Design of Enzymatic Systems for Redox Reactions

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Abstract

Oxidases are enzymes capable of carrying out redox reactions involving molecular oxygen and have huge industrial potential. Their applications usually involve additional agents that can prevent deactivation of enzyme and recover the final product. Tyrosinase and Glucose oxidase (GOX) are two such oxidases, capable of using molecular oxygen and oxidising monophenols such as p-cresol, and glucose, respectively to 4-methyl-quinone and gluconic acid. The o-quinone, so formed can function as electron acceptor for GOX, thereby reducing poisoning of the enzyme and also converting it to catechol derivative. The present work, therefore, attempts to integrate the two enzymes and their reactions resulting in 4-methyl catechol and gluconic acid as end-products. The methodology involves study of immobilized GOX for conversion of glucose in oxygen saturated and oxygen-depleted p-benzoquinone solutions, followed by studies with 4-methyl orthobenzoquinone and finally integration into the tyrosinase reaction system. Immobilized on suitable resin, GOX shows 80% conversion of glucose in oxygen-depleted benzoquinone solutions with gluconic acid and hydroquinone as products, both in batch and column modes. Studies are being carried out to extend the system to other catechol.

Keywords: Oxidases, Tyrosinase, Glucose Oxidase, p-cresol, glucose, gluconic acid, catechol, p-benzoquinone, Immobilization.

1. Introduction

Oxidoreductases comprise the large class of enzymes that catalyze biological oxidation/reduction reactions. Because many chemical and biochemical transformations involve oxidation/reduction processes, developing practical biocatalytic applications of oxidoreductases has long been an important goal in biotechnology. Oxidoreductases (Redox enzymes) catalyze the transfer of electron from one molecule to another.

$A^- + B \rightarrow A + B^-$... where, A is the reductant and B is the oxidant.

The analytical, food, and environmental applications are the only miniscule markets with a significant oxidoreductase commercialization. Innovative efforts at cost-competitiveness and overriding issues of co-factor regenerations and inhibition for existing oxidoreductase applications will fuel the future growth of industrial oxidoreductase biocatalysts. In the following work, we attempt to address the issue of inhibition and poisoning of the enzyme employing two oxidoreductases that can work in synchrony under optimized experimental conditions. Tyrosinase and Glucose oxidase are the two enzymes that have been used for this work.

Tyrosinase (monophenol monooxygenase EC 1.14.18), a bicupric oxygenase is widely distributed in nature and catalyses hydroxylation of monophenols[10] and oxidation of o-diphenols to o-quinones. This can be used for the conversion of p-cresol to 4-methyl catechol and further to benzoquinones. However, the second segment of the reaction is autocatalytic and in the presence of oxygen, leads to quinone polymerization reactions leading low recovery of 4-methyl catechol, as the product. Use of antioxidants as ascorbic acid helps in increasing the yields of 4-methylcatechol in the reaction system by further reduction of 4-methylorthobenzoquinone. However, ascorbic acid consumed in the reaction does not work in favor of the process economics.

Glucose oxidase (β-d-glucose: oxygen 1-oxidoreductase; EC 1.1.2.3.4) catalyzes the oxidation of β-d-glucose to gluconic acid, by utilizing molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide. Industrial applications of GOX have been limited due to the fact that hydrogen peroxide is reported to poison GOX and effectively reduce activity over the period of time. At smaller scales of applications this is counteracted by the enzyme catalase that decomposes hydrogen peroxide to water and oxygen, thus maintaining the GOX activity. However, this strategy does not seem to work at increased substrate concentrations as increased concentrations of localized H_2O_2 creates a poisonous microenvironment thereby deactivating GOX and rendering it ineffective. It has been reported that benzoquinones can function as the oxidizing substrate in place of molecular oxygen and catalyze the conversion of glucose to gluconic acid[1]. The advantages of such a system would be reduced poisoning of the enzyme and longer shelf-life associated with the process.

