonuclease (5 U/µg DNA, 15 min, 37°C) in order to generate complementary overhangs of ~20 nucleotides. T7 Gene 6 Exonuclease was subsequently inactivated by heat treatment (10 min, 80°C). Ligation was then performed by mixing digested PCR product and linearized pYES2 plasmid in a ratio of 12:1 (3000 ng : 250 ng). The ligation mix was heated (15 min, 75°C) and slowly cooled down to ambient temperature before transforming into *E. coli* strain DH5α using the TSS method (Chung et al., 1989).

2.5.4 Generation of mutant library by mutator strain

pYES2 vector harboring the *gox* gene was transformed into a mutator strain *E. coli* XL1-Red using the TSS method. Mutagenesis procedures by mutator strain were provided by Stratagene with the following modifications: 100 µl competent *E. coli* XL1-Red cells (Strategene) was transformed with 60 ng of purified plasmid pYES2-*gox* and selected on LB plates containing 100 µg ampicillin ml^{-1}. Resulting colonies were inoculated into 2 ml LB broth containing 100 µg ampicillin ml^{-1} and incubated overnight at 37°C with shaking. The cultures were diluted 1:100 and re-incubated. After two, three or four such cycles through XL1-Red, plasmids were isolated from each sub-culture and pooled. Plasmid of

pYES2-GOx from each cycle was extracted and retransformed to yeast competent cells. Improved mutants which were obtained from mutant library must be re-checked by following procedures: Plasmid of pYES2-*gox* and Plasmid of pYES2 were extracted using the QIA prep Miniprep Kit (Qiagen) and digested by two restriction enzymes KpnI and XbaI using double digestion conditions (5 U/µg DNA, KpnI buffer, 0.2 U XbaI, 0.1 U KpnI 6 h, 37°C) and purified with QIAquick Gel Extraction Kit (Qiagen). *gox* gene was re-ligated with a new double digested pYES2 vector (KpnI and XbaI digestion). After yeast transformation and GOx assay detection, if the mutant still showed improved activity, it was re-confirmed the mutation was generated from *gox* gene not from vector.

2.5.5 Construction of saturation mutant library

The saturation mutagenesis library of mutant T54S was created by employing the following modified QuikChange protocol (Stratagene). For the mutagenic PCR (95 °C for 4min, 1 cycle; 95 °C for 90 sec/62 °C for 75 sec/68 °C for 17min, 20 cycles; 68 °C for 17min, 1 cycle), 3.125 U *Pfu* DNA polymerase (Fermentas, St.Leon-Rot, Germany), 0.25mM dNTPmix (New England Biolabs, Frankfurt, Germany), 17.5 pmol of each primer (5′-ATCGCTGGTGGAGGTCTGNNKGGACTCACCACCGCTGCT-3′/5 AGCAGCGGTGGTGAGTCCMNNCAGACCTCCACCAGCGAT-3′), and 50 ng of template (pYES2 harboring mutant gene) were used. Then, 40 U of *Dpn*I (New England Biolabs) were added after PCR and incubated for 3 to 4 h at 37°C. The PCR product was purified using QIAquick Gel Extraction Kit (Qiagen) and transformed into *E. coli* DH5α. The plasmid library was isolated by resuspending ~400 colonies from the LBamp plate in 1mL LB media and using the QIAprep Miniprep Kit (Qiagen, Hilden, Germany). The

plasmid with a diversity of mutant library was retransformed into INVScI *S. cerevisias* yeast cell using S.c. EasyComp transformation kit (Invitrogen).

2.6 Screening methods of mutant library

Liquidator (Steinbrenner GmbH) was used for all liquid handling in the GODA, ABTS O_2 consumption and ferrocenemethanol assays in 96-well flat-bottom microplates (Greiner Bio-One GmbH, Frickenhausen, Germany).

2.6.1 Prescreening method

The solid phase screening of mutant libraries on agar plate was greatly simplified by establishing a direct physical link between gene, protein expression, and desired function. After yeast transformation, *S. cerevisiae* cells were grown on SC-induction agar plate which was mixed with 1 % pure agar agar for 2 days.1 ml ABTS reaction mix (4 mM ABTS, 1.25 U/ml HRP, 500 mM D-glucose) was added to an empty sterile Petri dish. A sterile filter paper was soaked into ABTS assay mix, following it was carefully transferred from Petri dish to the yeast cells agar plate and covered on the top of the cells. After 2-3 min, a green color development was observed around the active colonies. The filter paper was peeled off carefully; the colony which was around green color was picked into a deep 96-well microtiter plate for following liquid phase screening.

2.6.2 O_2 consumption assay

The O_2 consumption assay was carried out using a Fibox 3 fiber optical probe (PreSens, Regensburg, Germany), the assay mix condition was 100 µl D-Glucose (final

concentration 333 mM) mix with 100 µl sodium acetate buffer (100 mM, pH 5.5, ionic strength 158 mM) and the reaction was initiated by adding 100 µl rGOx (pH 5.5, ambient temperature)

O_2 consumption assay for screening mutant library can be carried out in 96 format OxoPlate® (PreSens Precision Sensing GmbH, Regensburg, Germany) which has 96 integrated oxygen-sensor layers immobilized on the bottom of each well (read out from bottom side).

2.6.3 ABTS assay in 96-well format

ABTS assay was used (Sun et al., 2002) with the following modifications to determine GOx activity. 100 µl of supernatant (containing recombinant GOx) was transferred to a 96-well flat-bottom microplate (Greiner Bio-One GmbH) and 200 µl assay mix was added into each well resulting in the following final concentrations: 330 mM ß-D-glucose, 3.3 mM ABTS, and 0.25 U horseradish peroxidase (HRP) in acetate buffer (100 mM, pH 5.5). Activity was measured by following the absorbance at 414 nm at ambient temperature using a FLASHScan S12 microplate reader (Analytik Jena AG).

2.6.4 Glucose oxidase detection assay (GODA)

100 µl supernatant containing recombinant GOx was added to 100 µl acetate buffer (30 mM, pH 5.5). The bioconversion of ß-D-glucose to D-gluconolactone was initiated by adding 100 µl ß-D-glucose (1 M) and quenched after 2-3 h by adding 20 µl of sodium hydroxide 200 mM to reach pH 10 (final concentration 12.5 mM). D-gluconolactone hydrolyzes to D-gluconate at pH 10 within 1 h at ambient temperature. To detect

NADPH formation, 100 µl of hydrolyzed reaction mixture was pipetted into a fresh microtiter plate containing 200 µl assay mix (0.37 M triethanolamine, pH 7.5; 0.99 mM $NADP^+$; 3.62 mM ATP; $MgSO_4$ (concentration not specified in the kit); 72 mU/ml 6-phosphogluconate dehydrogenase; 8.55 mU/ml gluconate kinase). This D-gluconate detection procedure based on NADPH formation follows the D-gluconic acid detection kit (R-Biopharm AG, Darmstadt, Germany), except that a 20-fold lower concentration of both 6-phosphogluconate dehydrogenase and gluconate kinase were used. In these coupled enzymatic reactions, the kinetic of NADPH formation was recorded at 340 nm (ambient temperature; $\varepsilon = 6300\ M^{-1}\ cm^{-1}$) using a FLASHScan S12 microplate reader (Analytik Jena AG, Jena, Germany).

2.6.5 Ferrocenemethanol based assay

The clear GOx-containing supernatant (100 µL) was transferred from each well of the deep well plate into a 96-well flat-bottom microplate (Greiner Bio-One GmbH), and 100 µL ferrocenemethanol (ox; pH 8, 100 mM KxPO4) was added per well. The conversion in the continuous ferrocenemethanol screening system was initiated by supplementing 100 µL β-D-Glucose (333 mM; pH 8, 100 mM KxPO4). Reaction kinetics was recorded at 625 nm using a FLASHScan S12 microplate reader (Analytik Jena AG, Jena, Germany).

2.7 Enzyme characterization

Maximum reaction rate (V_{max}), Michaelis–Menten constant (K_m) and turnover number (k_{cat}) were determined for β-D-glucose and oxygen consumption using the ABTS assay, ferrrocenemethanol based assay based on Michaelis–Menten model.

2.7.1 Protein content determination

After purification, concentration of rGOx was measured according to BCA™ assay kit (Pierce) using BSA for calibration within a detection range from 0 to 250 µg/ml. This concentration of rGOx was further used for determining kinetic constants.

2.7.2 K_m value for β-D-glucose

ABTS based kinetic measurements of purified wild-type GOx and mutant were performed identically to the ABTS assay in 96-well format, except that assays were carried out respectively with final concentrations of β-D-glucose ranged from 0 mM to 333 mM in acetate buffer (100 mM, pH 5.5, ionic strength 158 mM). The reactions were recorded at 414 nm at ambient temperature for 5 min using a FLASHScan S12 microplate reader (Analytik Jena AG).

Ferrocenemethanol based kinetic measurements of purified wild-type GOx and mutant were performed identically to the ferrocenemethanol assay in 96-well format, except that assays were carried out respectively with final concentrations of β-D-glucose ranging from 0 mM to 333 mM in phosphate buffer (100 mM, pH 8, ionic strength 158 mM). The reactions were recorded at 625 nm at ambient temperature for 30 min using a FLASHScan S12 microplate reader (Analytik Jena AG).

2.7.3 K_m value for O_2

Kinetics of oxygen consumption were recorded using a Fibox 3 fiber optical probe (PreSens, Regensburg, Germany) within a sealed gas tight vial of 25 ml total volume. Oxygen concentration in the liquid phase inside the sealed vial has been controlled (0–1100 µM) via oxygen partial pressure by supplying pure nitrogen or pure oxygen. The steady state oxygen consumption was initiated by injecting 100 µl of supernatant (containing equal amounts of purified wild-type GOx and mutant) into the sealed vial containing 8 ml of 500 mM D-glucose solution in acetate buffer (100 mM, pH 5.5). The reaction was recorded for 5 min at ambient temperature.

2.7.4 Turnover number

Turnover number (also termed k_{cat}) is defined as the maximum number of moles of substrate that an enzyme can convert to product per catalytic site per unit time and can be calculated as follows: $k_{cat} = V_{max}/[E]_T$, V_{max} (maximum rate of reaction) was obtained from K_m determination curve, [E] is molar concentration of enzyme which was calculated from result of BCA protein determination and molecular weight of enzyme.

2.7.5 Thermostability

Thermostability of purified WT and mutant in sodium acetate buffer (100 mM, pH 5.5, ionic strength 158 mM) was assessed by measuring residual activity/initial activity over the temperature range 20-60°C. The same amount of WT and mutant (50 µl, 26 µg) were incubated at each temperature for 2 hours in a thermal cycler and cooled down to room

temperature before they were assayed in a 96-well microtiter plate. The assays were measured by ABTS assay (pH 5.5) and ferrocenemethanol assay (pH 8).

The same amount of purified WT and mutant was aliquoted to 7 PCR tubes; they were incubated in a thermal cycler at specific temperature (57°C). Aliquots were taken at various time intervals (1 to 7 hours) and cooled down to room temperature before they were assayed in a 96-well microtiter plate. The assays were measured by ABTS assay (pH 5.5) and ferrocenemethanol assay (pH 8).

Table 4 Composition of ABTS assay for thermostability

	β-D-glucose	ABTS	HRP	WT/ Mutant	Sodium acetate
Concentration	333 mM	3.3 mM	0.83 U/ml		100 mM
pH	5.5	5.5	5.5	5.5	5.5
Volume	100 µl	50 µ	50 µl	40 µl	60 µl

Table 5 Composition of ferrocenemethanol assay for thermostability

	β-D-glucose	Ferrocenemethanol	WT/ Mutant	Phosphate buffer
Concentration	333 mM	3~6 mM		100 mM
pH	8	8	5.5	8
Volume	100 µl	100 µl	40 µl	60 µl

2.7.6 pH stability

Purified WT and mutant in sodium acetate buffer (100 mM, pH 5.5, ionic strength 158 mM) were aliquoted, diluted to 1: 50 with buffers (Table 6) in order to reach pH range 3-10 and incubated for 2 hours in room temperature. They were assessed by measuring residual activity/initial activity with ABTS assay (pH 5.5) and ferrocenemethanol based assay (pH 8).

