

ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**A NOVEL METHOD FOR PRODUCTION OF NEW TEXTILE DYES VIA
LACCASE-CATALYZED OXIDATION**

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JUNE 2008

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**Date of submission: 5 May 2008
Date of defence examination: 11 June 2008**

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**LAKKAZ KATALİZLİ OKSİDASYON VASITASIYLA YENİ
TEKSTİL BOYALARININ ÜRETİMİ İÇİN YENİ BİR METOT**

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Tezin Enstitüye Verildiği Tarih : 5 Mayıs 2008

Tezin Savunulduğu Tarih : 11 Haziran 2008

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HAZİRAN 2008

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the enthusiastic supervision, guidance and support of Prof. Dr. Candan Tamerler.

I also thank to Dr. Mehmet Şener, Dr. Rezzan Karaarslan and Ismail Yakın (Setaş Kimya San. AS. Istanbul/Turkey) for the help with HPLC, industrial quality test of dyes and relevant technical discussions.

I would like to thank Wetlands Engineering S.P.R.L (Belgium) for performing toxicity tests for our dyes.

Special thanks to M.Sc. Koray Yesiladali for sharing his ideas and helping me as a labmate.

I also thank Abdullah Sert, Volkan Demir, Onur Ercan, Hasan Kahraman and Emrah Yelboga for their lovely friendship.

Finally, I am forever indebted to my family and Aslihan for their understanding, patience and encouragement.

This study is a part of ‘Novel sustainable bioprocesses for the European colour industries (SOPHIED) EU, FP6 Integrated Project’ and is funded by European Union

MAY 2008

Mustafa KAHRAMAN

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ABBREVIATIONS

ABTS	: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
Caco-2 cells	: Human intestinal cell line
DNA	: Deoxyribonucleic acid
HPLC	: High Performance Liquid Chromatography
ISO	: International Standard Organization
ITU22	: Colored solution obtained from S ₁ and S ₂ in the presences of laccase
LB Media	: Luria-Bertani Media
MS	: Mass Spectrometry
NMR	: Nuclear Magnetic Resonance
NR	: Neutral Red Dye
NRU	: Neutral Red Uptake
REACH	: Registration, Evaluation, Authorization and Restriction of Chemical substances
RTG-2 cells	: Fish cell line
S₁	: 2-Aminophenol-4-sulphonic acid, precursor I
S₂	: 3,4,5-Trihydroxybenzoic acid (Gallic acid), precursor II
TLC	: Thin Layer Chromatography
TvL	: <i>Trametes versicolor</i> Laccase
UV	: Ultra-violet

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LAKKAZ KATALİZLİ OKSİDASYON VASITASIYLA YENİ TEKSTİL BOYALARININ ÜRETİMİ İÇİN YENİ BİR METOT

ÖZET

Oksidatif enzimlerin sentetik kimyada kullanımına yönelik çalışmalar hem akademik hem de endüstriyel açıdan ilgi toplamaktadır. Lakkaz oksitleyici enzimler sınıfında olan içerdiği dört bakır molekülü sayesinde bir moleküler bataryayı andıran güçlü bir oksitleyici enzimdir. Lakkaz enzimi fenolik substratların oksidasyonunu ve aynı anda gerçekleşen moleküler oksijenin suya indirgenmesi reaksiyonunu katalizler. Reaksiyon sonucunda substratın çeşitli rezonans yapılarına ilişkin serbest radikaller ortaya çıkar. Daha sonra oluşan serbest radikaller ya diğer moleküllerle eşleşip kovalent bağ oluşturur ya da polimerleşme meydana gelir. Lakkazlar katalizledikleri reaksiyonu hem hızlandırdıkları hem de yan ürüne sebep olmadıkları için kimyasal oksidasyon katalizörlerine alternatif yeşil katalizördür.