The issue of autocatalytic polymerization associated with benzoquinones for tyrosinase mediated reactions and poisoning of GOX due to oxygen can be resolved by integrating the two systems. This would ensure that quinones formed during the tyrosinase reaction is consumed by GOX and reduced to catechol, while GOX catalyses the conversion of glucose to gluconic acid without being poisoned by the H_2O_2 formed. The work presented in this paper therefore involved assessment of immobilized GOX to utilize benzoquinones and catalyze the conversion of glucose to gluconic acid and integrate the two immobilized enzyme systems for optimum yields of catechol.

2. Materials and Methods

2.1. Materials

The enzyme preparation GLUZYME BG10000 was procured from

Novozyme (Zytex, Mumbai). Glucose analysis was carried out using Eco-pack glucose obtained from Accurex Biomedical Pvt. Ltd. (Mumbai, India). Dextrose was purchased from HIMEDIA, Mumbai. Dinitrosalicylic acid (DNSA), for analysis of reducing sugars was procured from Sigma Aldrich Co., U.S.A. Bradford protein assay kit was purchased from Bio-Rad, U.S.A. Bovine serum albumin (BSA) fraction V was purchased from Hi-Media Laboratories Ltd., Mumbai. Diaion CR-20 was gift from Resindion Srl, Italy. All other chemicals used in the experimental work were of AR grades unless stated and purchased from Sisco Research Laboratories (SRL), Mumbai.

2.2 Analytical Methods

Dinitrosalysilic acid (DNSA) (Miller, 1959) and GOD-POD (ref) assay were used to estimate the amount of glucose in the reaction system. The protein content of the soluble enzyme was determined using Bradford's method (Bradford, 1976). SDS-PAGE was carried out to ascertain the enzyme purity after extraction for both GOX and Tyrosinase. Hydroquinone and Benzoquinone formed were monitored by HPLC analysis on UV (DAD) detector G1315D with C18, 5μ, RP, 25cm, Purospher Star (Merck) column at 25°C under gradient conditions.

p-Cresol and 4-Methylcatechol were analysed on a C18, 5μ, RP, 25cm, Purospher Star (Merck) column at 25°C. The elution was carried out under gradient conditions with 5mM H_2SO_4 (A) and 100% acetonitrile (B) at a flow rate of 1ml/min. The conversion yields were determined from peak areas of the substrate and product and expressed as percentage product formed with respect to the concentration of the substrate employed.

2.3 Experimental Methods

2.3.1 Immobilization of GOX

The immobilization of GOX involves two major steps; namely extraction of enzyme and coupling of enzyme to the matrix. Glucose oxidase was extracted from GLUZYME 10000BG powder using acetate buffer with pH 5.23. This yielded maximum recovery of active enzyme from the powder. SDS-PAGE gels were carried out to check the efficacy of extraction and also the purity of the extracted protein. Immobilization of the enzyme was further done on Diaion CR-20 pre-activated with glutaraldehyde. The activated matrix was contacted with 5 ml of enzyme stock solution and mixed on shaker at 30°C for 4 hours. The supernatant was then analyzed for unbound enzyme activity and protein content. The resultant preparation was then repeatedly washed with water to remove unbound protein and stored under refrigeration conditions till use.

2.3.2. Immobilization of Tyrosinase

Tyrosinase extracted from homogenized mushroom (Agaricus bisporus) with 10mM ascorbic acid in 100mM phosphate buffer (pH 7.0) was stored as crude enzyme at -60°C. SDS-PAGE gels were carried out to check the efficacy of extraction and also the purity of the extracted protein. This was then was immobilized on Sepabeads ECHA by covalent immobilization via glutaraldehyde. The activated matrix was contacted with 5 ml of enzyme stock solution and mixed on shaker at 30°C for 4 hours. The supernatant was then analyzed for unbound enzyme activity and protein content. The resultant preparation was then repeatedly washed with water to remove unbound protein and stored under refrigeration conditions till use.