Table 6 Buffer conditions for pH stability of GOx

Name	Concentration	pH value	pKa	Ionic strength (mM)
Phosphate buffer	0.1 M	3	2.15	0.158
Acetate buffer	0.1 M	4	4.76	0.158
Acetate buffer	0.1 M	5.5	4.76	0.158
Phosphate buffer	0.1 M	7	7.2	0.316
Phosphate buffer	0.1 M	8	7.2	0.316

CHES buffer	0.1 M	9	9.41	0.308
CHES buffer	0.1 M	10	9.41	0.308

2.8 Pyranose oxidase assay

Ferrocenemethanol was used as electron acceptor to drive recombinant pyranose oxidase (rPOx) from *E. coli*. rPOx from lysed cell extract had been obtained from Josefa Caballero Hernandez. The control and normal assay were carried out in following conditions:

Table 7 Ferrocenemethanol based assay for detecting rPOx

Composition	Control (without β-D-glucose)	Control (Without POx)	Measurement
β-D-glucose (333 mM)	0 µl	100 µl	100 µl
Pyranose oxidase	100 µl		100 µl
Ferrocenemethanol (3 mM)	100 µl	100 µl	100 µl
Phosphate buffer (pH 8)	100 µl	100 µl	0

2.9 Preparation methods

2.9.1 Plasmid recovery from yeast and sequencing

Plasmids harboring mutated *gox* genes that encode GOx variants with improved activity were isolated from *S. cerevisiae* INVScI and retransformed for amplification into *E. coli* strain DH5α using the TSS method. The clones harboring the plasmids were cultivated in LBamp media (2 ml, 12 h, 37 °C, 220 rpm). Plasmids were subsequently purified using QIAprep Spin MiniPrep Kit (Qiagen) and sequenced (MWG-Biotech AG; Ebersberg, Germany) using the following sequencing primers:

1) Forward primer 1: 5'-GCAGACTCTCCTTGTGAGCTCG-3'

2) Forward primer 2: 5'-GACACCGGCGATGCTATTCTCC-3'

3) Forward primer 3: 5'-GATTCCACAACACCACCGCCTTGC-3'

4) Reverse primer 1: 5'-GCCACTTTCGATGACGAAGCACACT-3'

2.9.2 Ferrocenemethanol (ox) preparation

Ferrocenemethanol [reduced (red), 6 mM] were partially dissolved under stirring in phosphate buffer (100 mM; pH 3, room temperature). The yellow suspension exhibits an absorbance maximum at 430 nm. Ferrocenemethanol (red) was enzymatically oxidized by adding laccase from *Trametes versicolor* (Fluka, 7.2 mU/mL in 50 mM phosphate buffer; 1 h). During the oxidation the 6 mM ferrocenemethanol (red) suspension dissolves completely. Ferrocenemethanol (ox) is at pH 3 blue in color with an absorbance maximum at 625 nm. After >90% conversion, the laccase catalyzed oxidation of ferrocenemethanol was stopped by adjusting the pH to 8 (NaOH; 0.2 M). Laccase was subsequently removed from the oxidized ferrocenemethanol solution by ultrafiltration using a stirred cell (200 mL, Amicon 8200, Millipore, Schwalbach, Germany) with a 30-kDa cut-off membrane (PBTK, Millipore).

2.9.3 Molar extinction coefficient determination

6 mM oxidized Ferocenemethanol was diluted to a concentration range 0-6 mM using phosphate buffer (pH 8.0, 100 mM, ionic strength of 316 mM). The absorbance was measured at 625 nm using a FLASHScan S12 microplate reader (Analytik Jena AG). The absorbance values of oxidized ferocenemethannol at different concentrations were then plotted and molar extinction coefficient ε ($M^{-1}cm^{-1}$) was calculated using the following formula: ε = A/Cl (A: absorbance at 625 nm, l: pathlength C: molar concentration of oxidized form ferrocenemethanol)

2.9.4 Linear detection range of ferrocenemethanol based assay

Ferrocenemethanol was used as an electron acceptor for reduced form glucose oxidase to determine the linear detection range under screening conditions in the 96-well format. Various concentrations of ferrocenemethanol (0-2 mM final concentration) were pipetted into 100 µl of phosphate buffer (100 mM, pH 8) with a 96-well microtiter plate. A solution consisting of 100 µl 1 M β-D-glucose in phosphate buffer (100 mM, pH 8) was mix with ferrocenemethanol, finally 100 µl WT GOx was immediately added to initiate the reaction, the bubbles were removed using a Bunsen burner, and absorbance was measured at 625 nm for 30 min to quantify the reaction kinetics using a FLASHScan S12 microplate reader (Analytik Jena AG).

3 RESULTS

3.1 Gene cloning of recombinant GOx

Gene cloning methods of rGOx were explained in the Materials and Methods section 2.2. *gox* from *A. niger* and α-pheromone mating have been cloned into pYES2 via KpnI–XhoI restriction sites and expressed in yeast *S. cerevisiae* in active form (Figure 12)

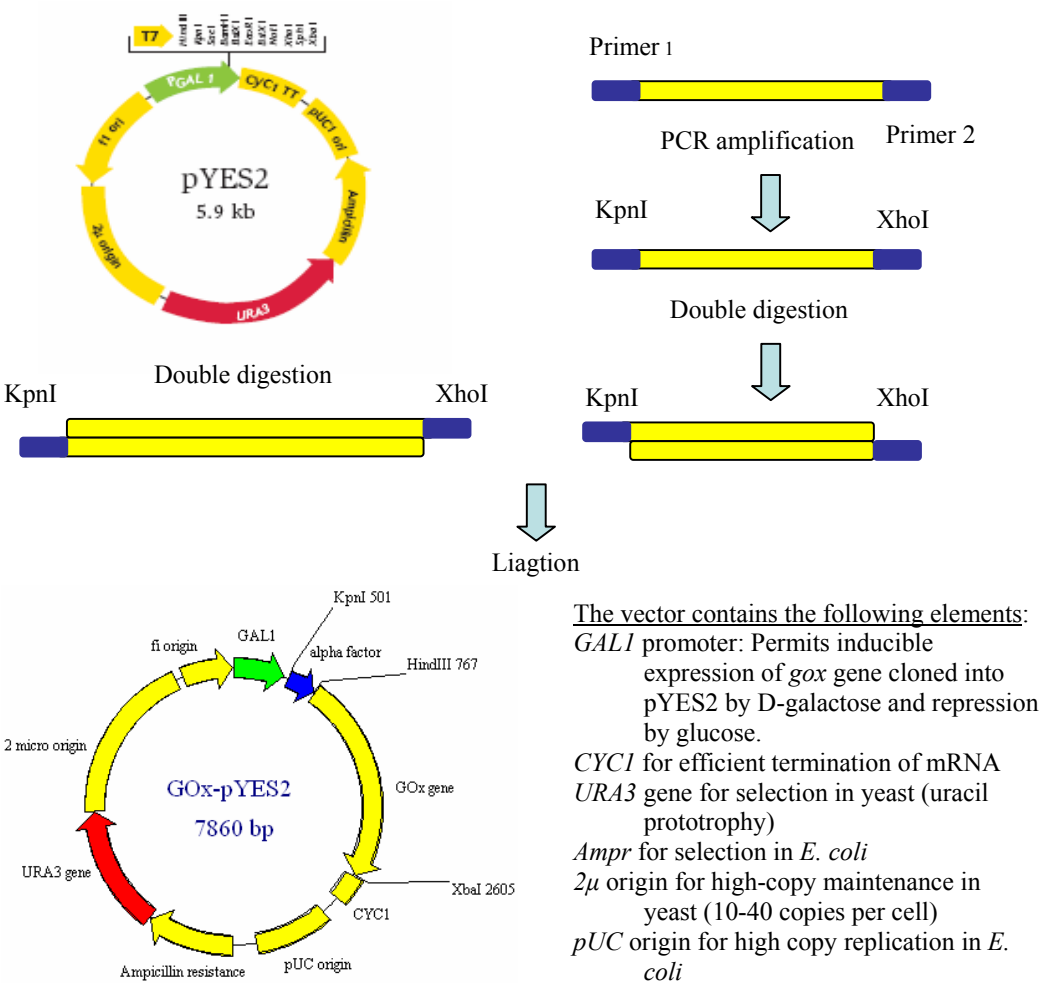


Fig.12. Cloning scheme of rGOx in pYES2 vector using classic double digestion method

pYES2 is a 5.9 kb shuttle vector designed for inducible expression of recombinant proteins in *S. cerevisiae* via GAL1 promoter. This classic DNA cloning procedure suffers

from several limitations including poor ligation as well as the availability of unique restriction sites in both insert and vector. Another alternative cloning method named as ligase free method was develop to solve the above problems: pYES2 vector and insert *gox* gene could form complementary sticky ends of a length of around 20-25 nucloetides, they will find each other at a special temperature and no ligase is needed. Primers for PCR amplification of *gox* gene contain phosphorothioate bonds, T7 Gene6 exonuclease hydrolyzed double stranded 3'overhanged DNA, non-prossessively in the 5′→ 3′ direction. It also degraded nucleotides at the gaps and nicks of double-stranded DNA from the 5′ termini. The T7 gene 6 exonuclease removed nucleotides and stopped at the phosphorothioate bonds (*). During this step sticky ends with long overhangs (20 – 25 nt) were formed (Figure 13).

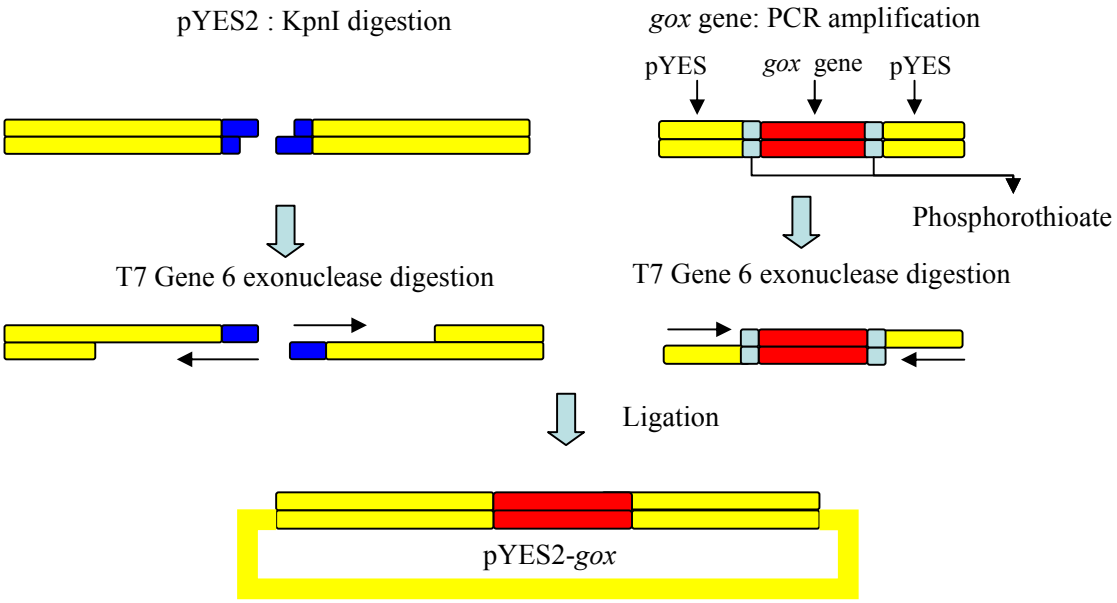


Fig.13. Cloning scheme of rGOx in pYES2 vector using ligase free method

The ligase-free cloning method with T7 Gene6 Exonuclease has shown higher transformation efficiency comparing to the classic method and it was successfully used for creating large mutant libraries (~2000 clones in 20 x 20 cm plate). However the both

gene cloning methods suffered a common problem: re-ligation of the plasmid without gene insert. This problem was solved by implementing an additional gel extraction step in order to remove self-religation of vector, only vectors carrying *gox* genes were transformed to *S. cerevisiae* (Figure 14). The religated products not harboring the *gox* gene was eliminated by moving from electrophoresis agarose gel which boosted the number of active clones by 20 to 30 %. For the classical gene cloning with ligase, four restriction enzymes (KpnI-XbaI, and EcoRI-XhoI) digestions were also used to completely avoid relegation of plasmid pYES2.

Mutagenic libraries were created by epPCR methods using different $MnCl_2$ concentration, followed by cloning of the mutant library into the shuttle vector pYES2 which was subsequently transformed into *E. coli* cells for amplification. The mutant library was isolated from *E. coli* and in the next step transformed into *S. cerevisiae* for active expression.