Çalışmamızın temelini oluşturan, lakkaz katalizli oksidasyon vasıtasıyla tekstil boyalarının üretimi fikri tamamıyla yeni bir yaklaşımdır. Çalışmamızda, belirli kıstaslara uyularak seçilen on beş farklı substitütif fenolik prekürsörlerin elisa mikro-kuyucuklarında lakkaz için uygun koşullarda lakkaz oksidasyonu vasıtasıyla oksitlenmeleri sağlanarak bunun sonucunda oluşan fenolik serbest radikallerin birbiriyle veya kendi aralarında eşleşmesi sayesinde renkli maddelerin oluşturulmaları sağlandı. On beş prekürsör için ikili eşleşmeler ve kendi aralarında eşleşmeler dahil 121 mikro-kuyucuk kullanıldı. Mikro-kuyucuk reaksiyonları içerisinde uygun bulunan renklerdeki kuyucuk reaksiyon ekstraktları kontrol reaksiyonlarıyla da kıyaslanarak seçilmiştir. Lakkazın oksidasyon gücünü ve reaksiyondaki maddeleri eşleştirme yeteneğini mukayese edebilmek için başka bir kuvvetli oksitleyici madde olan hidrojen peroksit pozitif kontrol olarak kullanılmıştır. Reaksiyonun ürünleri ve ürün oluşum öngörülleri için, spektral analiz, ince tabaka kromatografisi ve HPLC yöntemleri kullanıldı. Sonraki adımda, renkli maddeler endüstriyel tekstil boya kalite testlerine ve toksikoloji testlerine tabi tutulması için erlen-mayer ölçeğinde üretimleri gerçekleştirildi. Endüstriyel tekstil boya kalite testleri Setaş Kimya San. AŞ. tarafından gerçekleştirildi. Sitotoksikoloji testleri ise Wetlands Engineering S.P.R.L (Belçika) tarafından yapıldı, Sentezlediğimiz maddelerden birçoğu bu testlerden başarı ile geçti, söz konusu bu maddelerin yeni tekstil boyaları olarak fikri ve ticari haklarının korunması için patent başvurusunda bulunulacaktır. Bu sebeple tez kapsamında, ITU22 adını verdiğimiz yalnızca bir boyanın sentez ve üretim çalışmaları anlatılmıştır. Tekstil boya kalitesi testleri sonunda, ITU22 ortalama bir kaliteye sahip olduğu belirlendi. Fakat Wetlands ITU22'nin sitotoksik olduğunu tespit etmiştir.

A NOVEL METHOD FOR PRODUCTION OF NEW TEXTILE DYES VIA LACCASE-CATALYZED OXIDATION

SUMMARY

The studies aiming at the use of oxidative enzymes as a biocatalyst in synthetic chemistry have attracted attention from academic and industrial area. Specifically, the ability of laccases to catalyze the oxidation (by O₂) of various substances is remarkable for synthesis of novel or existing chemicals. The laccases store four electrons on coppers which directly involved at active site. Thus, it can be called as ‘molecular battery’. In laccase-mediated oxidation, the substrate loses a single electron and forms a free radical. Substrates such as phenols form semi-quinone free radicals in this process. The unstable free radicals may undergo further laccase-catalyzed oxidation, coupling other phenolic structure or non-enzymatic reactions such as hydration and polymerization.

Production of novel textile dyes by laccase-catalyzed oxidative biocatalysis is a completely new approach in terms of biocatalysis technology. The idea of the use of laccase in oxidative biocatalysis aiming at coupling of substitute phenolic substances by laccase is the basics of our studies. For this purpose, fifteen dye precursors which commonly used in textile dyes production have been selected. Laccase-catalyzed micro-plate screening reactions were designed in order to find out color alteration in each micro-well which contains only two precursors of interest. At this stage, we expected that the oxidative biocatalytic reactions in wells result in coupling between two precursors and colorful extracts such as green, brown, orange, red and black. As a result of micro-plate screening reactions in the presence of laccase, we have obtained a few industrially-acceptable and non-toxic novel textile dyes. However, in order to protect proprietary rights of novel dyes, this thesis comprises only one of the micro-plate reactions and its dye, ITU22, catalyzed by laccase and analytical studies to be mentioned.

Thin layer chromatography, spectrum analysis and HPLC of the ITU22 were performed for determination of color contents and products analysis. The positive reactions in micro-plates were scaled-up at bench-scale and then antimicrobial activity tests were carried out. Finally, industrial dye quality tests and toxicity tests of selected colorful extracts were tested by Setas Kimya San. AS. (Turkey) and Wetlands Engineering S.P.R.L (Belgium), respectively. ITU22 has moderate quality in terms of industrial dye quality, however, it was determined as a cytotoxic dye for Caco-2 cells, while there was no cytotoxicity for RTG-2 cells.