2.3.3. Batch reactions for GOX

Conversion of glucose to gluconic acid was experimented and samples were analyzed for glucose by GOD-POD assay kit, DNSA analysis and HPLC for substrate and product. Varying concentrations of soluble and immobilized GOX were contacted with glucose (50mg/ml) and oxygen under different reaction conditions in a reaction volume of 40 ml. p-benzoquinone was used as the substrate to assess GOX activity in the presence of quinones. The reaction system was made oxygen-free by passing N_2 gas and 0.5 ml soluble enzyme (3 mg/ml) was reacted with p-benzoquinone (0.75gm) in molar ratio with glucose under different stirring conditions. pH optima for the reaction was assessed by preparing the glucose solution (12.5mg/ml) in the respective buffer and carrying out the reaction using soluble enzyme on orbital shaker at 180rpm and 30°C. Varying enzyme concentrations were also tested to find optimum enzyme concentration required for the reaction. Comparison of reactivity of GOX for glucose was carried out with catalase and p-benzoquinone. Glucose solutions (15mM) prepared in distilled water was reacted with soluble GOX, soluble GOX and soluble catalase enzyme and also with soluble GOX and p- benzoquinone 180rpm and 30°C. Samples withdrawn from the reactions were used to assess the progress of the reaction under the different conditions.

2.3.4. Packed Bed Reactor for GOx

5ml immobilized enzyme packed in 1cm diameter glass column was contacted with reaction mixture at a various flow rates. Samples from the column flow through were analyzed by GOD-POD kit and HPLC periodically. Immobilized enzyme was also used for evaluating conversion of p-benzoquinone to hydroquinone at 0.5ml/min flow rate and 100mM glucose and p-benzoquinone. Varying flow rates of substrate mixture (0.25ml/min, 0.5ml/min, 1.0ml/min, 1.5ml/min and 2.0ml/min) were tried on packed bed reactor with 100mM glucose and p-benzoquinone. Samples were collected periodically and were analyzed for hydroquinone and p-benzoquinone concentration using HPLC analysis.

2.3.5 Integration of GOX and Tyrosinase Reactions

Reactions were carried out with 100mM glucose, 10mM 4-methylcatechol and 10mM p-cresol in presence of soluble GOX (1.0ml) and Tyrosinase (0.5 ml) in 40ml reaction volume. Samples were collected periodically and analyzed for catechol, p-cresol and 4-methyl catechol conversion using HPLC analysis. Varying concentrations of hydrogen peroxide (1mM, 2.5mM and 5mM) were added to the reaction to evaluate tyrosinase activity in the presence of Hydrogen peroxide. The reaction system was flushed with nitrogen gas to remove traces of oxygen from the reaction. Tyrosinase immobilized on ECHA and immobilized GOX were packed in two separate columns and mixture of 1mM p-Cresol and 10mM glucose (pH 7.0) was passed through of them. Samples were collected periodically and p-Cresol and 4-methylcatechol were tested by HPLC analysis.

3. Results and Discussions

The prime purpose of the proposed work was to eliminate the use of catalase for catalyzing breakdown of H_2O_2 in case of GOX and the use of ascorbic acid as an antioxidant for tyrosinase mediated conversion of p-cresol to 4-methyl catechol. The work carried out involved the study of immobilized GOX for conversion of glucose in oxygen-saturated and oxygen-depleted p-benzoquinone solutions, followed by studies with 4-methyl orthobenzoquinone and finally integrating the GOX reaction with tyrosinase reaction system. The combined system would help design a process to produce gluconic acid and 4-methylcatechol simultaneously in an environmentally viable and cost-effective manner.