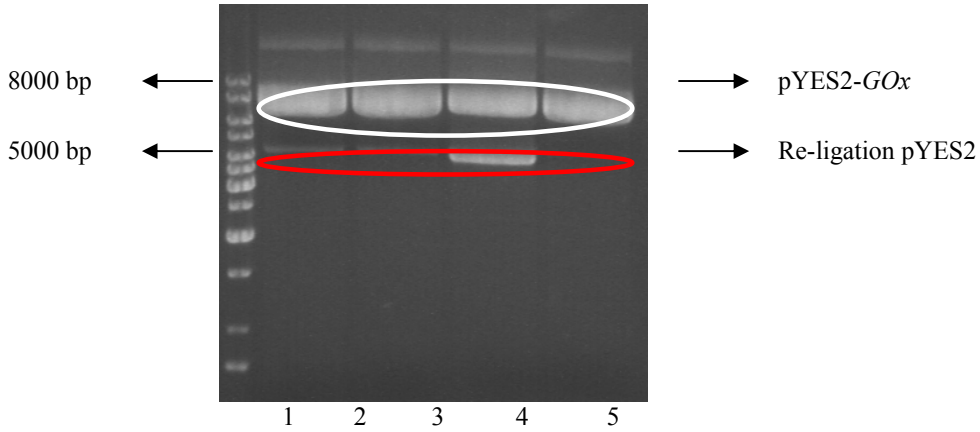


Fig.14 DNA electrophoresis analysis 1 - DNA ladder, pYE2-*gox* plasmid using different $MnCl_2$ concentration for generation libraries of GOx 2 - 0.04 mM, 3 - 0.08 mM 4 - 0.1 mM 5 – 0.2 mM

3.2 Production of rGOx

First pYES2-GOx was transformed in *E. coli* to select and multiply properly ligated plasmid, and then it has been transformed in yeast *S. cerevisiae*. The yeast *S. cerevisiae* cells which containing recombinant GOx were grown in SC-U growth medium for 30-40 hours to reach stationary phase (OD_{600}~3.0). Optimization of induction period is 11 hours for flask-scale and 24 hours for 96-format deep-well plate. After the optimizing induction time, it was observed that activity of GOx was slowly reduced to zero; pH dropped to ~ 3.

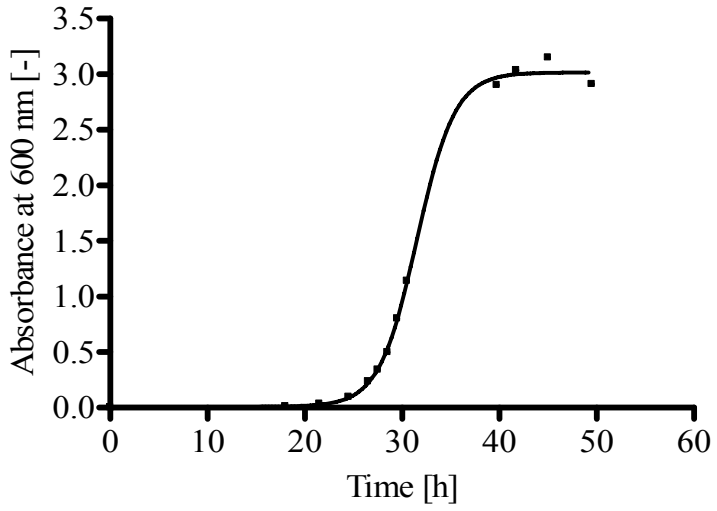


Fig.15. Growth curves of *S. cerevisiae* cells which containing rGOx in 300 ml flask scale (50 ml, SC-Growth medium, 250 rpm, 30°C)

rGOx was produced in large scale by batch fermentation in 16 l stirred tank bioreactor (Figure 16). Enzyme production and glucose consumption during the fermentation was monitored by ABTS assay and β-D-glucose detection kit. The fermentation was started in SC-growth medium with 2 % glucose. When concentration of β-D-glucose was lower than 1 g/l, inducer galactose was added to fermentor, consequently increase of activity of

GOx was observed by ABTS assay. After 3 days of fermentation, rGOx was expressed from supernatant.

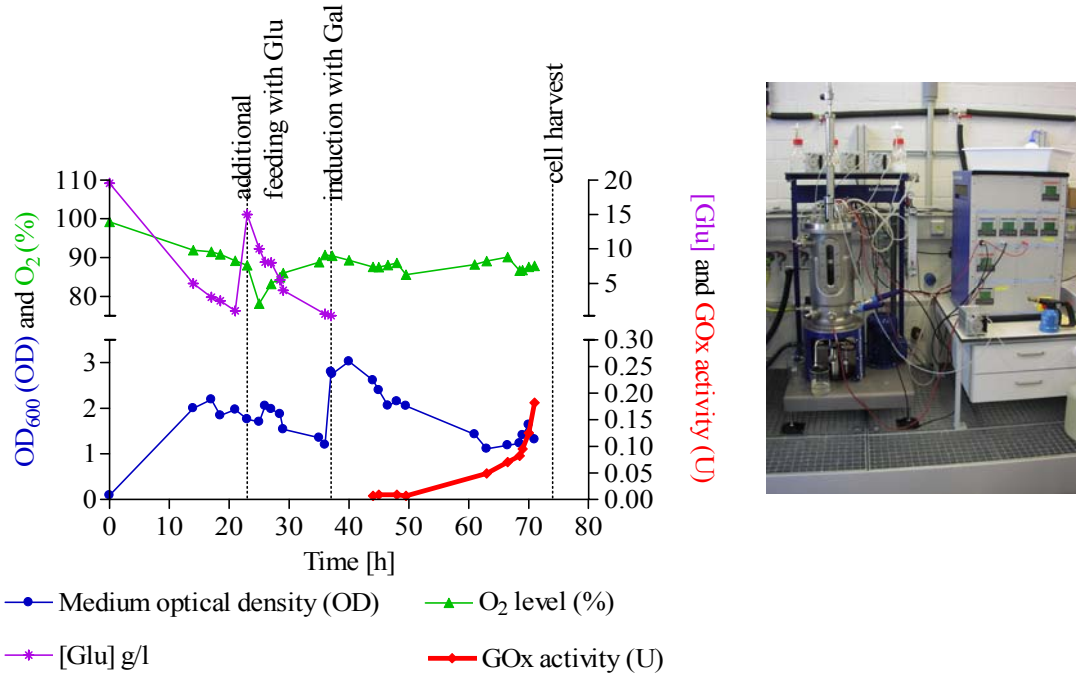


Fig.16. 10 l Fermentation of rGOx in 16 l stirred-tank bioreactor (Bioengineering AG, Switzerland). Fermentation parameters were: 30°C, pH 5, 300 rpm stirrer speed, air flow of 3-5 l/min, pO_2 was maintained at 80%, pressure of 2 bars. Media contains: Yeast Nitrogen Base, essential aminoacids and glucose).

3.3 Purification of rGOx

GOx was collected directly in supernatant after 11 hours induction; procedures of concentration of GOx and size exclusion chromatography were explained in Material and Methods section. The concentration of GOx in 1 liter flask scale was measured by BCA kit after purification and concentration. Figure 17 showed the three peaks observed on the chromatogram, which were recognized by wavelength 280(Protein), 452(FAD), 382(FAD). Sample of the all peaks were collected and checked using ABTS assay, finally the second peak was confirmed to be GOx. The absorption spectrum of purified

rGOx and cGOx was compared with Figure 18, the peaks are showed at 280 nm, 382 nm, and 452 nm, and absorbance values in the ratio 2.72:0.79:1 (cGOx) and 3.89:0.61:1 (rGOx). Figure 19 commercial GOx from *A. niger* migrated as protein with an apparent molecular weight of 110~120 kDa on SDS-polyacrylamide gel electrophoresis; rGOx from *S. cerevisiae* showed a 30-40 kDa higher molecular weight than cGOx from *A. niger*.

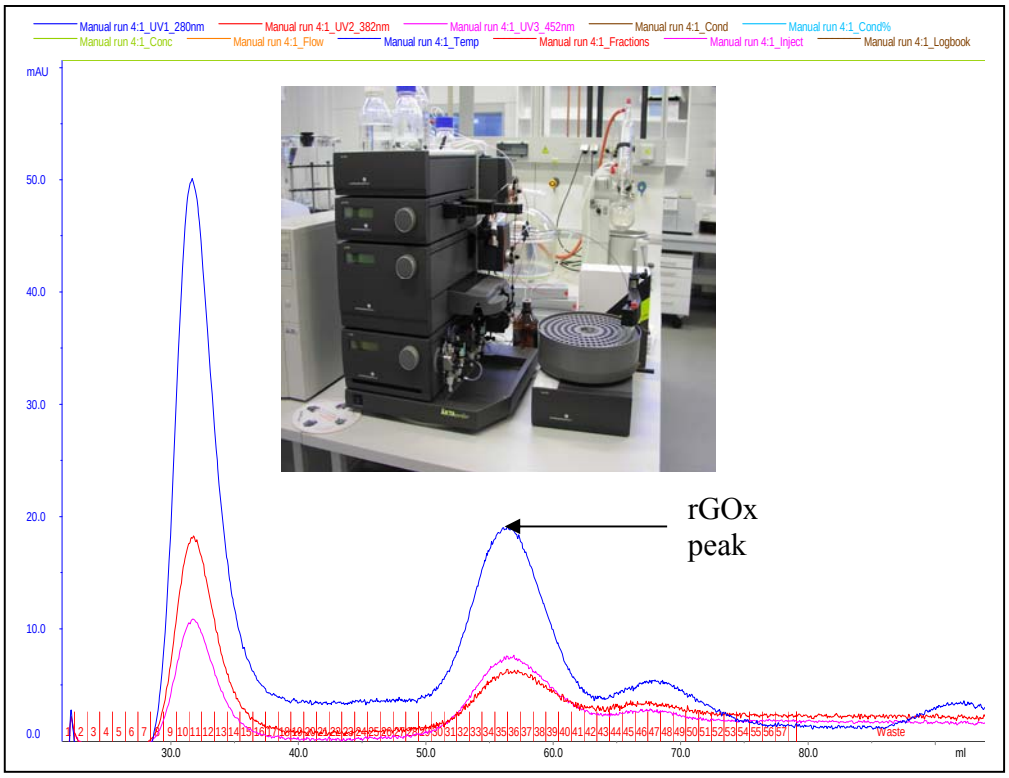


Fig.17. Size exclusion chromatography of rGOx, 1 ml concentrated rGOx was injected and monitor by at 280 nm, 382 nm and 452 nm. Enzyme activity was monitored using ABTS assay

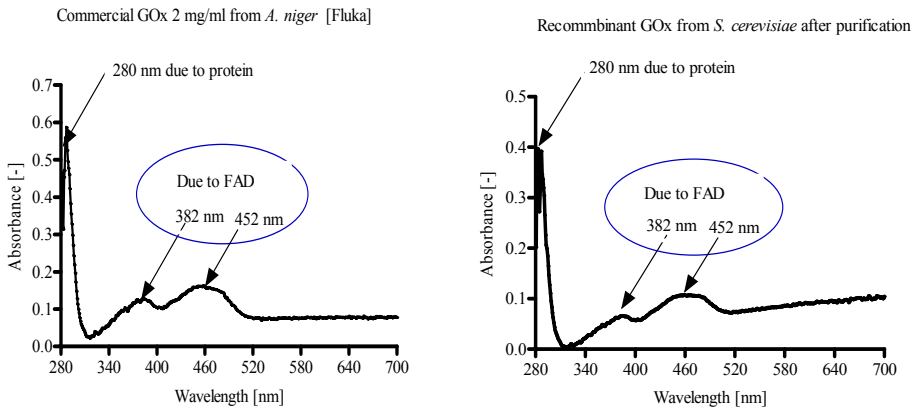


Fig.18. Absorbance spectra of cGOx (2 mg/ml) from *A. nger* and rGOx from *S. cerevisiae* following gel filtration

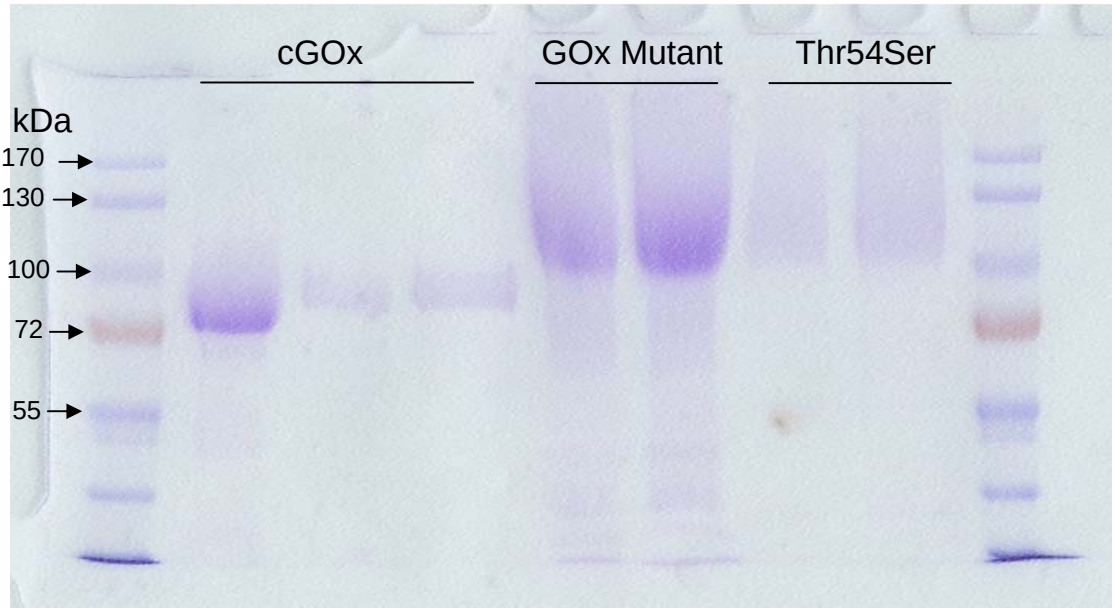


Fig.19. SDS-polyacrylamide gel electrophoresis of rGOx from *S. cerevisiae* and cGOx from *A. niger*

3.4 Screening methods of GOx

3.4.1 Solid phase screening

Figure 20 illustrates the result of ABTS assay by using a filter-paper based pre-screen on the agar plate. Filter paper soaked with ABTS assay mix was carefully laid on the top of colony. The colorimetric development immediately occurred. Actives clones were

subsequently picked directly from the pre-screen plates. Only active clone was picked for following liquid phase screening. It proved to be an effective and fast prescreening method which save time (5000 colonies per day) and assay compounds (1 ml assay mix each agar plate).