1. INTRODUCTION

1.1. Current Industrial Dye Production and Dyestuff Market

A dye can basically be described as a colored substance that has an affinity to its substrate. Dyes can be classified concerning their chemical structure (azo dyes, anthraquinonic dyes, xanthene dyes, triphenylmethane dyes etc.), usage or application method (textile dyes, food dye etc.) [1]. Mauveine is known as the first synthetic dye which discovered by William Henry Perkin, an English chemist, in 1856 when marks the beginning of synthetic dyestuffs studies. As a result of Perkin's studies, the synthetic dye manufacturing industry was founded by Perkin in 1857 [2]. Therefore, the dyestuff industry was an important activity in European economic until the end of 20th century. However, dye stuff industries have shifted to the developing countries in last two decades, due to increasing labour and production costs in Europe.

Currently, dyes are synthesized in a reactor by means of conventional chemical catalysts. There are usually several steps in dye manufacturing process, including reactions in reactor, separation, drying and grinding [1]. The chemical synthesis step involves reactions such as sulfonation, halogenation, amination, diazotization, and coupling, followed by separation processes that may include distillation, precipitation, and crystallization.

In the recent years, dyestuff production in the world is kept around 1-1.5 million tons in per year. Dyes can be segmented by their usage for printing inks, plastics, textiles, paper and foodstuff. Textile segment is responsible for great part of the dye consumption [3]. Today, Asian countries are the major dyestuff producing countries in the industry. In terms of market share Europe is the leading producer due to its allegiance towards specialty products and the countries in Europe have remained the largest players owing to specialty products.

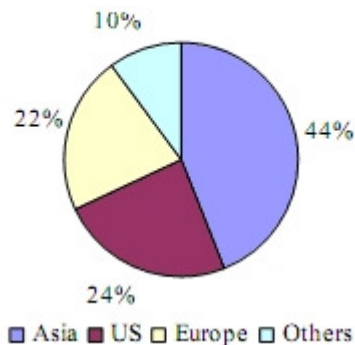


Figure 1. 1 Market share in dyestuffs industry [3]

Nowadays, European legislation implements a new system called REACH for Registration, Evaluation, and Authorization of Chemicals in Europe to ensure the protection of human health and environment. The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances [4]. It's known that some dye intermediates and dyes are toxic and the conventional dye synthesis pathways and the dying of fibres are not fully worker and environmentally friendly [5]. These processes have certain risk to living organisms and their environments. Hence, the regulation somehow is likely to affect on conventional dyestuff industry. We can clearly say that there is a need for alternative processes and products which are economic, non/less-toxic, environmentally and worker friendly.

1.2. A New Way to Synthetic Chemistry; Biotransformations and Biocatalysts

1.2.1. Historical Landmarks of Modern Biotechnology and Biocatalyst

Microorganisms have been of tremendous social and economic significance throughout the history of humankind. The humankind is known to have made fermented foods since Neolithic times, without being conscious of their existence. After observing microorganism and discovering microscopy in 16th century, scientists had the knowledge of fermentation of food and beverages. However, the production of chemicals by means of fermentation is relatively recent and the knowledge in the literature for fermentation process only appeared in the second half of the 19th century.

Table 1. 1 Dates and events in biotechnology and biocatalyst, period since 1944 [6]

SCIENTIFIC EVENT	TECHNICAL APPLICATION
1944 Avery et al.: Chemical nature of chromosomes: DNA	
1950 Chargaff: Rule of nucleotide ratios	
1953 Sanger: Sequence of insulin	
1953 Watson and Crick: Structure of DNA	1955 Industrial steroid transformation (prednisolone)
1955f Kornberg et al: Enzymatic DNA replication	
1955f Zamecnik and Hoagland: Amino acid activation, translation in protein synthesis	
1960/61 Jacob and Monod: Operon model of gene regulation; concept of mRNA	
1963 Merrifield: solid-phase protein synthesis	
1961-66 Nirenberg, Khorana et al.: Genetic code; first X ray enzyme structure	
1968 Arber and Linn: Restriction enzyme	1973 Industrial production of Amino Penicillanic Acid
1971f Nathans; Southern: DNA separation	
1972 Mertz, Davies: Recombinant DNA; Berg: First recombinant organism; Khorana: Oligonucleotide synthesis	
1973 Cohen, Boyer: Recombinant plasmid	1974 Glucose/fructose syrup
1975f Maxam and Gilbert; Sanger: Methods for DNA sequencing Köhler and Millstein: Monoclonal antibody	1976 Swanson, Boyer: Foundation of first biotech company: Genentech
Directed mutagenesis	
1975 Asilomar conference	
1977 Itakura et al. : Chemically synthesized gene	1978 Recombinant human insulin
1979 Mayer et al.: Recombinant penicillin acylase	1980 Chakrabarty : first patent for recombinant bacterium
1983f Frank and Blöcker ; Carruthers : mechanised DNA synthesis	1983: Schell, Montagu: First transgenic plant (tobacco)
	1984 Mechanised DNA sequencing
1988 Mullis: Polymerase chain reaction (PCR)	1988 Leder, Stewart: patent for transgenic mouse
1990: Start of Human Genome Project	
1994: First example for directed evolution of an enzyme (using DNA-shuffling)	1995 First complete bacterial genome sequence
1997 First cloned animal: Dolly	1996 Mass cultivation of recombinant seeds (commercial corn seeds)
1998 Argonne Structural Genomic Meeting	
	1999 Start of CELERA-industrial genome sequencing
	1999 Sequence of human chromosome-22
	1999 Vitamin C via whole cell biocatalysts
	2000 First approximate version of human genome