3.1. Conversion of Glucose using GOX

The conversion of glucose to gluconic acid via the formation of an intermediate lactone using GOX has been practiced commercially[6]. Conversions are very low for the soluble and immobilized enzymes under shaker/aerated conditions. This can be reasoned out as the oxygen concentrations are higher under these conditions, more of H_2O_2 gets formed and the enzyme is irreversibly damaged, leading to loss of activity. In reactions wherein, catalase is used to breakdown H_2O_2, 90% of glucose was converted to gluconic acid in two hours. The same results were observed for the immobilized preparation as well. Thus, conversion of glucose is clearly dependent on the removal of H_2O_2 and schemes to effectively remove this dependency or avoid the formation of H_2O_2 would be probable solutions to avoid enzyme denaturation and allow the reaction to proceed to completion. The use of alternate electron acceptors other than molecular oxygen would prevent the formation of H_2O_2 and prevent enzyme damage. Quinones have been reported to function as an electron acceptor for GOX in the place of molecular oxygen[4, 5]. These reports stress on the requirement of 'unusual' environment for the reaction to progress. This necessitated the assessment of conditions favorable for the conversion of glucose in the presence of quinones. The model electron acceptor selected for the reaction was p-benzoquinone. Reactions with p-benzoquinone in the presence of oxygen and in oxygen depleted conditions were carried out. In the presence of oxygen and p-benzoquinone, very low conversions for glucose were obtained. This was probably due to the preference of enzyme for oxygen as electron acceptor rather than p-benzoquinone. In presence of oxygen, the enzyme has higher affinity for oxygen and does not use p-benzoquinone, thereby necessitating the need for complete removal of oxygen from the reaction. This was achieved by flushing nitrogen into the reaction mixture before addition of the enzyme solution. With p-benzoquinone as electron acceptor, glucose showed up to 90% conversion, as compared to 30% conversion in the presence of oxygen. This highlights the fact that deoxygenated p-benzoquinone which gets converted to hydroquinone, avoids poisoning of glucose oxidase by hydrogen peroxide, and thus maintains the activity of the enzyme. All enzymatic reactions carried hereafter, therefore use deoxygenated p-benzoquinone to optimize the conditions required for reaction coupling.

3.3. Immobilization of Glucose Oxidase and SDS-PAGE Analysis

The commercial preparation Gluzyme BG10000 suffers from certain disadvantages and therefore it was necessary to reimmobilize the enzyme to commercially available solid supports using the protocol discussed in 2.3.2. The extracted soluble GOX, supernatant from immobilization step were analyzed for protein using the SDS-PAGE analysis (see Figure 3.1).

The supernatant from the immobilization reaction showed no protein

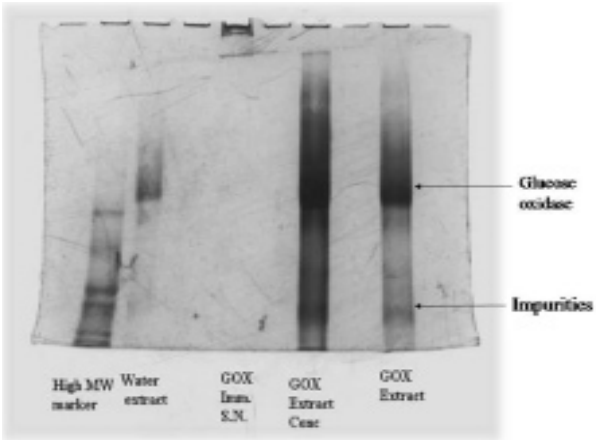

Figure 3.1 : SDS-PAGE Analysis for GOX

indicating complete immobilization of the protein to the adsorbent, which was verified further by estimation of residual protein content and also by enzyme activity. The potentiality of the immobilized preparation of GOX for the conversion of glucose to be used in conjugation with p-benzoquinone was also studied. The next array of experiments carried out were to optimize the different variable parameters in the reaction and to have an optimum concotion of substrate, enzyme, reaction environment.