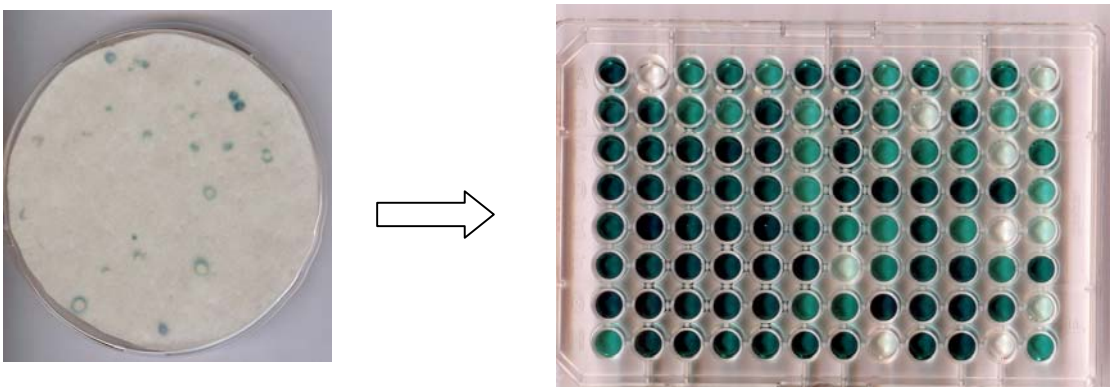


Fig.20. Solid phase screening and following liquid phase screening using ABTS assay

3.4.2 Glucose oxidase detection assay (GODA)

Figure 21A shows the reaction sequence of GODA leading to NADPH formation which was recorded at 340 nm (ε = 6300 M^{-1} cm^{-1}) (Mollering and Bergmeyer, 1967). Conversion of ß-D-glucose by GOx was terminated by pH shift to 10. At this pH the reaction product D-gluconolactone was hydrolyzed to D-gluconate, which was subsequently phosphorylated by gluconate kinase (GK) and oxidized by 6-phosphogluconate dehydrogenase (6-PGDH) to ribulose-5-P (Figure 21A). Figure 21B shows the sensitivity of GODA for detecting D-gluconolactone. A linear range of up 2.0 absorbance units (data not shown) and up to 3 mM D-gluconolactone (Figure 21B) has been recorded. Under assay conditions in microplates and two hour incubation time concentrations down to 0.5 mM could be monitored.

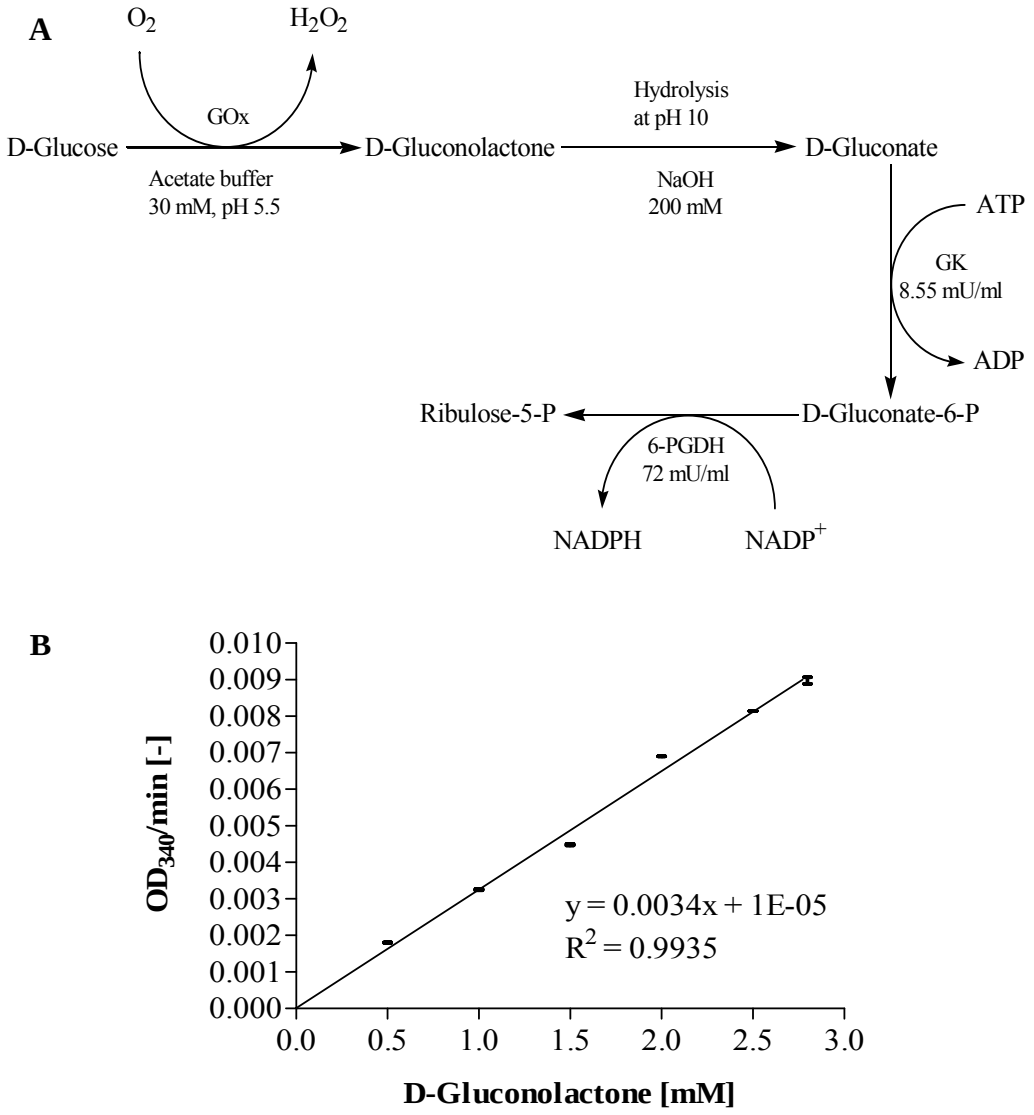


Fig.21. (A) Reaction scheme of GODA. (B) Calibration curve for detection of D-gluconolactone.

Standard deviation in 96-well plates

An important performance criterion for a screening system was the standard deviation of the wild-type enzyme in 96-well plate format. Figure 22 shows the standard deviation (SD) of wild-type GOx before (4.6 %; apparent SD) and after (10.7 %; true SD) subtracting the background absorbance.

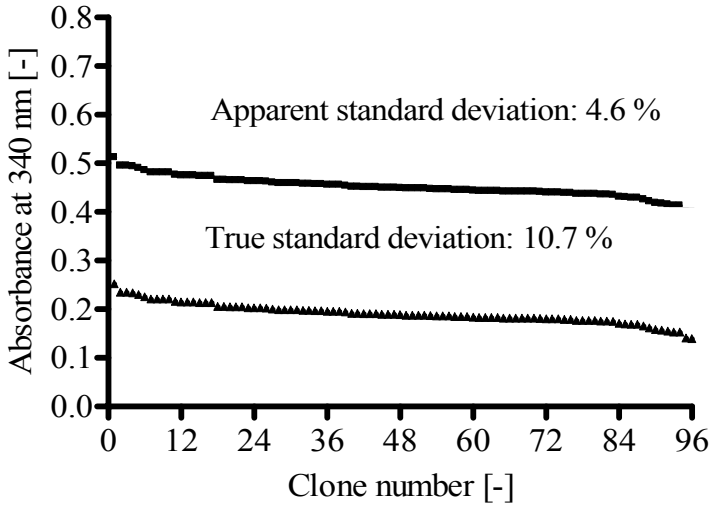


Fig.22. Standard deviation of the GODA in 96-well format with recombinant wild type GOx expressed in *S. cerevisiae*. Data are in descending order of absorbance values in a 96-well plate.

For validating GODA, a mutant library (2000 clones) was generated by epPCR and screened for improved activity towards β-D-glucose. Improved variants and potentially improved variants were combined on a 96-well master plate. Figure 23 shows for this 96-well plate the performance of GODA compared to a modified ABTS assay (see Material and Methods), which detects the side-product hydrogen peroxide. D-gluconolactone and hydrogen peroxide formation correlates well in a 96-well plate (Figure 23). GOx variants detecting from both assays have the same order of absorbance value (Figure 23) after at least 50 min incubation time. An incubation time of two hours proved to be a suitable compromise between conversion and time consumption for detection of GOx activities. The absolute absorbance values differ however significantly for the most active mutants in terms of absolute values.

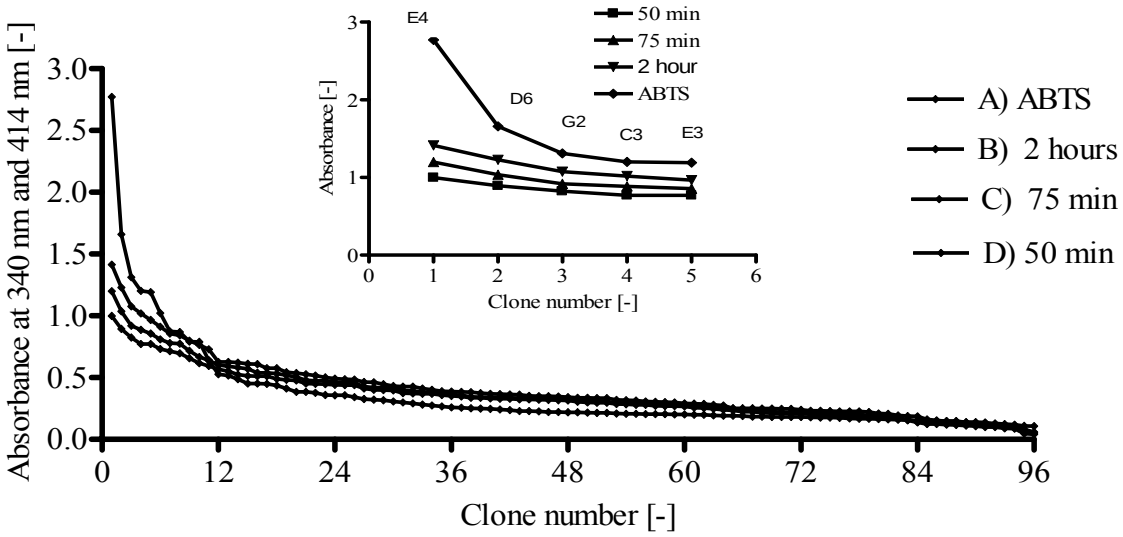


Fig.23. Comparison GOx activity in 96 well plate: A) ABTS-assay at 414 nm and GODA at 340 nm after various reaction times: B) 2 hour; C) 75 min; D) 50 min. The order of wells is identical for the improved variants of GOx and validates the GODA for evolving GOx.

3.4.3 Ferrocenemethanol ($FcCH_2OH$) based assay

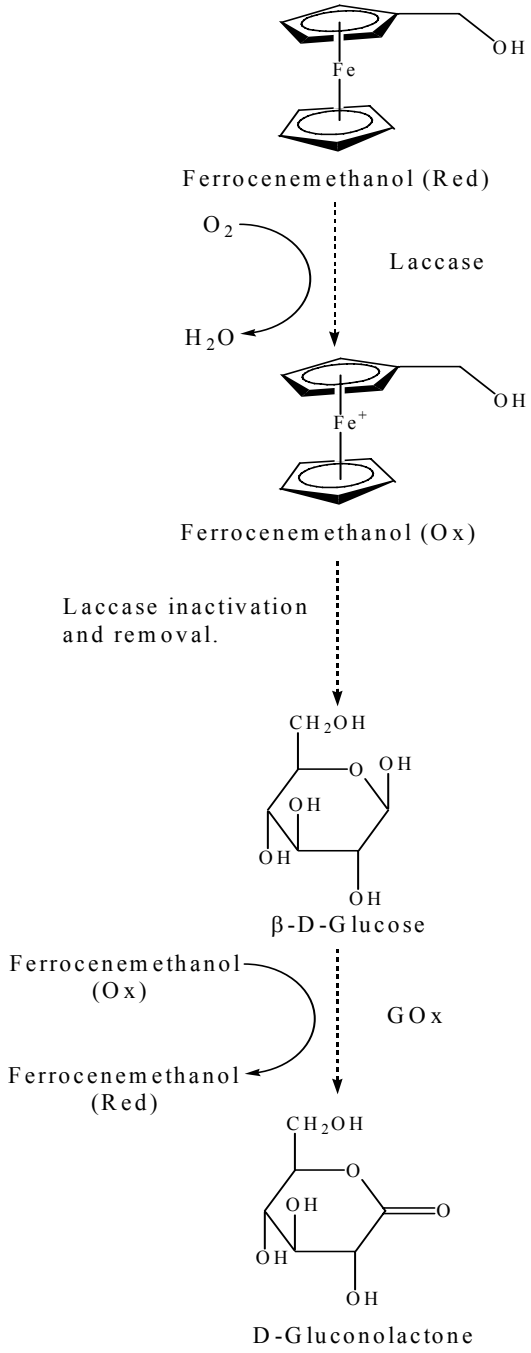
We aimed to use ferrocenemethanol as an effective mediator to screening mutant libraries of GOx in a 96 format.

The whole reaction scheme was illustrated in three steps (Figure 24A):

1) Formation of oxidized formed ferrocenemethanol: Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyzed oxidation of ferrocenemethanol with the concomitant reduction of molecular oxygen to water at pH 3. Figure 24B shows that more oxidized ferrocenemethanol was formed after adding laccase, which was observed by continuous absorbance increase at 625 nm (Figure 24B).
2) Laccase removal: the solution pH was adjusted to 8 to inactivate laccase. In the end, laccase was completely removed by ultrafiltration.
3) Ferrocenemethanol based assay: oxidized form of ferrocenemethanol was oxidizing reduced form of GOx and colorimetric development during redox

reaction was observed. Kinetics was monitored by absorbance decrease at 625 nm. Assay control measurement showed only at the normal assay condition, an immediate color change from deep green to yellow was observed (Figure 24C).

A



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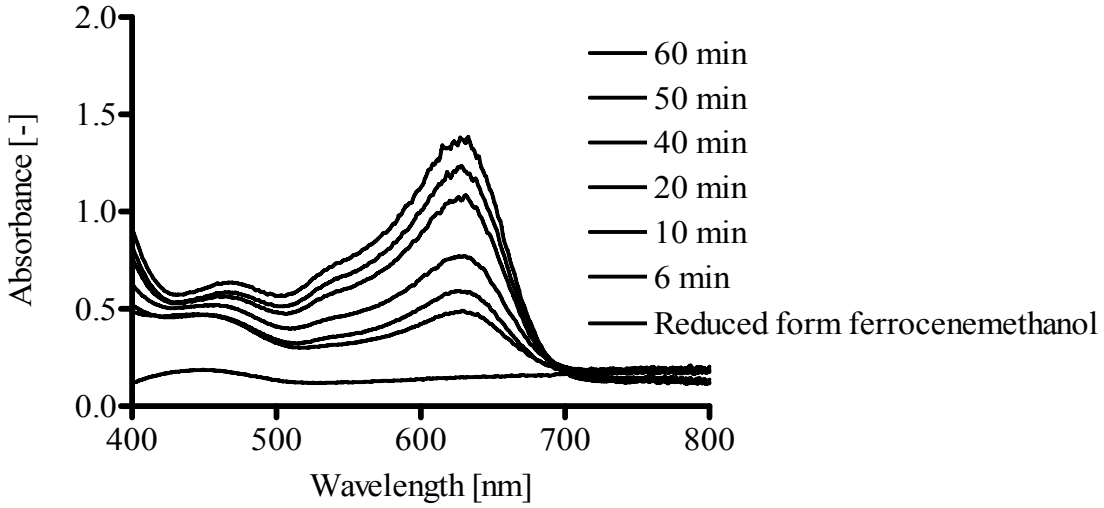


C

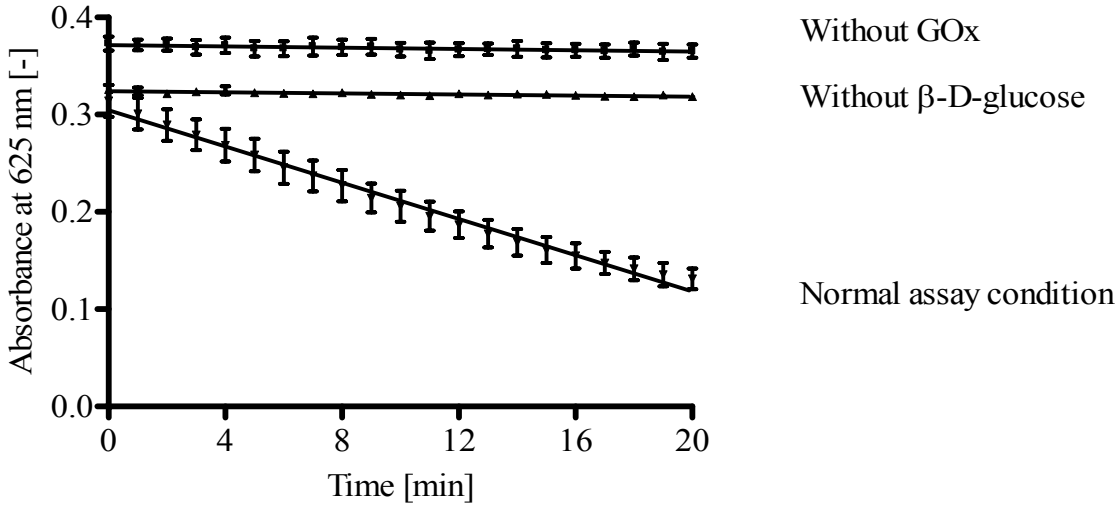


Fig.24A: Complete reaction scheme of ferrocenemethanol assay, 13B: Spectral change observed upon the addition of laccase in 100 mM phosphate buffer (pH 3) at 20 °C 13C: GOx assay with control measurements in 100 mM phosphate buffer (pH 8) at 20 °C in different conditions: a) without GOx, b) without β-D-Glucose and c) normal assay conditions.

The oxidized form of ferrocenemethanol exhibited an absorbance at 625 nm with molar extinction coefficient of 223 [$M^{-1}cm^{-1}$] as shown in Figure 25. $\varepsilon = A/Cl$ (A: absorbance at 625 nm, l: pathlength C: molar concentration of oxidized form ferrocenemethanol)

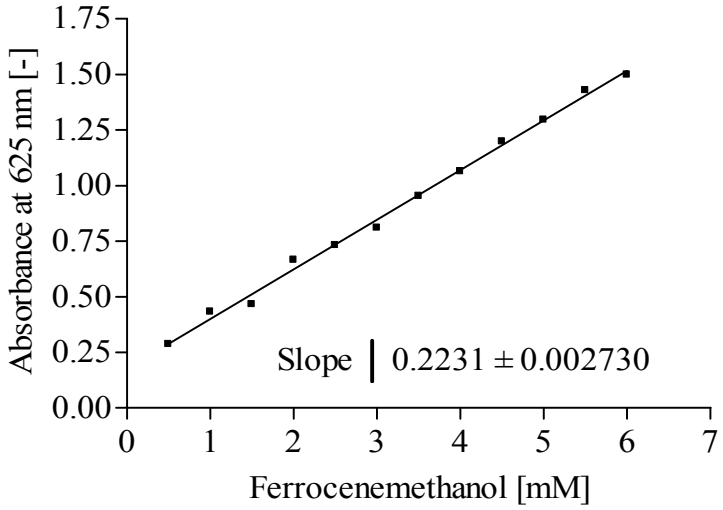
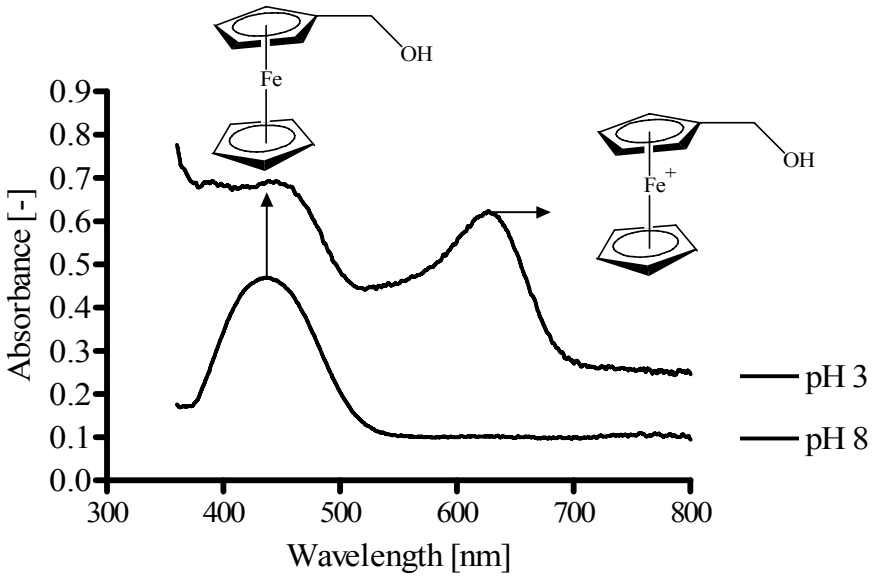


Fig.25. Molar extinction coefficient of oxidized form of ferrocenemethanol in 100 mM phosphate buffer (pH 8) at 20 °C

Figure 26A showed pH influence on the stability of reduced form of ferrocenemethanol. At low pH (pH 3), reduced form of ferrocenemethanol was not stable. It was slowly oxidized by O_2 in ambient condition, which was confirmed by a decrease absorbance at 430 nm and increase absorbance at 625 nm. Figure 26B showed a kinetic measurement to detect activity of rGOx. Electron acceptor ferrocenemethanol was oxidized by O_2 during 6-day slow reduction. There was still reduced form of ferrocenemethanol remained in solution, however partially oxidized formed ferrocenemethanol already showed the similar results as laccase did (Figure 26B), it proved that the colorimetric development was not coming from laccase but ferrocenmethanol itself. At high pH (pH 8), ferrocenemethanol was stable, it did not show any absorbance change during 6 day

measurements (Figure 26A). It was the reason why at pH 8, stable ferrocenmethanol could be selected as electron acceptor for developing an assay for detecting of GOx.

A



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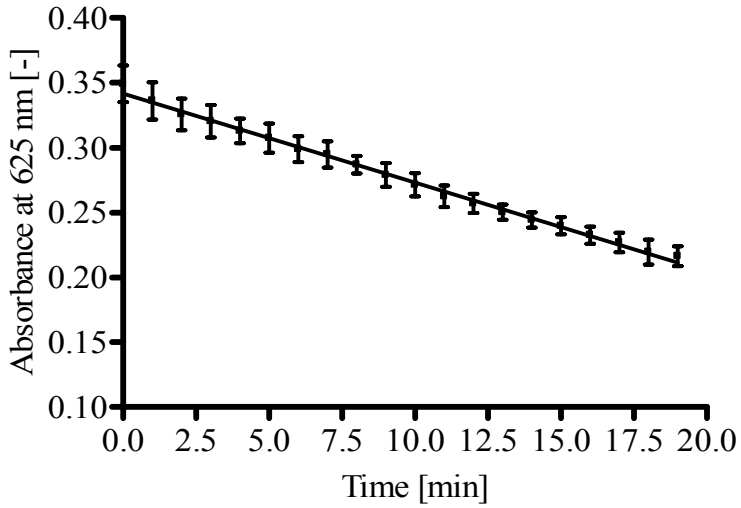


Fig.26A Spectral change of Ferrocenemethaol solution (3M) after 6-days in ambient condition B) Ferrocenemethanol based assay using 6-days ferrocenemethanol solution (pH 3) as electron acceptor in 100 mM phosphate buffer (pH 8)

Dioxygen is natural electron acceptor of GOx, that is the reason why GOx has been used as oxygen scavenger in industry. How to compete with dioxygen from air is the crucial point for building up a reliable mediator based assay. Figure 27 showed ferrocenemethanol based assay in oxygen-free environment: a nitrogen purging system (pink color) with O_2 probe monitor (blue color) was set up. An obvious color changed (green to yellow) was observed after adding commercial GOx (cGOx) to the solution containing ferrocenemethanol and β-D-glucose. A control measurement with normal air condition was compared with nitrogen purging condition. Both results are very similar, which is confirming that ferrocenemethanol based assay at pH 8 was insensitive for oxygen.