3.4. Optimization of Reaction Conditions

Different parameters effecting the reaction were studied to find the optimum conditions for the reaction to proceed. Substrate concentrations of 0-200 mM of glucose were used in the reaction with fixed and varying concentrations of p-benzoquinone. For both the substrates, it was observed that 100mM of glucose and p-benzoquinone gave optimum results in terms of % conversion. Controls to assess the negative effect of benzoquinone and gluconic acid, on the enzyme were also carried out. It was observed that the enzyme was not affected by the presence of these chemical moieties. All these optimization studies have been done on soluble enzyme with deoxygenated substrate solutions. No significant changes were seen when the reaction was carried out in pH controlled systems from that carried out in distilled water. Hence, all further reactions were carried out using distilled water as diluting agent. It was observed that 0.7 ml enzyme volume was optimum for 15 mM of glucose concentration.

3.4.1 Effect of Benzoquinone on Reaction Conditions

As seen in figure 3.2, glucose gave 90% conversions in catalase and p-benzoquinone system, while glucose oxidase and oxygen system gave very low conversion up to 20%. The conversions in case of p-benzoquinone were faster and comparable to that of catalase system and so the proposed system was further optimized.

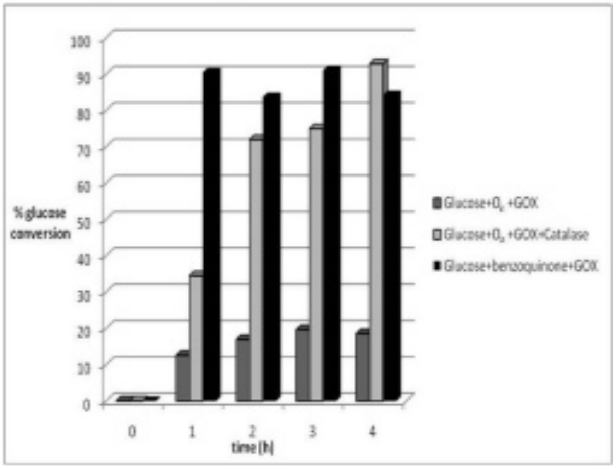

Figure 3.2 : Comparison of Reaction Conditions

3.5. Glucose Oxidase Reaction in Packed Bed Reactor

Immobilized glucose oxidase in packed bed reactor showed up to 90% constant conversion of benzoquinone till 4 hours at 0.5 ml/min with initial glucose and p-benzoquinone being 100 mM(see figure 3.3). The yields of hydroquinone were lower compared to that of benzoquinone conversions. This can be due to many reasons. The adsorbent used for immobilization when packed in a column caused either the substrate or the product to adsorb to itself, hereby reducing the amount of detectable hydroquinone. Further experiments need to be carried out to prove which of the two moieties adsorbed to the matrix and methods need to be optimized to reduce this adsorption.

3.6. Reusability of immobilized GOX enzyme

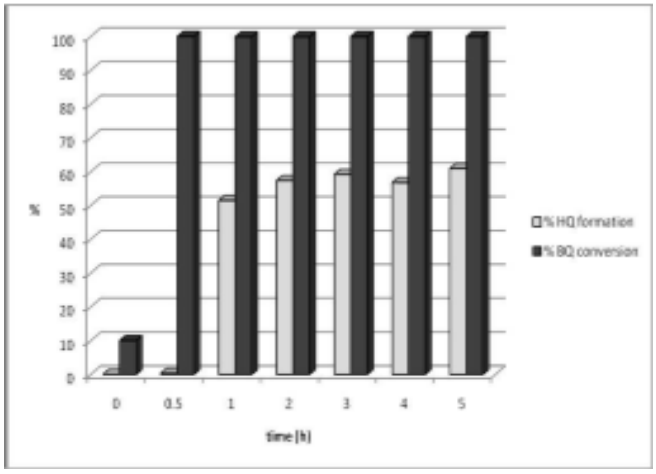

Figure 3.3 : Reaction in Packed Bed Reactor

p-benzoquinone was observed to get absorbed on the immobilized enzyme matrix. However, it was found that the product formation did not drop below 55% and remained almost constant for another 4 hours. This gave clear evidence that the enzyme immobilized adsorbent can be used for repeated continuous runs after the adsorption problem could be addressed.