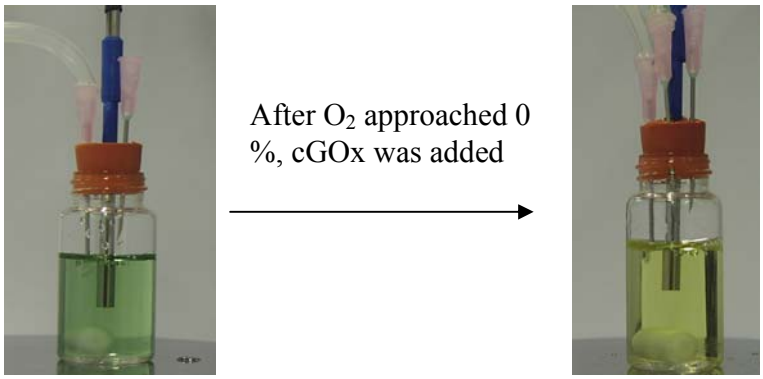


Fig.27. Colorimetric development of ferrocenemethanol based assay in oxygen-free system in 100 mM phosphate buffer (pH 8) at 20°C

Figure 28 showed that dioxygen changes were monitored in the completed reaction scheme using oxygen probe. The reduced formed ferrocenemethanol was the starting point for measurement, after adding laccase and applying a stirring condition, oxygen level was immediately reduced from 100 % to 0 %. Most of reduced formed ferrocenemethaol was oxidized with an obvious color change (yellow color to green-blue). After 15 min, rGOx and β-D-glucose were added into the system and immediately

initiate ferrocenemethanol based assay and O_2 based assay at pH 3, the oxidized form of ferrocenemethanol and dioxygen started to oxidize reduced form GOx, and at the same time, oxidized formed ferrocenmethanoll was regenerated by laccase. However, at low pH, Ferrocenmethanol was difficult to compete with dioxygen. Oxygen level started to slowly increase, because formation reaction rate of oxidized form of ferrocenemethanol catalyzed by laccase was much faster than the consumption reaction rate of oxidized form of ferrocenemethanol catalyzed by GOx. After 1 hour reaction, NaOH was added and adjust pH to 8. Oxygen level was increasing much faster than before. It could confirm that laccase was inactivated at pH 8 and dioxygen was inhibited as electron acceptor to drive GOx at pH 8. Figure 29 was further proved that the oxidation of β-D-glucose by dioxygen at pH 5 was much faster that at pH 8; ferrocenemethanol was reversed. So ferrocenemethanol based assay could compete with dioxygen at pH 8.

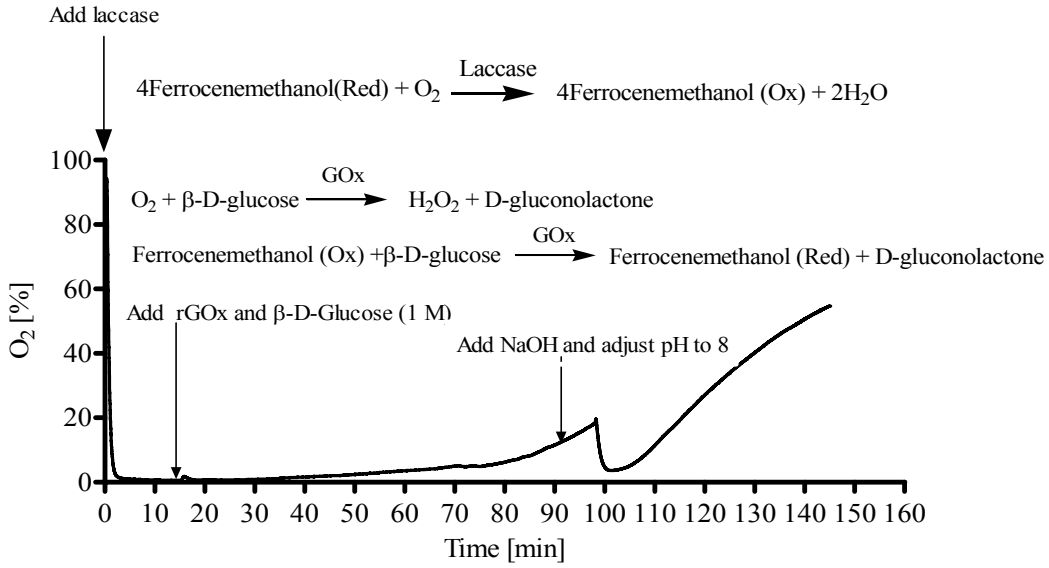
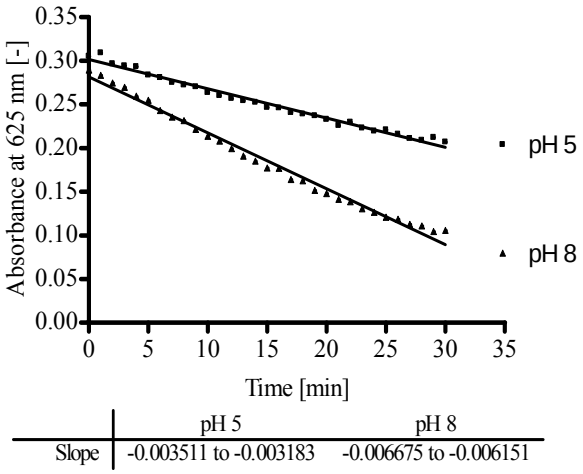


Fig.28. Scheme of oxygen change during laccase and rGOx enzymatic reactions at 20 °C

A



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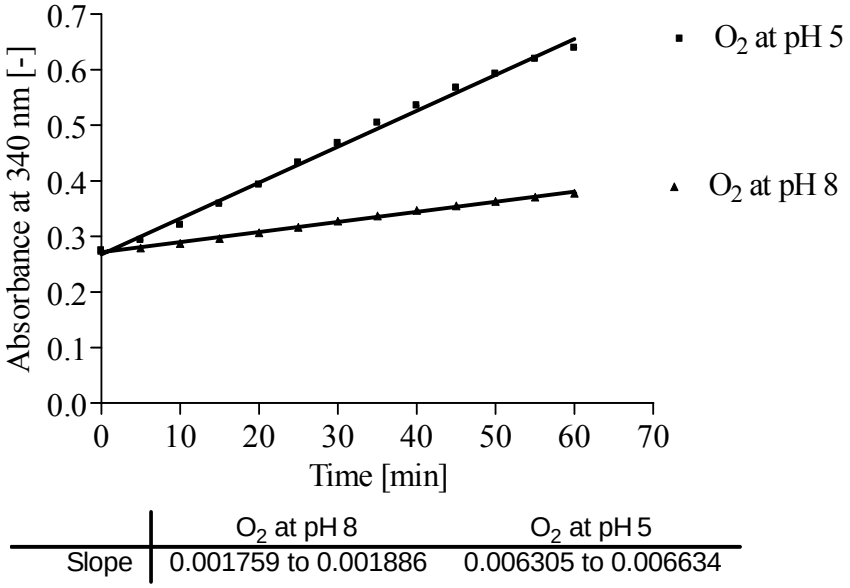


Fig.29. pH effect on assay systems for detecting rGOx in 100 mM acetate buffer (pH 5) and 100 mM phosphate buffer (pH 8) at 20 °C A) Ferrocenemethanol based assay B) GODA assay using dioxygen as electron acceptor

After optimizing the assay conditions, a linear detection range of ferrocenemethanol was measured (Figure 30). A wide linear detection window was important for identifying

beneficial mutants, and screening systems for evolved mutants have to be constantly adapted for hitting the linear detection window.

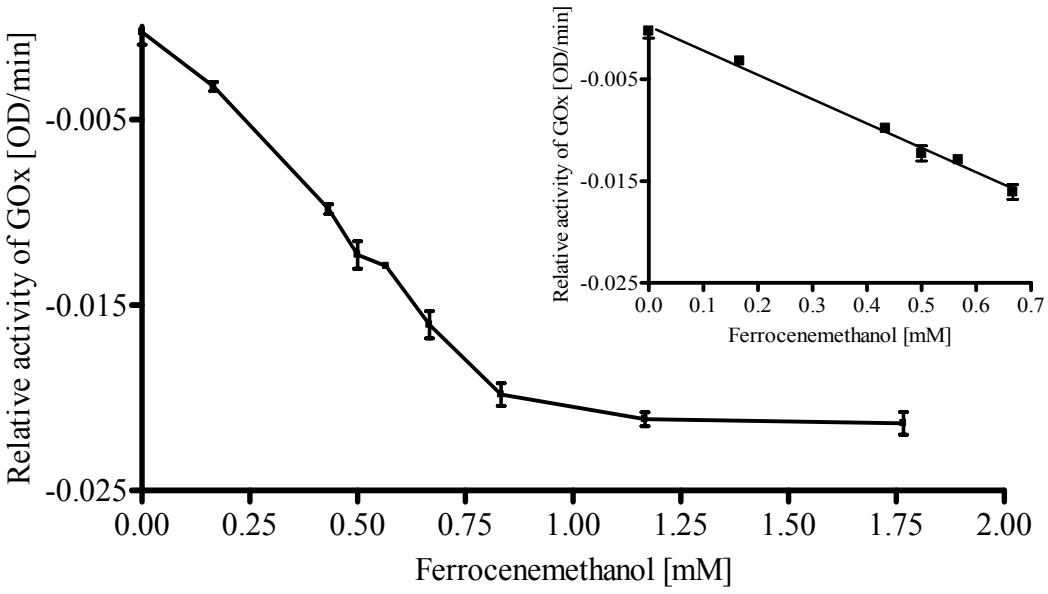


Fig.30. Linear detection range of ferrocenemethanol based assay in 100 mM phosphate buffer (pH 8) at room temperature, close-up figure showed a linear response was achieved between 0-700 µM ferrocenemethanol.

After setting up a single ferrocenmethanol based assay, mutant libraries of GOx (4000 clones) were screened with this assay for directed protein evolution. For a successful directed evolution experiment three tasks have to be accomplished: a reliable assay system (Coefficient of variant < 20 %), epPCR conditions with ~50 % dead clones and large libraries of 4000-10000 clones are accepted for screening.

Standard deviations have been reduced by optimizing growth conditions and culture volume. The preferred combination of 1 ml SC-media in 2-ml deep well plates at 700 rpm in a microplate shake resulted in a true coefficient of variance 16 % after subtracting the background.

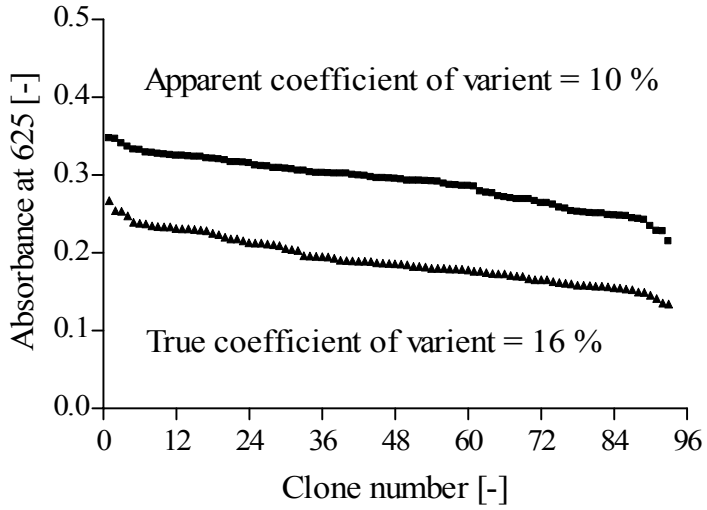


Fig.31. Ferrocenmethanol based assay in 100 mM phosphate buffer (pH 8) at 20 °C in descending order of activity value

To validate the assay, ABTS assay was used as a standard model to compare with ferrocenemethanol based assay. Figure 32 showed that color distribution of ABTS assay was highly correlated with ferrocenemethanol based assay in 96 formats. Slightly green and deep green in ABTS assay was correlated with green-blue and yellow in ferrocenemethanl based assay.

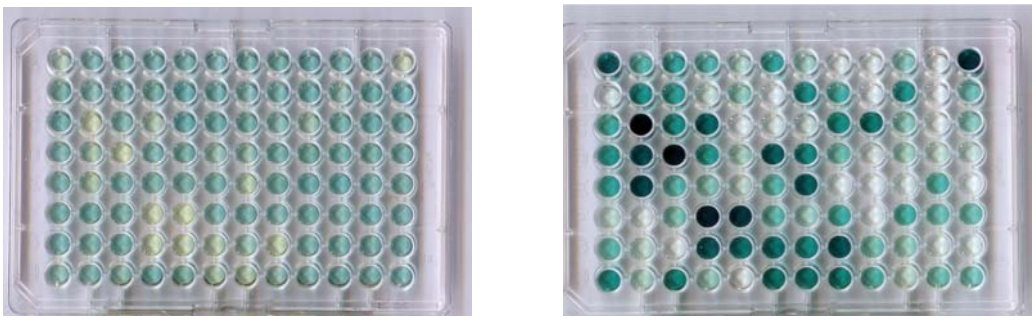


Fig.32. Colorimetric development of ferrocenemethanol based assay in 96 format (left) in 100 mM phosphate buffer (pH 8) and ABTS assay (right) in 100 mM acetate buffer (pH 5.5) at 20 °C

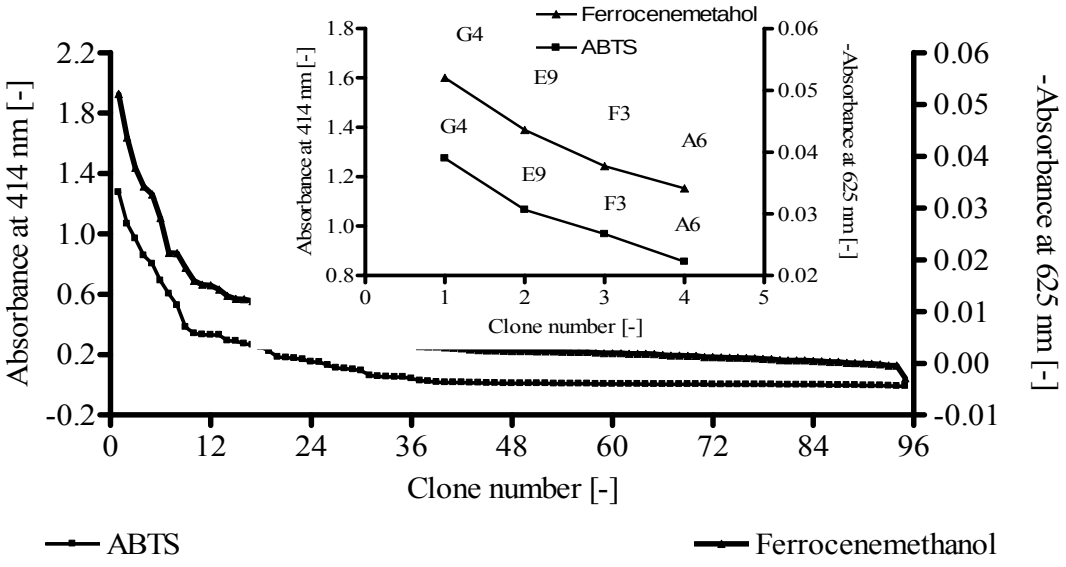


Fig.33. Comparison GOx activity in 96 well plate format: A) ABTS-assay at 414 nm B) ferrocenemethanol based assay at 625 nm. The order of wells was identical for the improved variants of GOx and validates the ferrocenemethanol for evolving GOx.

Figure 33 shows absorbance from validating assays (ferrocenemethanol and ABTS) in descending order. Ferrocenemethanol based assay is a decolorization assay, absorbance values have to minus one to compare absorbance using ABTS assay.

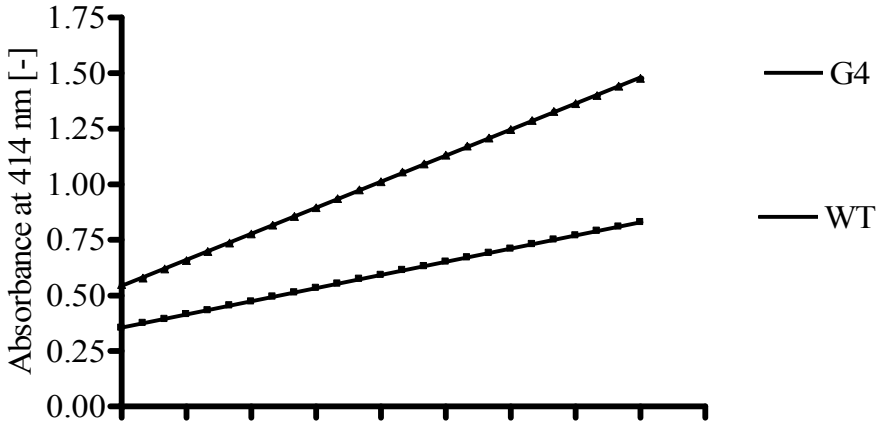
3.5 Enzymatic characterizations

Screening 2000 mutants in the first round of mutagenesis of GOx library generated two variants with 1.3~1.5 higher total activity toward β-D-Glucose than the wild type. The most active one E4 was used as a template for the second generation. Screening another 2000 clones in the second generation identified one variant G4 with improved activity 2-fold with both assays (Figure 34) and enhanced thermostability (Figure 35).

Screening of a GOx mutant library resulted in variants with increased V_{max} and preserved K_m values. The most active mutant (G4) was isolated, sequenced, purified and the kinetic data for glucose conversion were determined. The GOx mutant G4 carries a T51S

substitution and kinetic parameters were determined as following Table 8. The difference to reported kinetic constants of GOx from *A. niger* (Bohmhammel et al., 1993) can be attributed to the extend of FAD incorporation in recombinant GOx expressed in *S. cerevisiae* (data not shown). The thermostability of the purified WT and mutant GOx was assessed by measuring residual activity over a temperature range 24-75 °C. The T_{50} was increased from 53°C (WT) to 56°C (Mutant).

A



	WT	G4
Slope	0.05922 ± 0.0005676	0.1174 ± 0.0002179

B

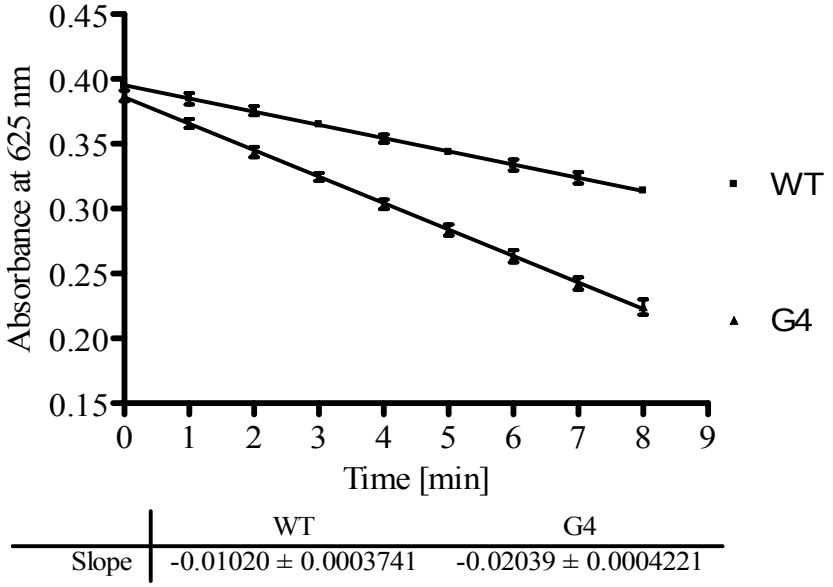


Fig.34. Kinetics of rGOx WT and mutant G4 at 20 °C A) ABTS assay in 100 mM acetate buffer (pH 5.5); B) Ferrocenemethanol based assay in 100 mM phosphate buffer (pH 8)

Table 8 Kinetic parameters of WT and mutant G4. The purified enzyme was normalized to the same concentration at 20 °C.

GOx	K_m for β-D-glucose [mM]	k_{cat} [sec^{-1}]	k_{cat} / K_m [$mM^{-1}sec^{-1}$]
WT	20.77	69.47	3.34
Mutant (T51S)	21.32	137.7 4	6.46

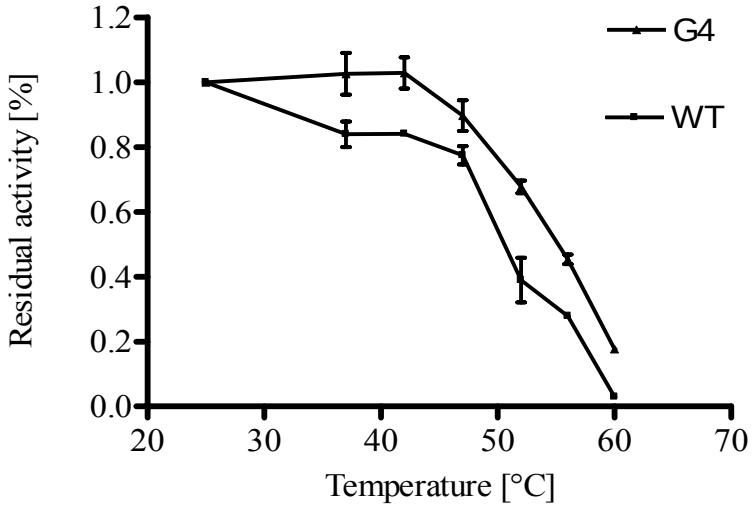


Fig.35. Temperature effect on GOx A) Thermostability measured by ferrocenemethanol based assay in 100 mM phosphate buffer (pH 8), the same amount purified proteins were incubated 2 hours before assay reaction.

3.6 Other assay systems

Beside O_2, H_2O_2, GODA and ferrocenemethanol based assay, other mediators such as 2,6-dichloroindophenol (DCPIP), Iron(III) acetylacetonate/phenanthroline mixed complex, thionine, phenazine methosulfate (PMS), ABTS cation radical, all failed to detect rGOx in 96 format. The results showed cGOx was easily reduced by the mediators which were mentioned above, however, rGOx in supernatant after induction resulted in a strong interference background due to high pH dependence (such as thionine, DCPIP) and relatively high redox potential (Fe[III] mixed complex and ABTS cation). Other reducing compounds in supernatant were also oxidized by mediators, it made difficult to apply them in rGOx, which was confirmed by the immediate color change in control measurement (containing pYES2 without *gox* gene insert) after adding the assay mix. The results did not make any difference between control measurement (only plasmid) and

normal assay measurement (with GOx). The competition with O_2 and H_2O_2 interference were another two important reasons that make mediator assay insensitive.

Table 9 properties of other artificial electron acceptors

Mediator assay	Stability	Spectral properties	H_2O_2 interference	Optimizing assay condition	Redox potential
Thionine	Light sensitive, pH dependence	Blue to colorless	medium	pH 7.0; Room temperature	0.258 V Ag/AgCl
DCPIP	Light sensitive, pH dependence	purple to colorless	medium	pH 5.5; Room temperature	0.217 V Ag/AgCl
Fe[III] mixed complex	Not stable	yellow to red	high	pH 7.0; Room temperature	0.93 V Ag/AgCl
ABTS cation	Light sensitive	Green to colorless	low	pH 5.5; Room temperature	0.78 V Ag/AgCl

3.7 Pyranose oxidase (POx) assay

Ferrocenemethanol was used as electron acceptor to drive another glucose oxidizing enzyme: pyranose oxidase. rPOx was lysed cell extracts of recombinant *E. coli* expressed POx. rPOx assay was measured at 625 nm using two different control conditions: a) Without rPOx and b) without β-D-glucose (Figure 36). It showed promising results for using ferrocenemethanol based assay in 96-well format.

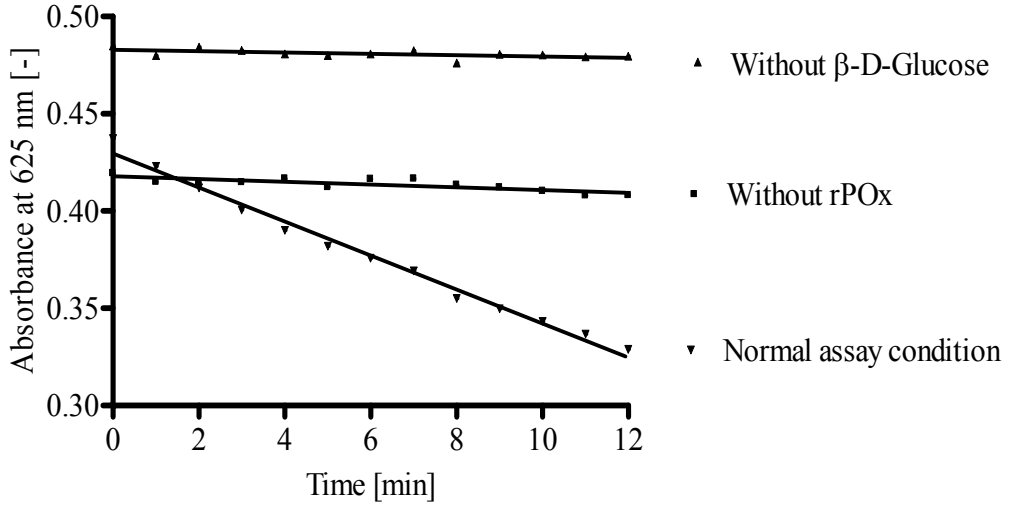


Fig.36. Kinetics of ferrocenemethanol based assay to detect rPOx from lysed cell extracts in *E. coli* in 100 mM phosphate buffer (pH 8)

4. DISCUSSIONS

For advancing the field of miniature biofuel cells (mBCs), it is important to boost power output, improve long-term stability and avoid hydrogen peroxide production that might cause tissue damage. Directed evolution allows improving biocatalysts for the objectives mentioned above.