3.8. Integration of GOX and Tyrosinase System

4-methylcatechol was not consumed significantly as in the absence of glucose-glucose oxidase system. This lead to two possibilities-

- ✓ Either 4-methylorthobenzoquinone formed from tyrosinase system was used as electron acceptor and reduced back to 4-methylcatechol or
- ✓ Tyrosinase activity was significantly reduced by glucose-glucose oxidase system product hydrogen peroxide which led to no consumption of 4-methylcatechol.

3.8.3 Packed Reactor Bed for Tyrosinase and GOX

As seen in figure 3.4, the packed bed reactor gave no formation of

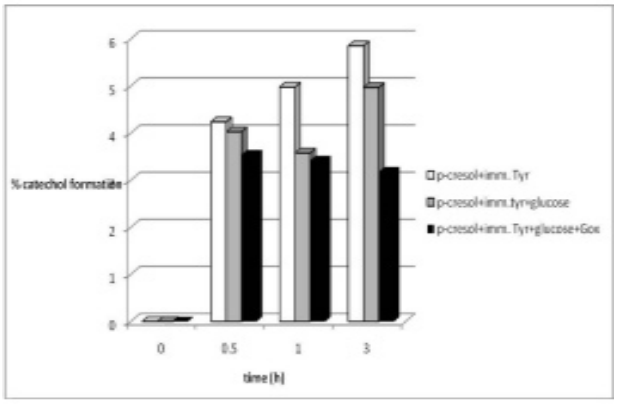

Figure 3.4 : p-Cresol in Integrated System with Immobilized Enzyme

4-methylcatechol.This was attributed to the fact that p-cresol was adsorbed on the ECHA resin which was seen from HPLC analysis. The presence of mild concentrations of oxygen in the reaction system would make the two reactions independent of each other. Such microenvironments would result in increasing concentrations of H_2O_2, thereby poisoning both the enzymes and resulting in loss of activity.

4. Conclusions

- ✓ In the absence of catalase, soluble and immobilized GOX gave low conversion of glucose to gluconic acid due to inhibition by hydrogen peroxide.
- ✓ p-benzoquinone can be used as a substitute to molecular oxygen and result in <90% glucose conversion.
- ✓ Soluble GOX and immobilized GOX, resulting in 70% hydroquinone formation with up to 90% glucose conversion, can be used successfully in combination with quinones. This system offers a very economic solution to overcome the harmful effects of hydrogen peroxide formed during reactions with GOX.
- ✓ Packed bed reactor of immobilized GOX can also be used continuously for eight hours and for at least two cycles. Issues of adsorption of substrate to immobilized enzyme matrix need to be addressed.
- ✓ Integration of tyrosinase and GOX system together in solution showed inhibition by hydrogen peroxide formed.

5. Comparison with Conventional Methods of Gluconic Acid and 4-methylcatechol Manufacture

- ✓ Conventional methods for Gluconic acid manufacture are as follows

1) Gluconates are advantageously produced from Glucose by electrolytic process.
2) Gluconic acid used in pharmaceutical industries is produced by the fermentation of glucose by strains of Aspergillus niger. Enzyme catalase is used to convert H_2O_2 to H_2O to avoid decrease in activity of GOX by H_2O_2.

✓ 4-methylcatechol is manufactured from p-cresol using enzyme Tyrosinase. Ascorbic acid is used as an antioxidant to convert 4-methylorthobenzoquinone back to 4-methylcatechol.

✓ Advantages of the Studied Method with Conventional Ones

1. The current method gives simultaneous production of both gluconic acid and 4-methylcatechol, eliminating the use of enzyme catalase and ascorbic acid.
2. Thus, the method becomes both cost-effective and environmental-friendly.

6. Future Scope

✓ Optimization of parameters for integrated p-Cresol-Tyrosinase and glucose-GOX system needs to be worked upon.
✓ Novel reactor designs to integrate both the systems need to be worked on.

7. Acknowledgment

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