4.1 Gene cloning

4.1.1 Ligase free method

A classical ligation method and a ligase free method were used for *gox* gene cloning. The classical ligation method showed rather low transformation efficiency and high re-ligation problems compared to a ligase free method. By generating customized complementary sequences, this method yields more frequent accurate joinings between the vector and the insert. With this ‘easy’ procedure, there is no need to pine over the availability of a restriction site. In addition, because the overhang sequences are custom-designed, symmetry is absent, thus reducing self-religation of the DNA fragments. This reduces the number of unwanted insert-insert and vector-vector joining, increasing the number of clones that successfully get copies of the GOx gene. The quality of the mutagenic libraries was also improved by implementing an additional gel extraction step in order to remove self-religation of vector.

4.2 Expression system

4.2.1 Disulphide bond formation

As a glycoprotein, rGOx is difficult to be expressed in *E. coli* in active form. According to literatures, the commonly used GOx in mBCs is from *A. niger* and cannot functionally be expressed in rapidly growing *E. coli* cells. GOx from *P. amagasakiense* can be expressed in *E. coli* as inactive inclusion bodies. The inability of *E. coli* strains to form disulfide bridges in GOx explains the non-functional expression in *E. coli*. Disulphide bridges are very important for proper protein folding, function and stability (Witt et al. 1998). The yeast *S. cerevisiae* has been used for directed evolution of a few biocatalysts (Bulter et al., 2003), allowing functional secretion of GOx into the media and representing a considerable advantage for downstream processing and purification. Therefore, in our study, the yeast strains *S. cerevisiae* was selected as host for directed evolution of GOx.

4.2.2 Production of rGOx

Production level of rGOx in *S. cerevisiae* was quiet low and could be explained by following reasons.

1) Ethanol formation

The yeast metabolism converted D-glucose to ethanol, which has inhibitory effect on the gene expression resulting in less production of GOx. The formed ethanol became as new carbon source which is taken by yeast cells. When O_2 consumption rate is higher, the more ethanol will be produced, and less protein will be expressed. So oxygen supply and carbon source will be key parameters for expression of GOx.

2) Hyperglycosylation

S. cerevisiae has the tendency to hyperglycosylate: For the production of heterologous proteins, hyperglycosylation results in a major inconvenience, resulting in loss of activity.

3) α-factor signal sequence

De Baetselier has been reported the expression level of GOx in *S. cerevisiae* was highly dependent on the signal sequence used. A 5-10 fold increase was observed, when the homologous *gox* signal sequence was used instead of α-factor presequence (Debaetselier et al. 1992).

4) Host strains

The level of GOx synthesis was found to be highly dependent on the genetic background of host strain, cultivation condition and medium composition, resulting in a 100-fold difference between the lowest and highest producing strain (Debaetselier et al. 1992).

4.3 Screening goals

Specific objectives for in-body-applications of GOx in mBCs are derived from conditions in blood vessels and comprise: A) improving activity of GOx for β-D-glucose, B) designing oxygen independent GOx, and C) improving thermostability.

4.3.1 Electron transfer rate

Laboratory evolution methods have very few successful stories for improving electron transfer from mediators to enzyme. Ferrocenemethanol based assay was developed for improving electron transfer rates between oxidized formed of mediator and reduced form of GOx. Electron transfer was monitored by colorimetric development during redox reaction. In GOx, a redox mediator must enable to shuttle electrons between the mediator and the active site of protein. Alvarez lcaza constructed a molecular model of the active

site of glucose oxidase complexed with ferrocene, and found that, in this model, the N5 atom of reduced coenzyme is 4.56 Å away from the ferrocene. Thus, this low-molecular mediator such as ferrocenemethanol can move all the way down to the active site where it can exchange electrons by the space jump (Leskovac et al. 2005). At pH 8, ferrocenemethanol based assay is insensitive to natural substrate dioxygen and the oxidation rate constant found at pH 8 is indeed larger than observed with dioxygen (Leskovac et al. 2005). Isoelectric point of GOx is 4.2; at pH 8, GOx is therefore a very anionic enzyme. The electrostatic interaction between negative charged GOx and positively charged ferrocenemethanol plays an important role in the efficiency of electron transfer at pH 8. Comparing with ferrocenmethanol based assay, conventional standard assay detects the byproduct H_2O_2, and does not allow detecting electron transfer.

According to electron-transfer theory, cleavage of the protein-bound carbohydrate moiety of the enzyme has been demonstrated to improve rates of electron transfer from its active site to an enzyme electrode. Since rGOx from *Saccharomyces cerevisiae* is hyperglycosylated compared to GOx from *Aspergillus niger*, removal of carbohydrate moieties of rGOx will be an idea to compare electron transfer rate between deglycosylated GOx and glycosylated GOx.

4.3.2 Oxygen independency

The determination of glucose using dioxygen is often undesired because it is subject to variations of dissolved dioxygen concentrations. The byproduct H_2O_2 has in addition deleterious effect on GOx. This problem can be either partially solved by replacing

dioxygen with alternative artificial electron acceptors, or by screening for GOx mutants with low binding affinities for oxygen.

Oxygen independency can be screened by combining two assays (ABTS and ferrocenemethanol). ABTS assay use dioxygen as electron acceptor and ferrocenemethanol assay is insensitive to dioxygen and uses ferrocenemethanol as electron acceptor. When both assays show positive results, it means that mutants do not affect O_2 binding. A negative result of only the ABTS assay means that mutants are still strong for O_2 binding; when both assays show negative results, it indicates that mutants have no activity.

Table 10 Screening scheme for oxygen independency of GOx

'Mutated' GOx	ABTS assay	Ferrocenemethanol assay
Oxygen independency	Negative	Positive
Oxygen dependency	Positive	Positive
Inactive biocatalysts	Negative	Negative

A really random mutagenesis method has to be set up to overcome mutation bias. Only with a highly diversified mutant libraries, oxygen independency can be screened in a reasonable way.

4.4 Screening methods

For the screening of mutants, two novel and reliable screening systems have been developed for medium-throughput screening. Additionally, re-ligated vector without gene insert was completely removed using gel extraction. Following solid phase screening, only active clones were selected for further liquid phase screening, which could greatly

reduce screening time and consumption of assay mix. The re-legation removal and solid phase pre-screening are methods to generate high quality of epPCR libraries.

4.4.1 Glucose oxidase detection assay (GODA)

For validating the GODA protocol we decided to improve the enzyme activity of GOx toward β-D-glucose. GOx variants with increased activity will improve power output in mBCs and allow a validation of GODA by hydrogen peroxide determination with the well-known ABTS assay (Sun et al., 2001). In contrast to the ABTS assay the GODA detects the product D-gluconolactone and allows improving bioelectrochemical properties in which GOx is not reoxidized by molecular oxygen as in biofuel cells. The product detection in GODA should further prevent directed evolution of GOx into an uncoupled hydrogen peroxide producer. Using surrogate substrates and conditions not close to application conditions resulted in bitter lessons that we learned in many directed protein evolution experiments (Woodyer et al., 2004).

GODA is a medium-throughput screening system with a SD of 10.7 % and detection limit of 0.5-1 mM D-gluconolactone under assay conditions in microtiter plates. SDs around 11 % have successfully been used for the directed evolution of monooxygenases, for example toward aromatic and heterocyclic compounds (Wong et al., 2005) and improved organic solvent resistance (Wong et al., 2004a). The reaction principle of the GODA assay is based on coupled enzyme reactions (Mollering and Bergmeyer, 1967) resulting in the formation of NADPH which is monitored at 340 nm. The respectively high sensitivity and low background is achieved due to secretion of GOx into the cultivation broth in which NADPH consuming side-reactions are minimized. GODA was

validated by improving beyond statistical errors the activity of GOx; mutant I115V: (V_{max} (β-D-glucose) 10.81 µmol min^{-1} mg^{-1} compared to 7.94 µmol min^{-1} mg^{-1} of the wild-type; differences between K_m values are below 2 mM. Additionally, the improved activity of mutant I115V was validated by oxygen kinetics, showing that the maximal oxygen consumption rates (V_{max}) have been improved by ~1.5 folds. Amino acid position 115 is in close proximity to the catalytic FAD centers of the GOx dimer and additional studies are in progress to understand the activity modulating role of this residue.

Similar like GODA, another enzymatic assay to detect D-gluconate which is hydrolysis product of D-gluconolacone will be an alternative to detect activity of GOx.

$$\text{D-gluconate + Oxidized DCPIP} \xrightarrow{\text{GDH}} \text{2-Dehydro-D-Glconate + Reduced DCPIP} \quad (6)$$

GDH: Gluconate dehydrogenase, not commercial available.

4.4.2 Ferrocenemethanol based assay

Ferrocenemethanol assay is a colorimetric medium throughput screening assay in 96-well format. Choosing a right mediator is certainly important to achieve high sensitivity and selectivity. The artificial electron acceptors have to satisfy the following requirement:

1) Mediators with an appropriate redox potential enables to oxidize reduced form FAD (E^0=-219 mV Ag/AgCl). Redox potential can not be too high in order to avoid any interferences in the supernatant.
2) Mediators are stable in reduced and oxidized forms.
3) The spectral properties of redox form are not pH dependent.
4) An obvious color change will be observed during the redox reaction.

5) A high reaction rate is required during half reductive reaction for electron transfer between mediator and reduced form GOx, and to minimize competition with dioxygen.

Ferrocenemethaol satisfy most of the criteria mentioned above. It was selected after an intensive mediator screening since ferrocenemethanol (ox) shows fast reaction kinetics with GOx at pH 8 and ferrocenemethanol (red) shows a “stable” color change during the time course of screening with negligible reoxidation by molecular oxygen. fm-GODA has, in contrast to GODA , been developed for improving mediated electron transfer properties. Compared with GODA, ferrocenemethanol based assay reflects screening conditions for bioelectrocatalytical applications that might lead to different solutions in directed evolution experiments. For the mediator ferrocencemethanol, one could expect novel binding sites or novel channels/openings in the isolating protein shell that speed up electron transfer rates between GOx and ferrocenemethanol (ox).

Based on E4 mutant, additional 2000 clones were screened by ferrocenemethanol assay for improving thermostability and activity. Coefficient of variation (CV) is a main performance criterion of a medium-throughput-screening. Screening systems with CV less than 20 % have been successful used for the directed evolution (Wong et al., 2005). Ferrocenemethanol based assay is a colorimetric medium-throughput screening system with a CV of 16 % and detection range of 0-700 µM of ferrocenemethanol under assay conditions in a 96 microplate. Ferrocenemethanol assay was also validated by standard hydrogen peroxide detection (ABTS assay).

Ferrocenemethaol assay can even be used to detect pyranose oxidase (POx) activity of lysed cell extracts of recombinant *E. coli* expressing POx. The ferrocenemethanol assay system has therefore the potential to screen FAD containing oxidases.

SUMMARY AND PERSPECTIVES

The objectives were to improve properties of GOx for its putative use in the anodic compartment of miniaturized biofuel cells (mBC). The activity of GOx for β-D-glucose conversion has been improved (k_{cat} (WT) 69.74 sec^{-1}; k_{cat} (Mutant) 137.74 sec^{-1}), an oxygen independent GOx assay has been developed for improving electron transfer properties, and thermostability has been enhanced (T_{50} (WT) 53°C; T_{50} (Mutant) 56°C).

Glucose oxidase detection assay (GODA) and ferroecenemethanol based assay were designed for screening catalytic activity and thermostability. GODA has been published and couple product detection to NADPH formation which is recorded at 340 nm (Zhu et al. 2006). The main advantage of validated GODA is that it detects the production (D-gluconolactone) instead of the side-product (hydrogen peroxide). Product based detection enables to avoid a common pitfall in directed evolution (‘you get what you screen for’) and evolve GOx variants that are peroxide producers. Ferrocenemethanol detection system is an oxygen independent GOx assay at pH 8, which can directly screen for mediated electron transfer. Compared to other artificial mediators, ferrocenemethanol is well behaved electrochemically and exhibits rapid kinetics for enzyme oxidation. Both assays are novel medium-throughput screening systems with SD (GODA 11.7 % Ferrocenemethanol based assay 16 %) and have been compared to the hydrogen peroxide detection ABTS assay. Ferrocenemethanol based assay can further be to detect pyranose oxidase (POx) activity, which will be an alternative to the GOx in use in the anodic compartment of mBC.

In the long run, the GOx technology platform will lead to biofuel cells that can power implantable device such as pacemaker and insulin pump. The two novel screening assays, in this regard, represent a breakthrough to evolve GOx for biofuel cell applications by directed evolution. All improved mutants from directed evolution will in the future be used to elucidate the molecular basis of enzyme for efficient electron transfer and broaden our knowledge in the field of bioelectrocatalysts.

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DECLARATION

I declare that this dissertation is my own account of my research and contains and the results have not previously published by any other person.

Signature: Ziwei Zhu

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