The first optically active substance industrially produced by fermentation was probably lactic acid [7]. After discovering of DNA in 1940's, period of modern biotechnology has been started (Table 1. 1). Restriction enzymes and synthesis of oligonucleotids were found in about 1970's. These caused recombinant DNA technology by the help of constructing recombinant plasmids in the same years. By means of the recombinant DNA technology, insulin was the first commercialized recombinant product in 1976. In terms of biocatalyst, the first recombinant enzyme at an industrial scale (10 m³ fermenter) was produced by Boehringer Mannheim (Germany) Company in 1982 [6].

1.2.2. Biotransformations and Biocatalysts

Life depends on a well-designed series of chemical reactions. However, many of these reactions proceed too slowly to sustain life on their own. Protein based catalysts, which referred as enzymes or biocatalyst, greatly accelerate the rate of these chemical reactions. Nowadays, their catalytic potential is also of great importance for the industrial purposes. Biotransformations have become a well-known tool specifically in fine chemical industry since the mid-1970s [8]. Biocatalytic systems which include crude and purified enzymes as well as whole-cell systems can perform highly selective reactions under mild conditions. As the results of the recent advances in large scale DNA sequencing, structural biology, protein expression, high throughput screening, directed enzyme evolution, metabolic engineering and advance in process development, biocatalysis is becoming a transformational technology for chemical synthesis (Table 1.2.) [9]. The biocatalysts are widely used especially in synthesis and production of biologically active compounds in the agrochemical, polymer and pharmaceutical sectors. Biocatalysis has already been proven in many cases to overcome specific synthetic problems.

Advantage of biocatalysts;

Chemoselectivity; biocatalyst can act on single type of functional group.

Regioselectivity, stereoselectivity and enantioselectivity; complex 3D structure of an enzyme can only interact with one type of region of substrate [10].

Preventing or limiting the use of the hazardous reagents; biocatalysts can use the alternative reaction mechanism or solvents.

Impurities and by-products; because of the selectivity of biocatalysts in reaction, impurities and by-products can be minimized.

High turnover number; biocatalytic reactions usually display characteristically high turnover numbers [11].

Energy Efficiency; biocatalysts generally work at low temperatures and can be regenerated in biocatalytic system.

Easy product purification; heterogeneous reaction medium in biocatalysts provide easy product purification.

Table 1. 2 Examples of current catalyst and process development studies [12]

			Areas	Strategy
Advances in catalyst development			Directed Evolution	Error prone PCR , saturation mutagenesis, genes/family shuffling, staggered, extension mutator strains
			Metabolic engineering	Molecular breeding of strains
			Bioinformatics	Proteome analysis using 2D PAGE, Transcriptome analysis using DNA microarrays
			Screening for new biocatalyst	High-throughput screening
			Exploitation of microbial adaptation	Selection following various cultivation procedures
Advances in process development			Two-phase systems	Emulsion process, in situ product extraction
			<i>In vitro</i> redox reactions including regeneration techniques	Electrochemical NADH regeneration
				Direct enzyme regeneration

In order to design economically feasible biotransformation, there are some critical steps in the biocatalysis cycle [13] (Figure 1. 2). Firstly, the synthetic routes can be biocatalysis or a combination of chemistry and biocatalysis. The availability of starting materials, number of steps in route, scalability, development time, product quality, and down-stream processing should be taken into consideration [14]. The main catalyst in biotransformation is enzyme which can be wild-type or recombinant. One or more enzymes can be used and the whole cells can also be used instead of the use of the enzyme alone.

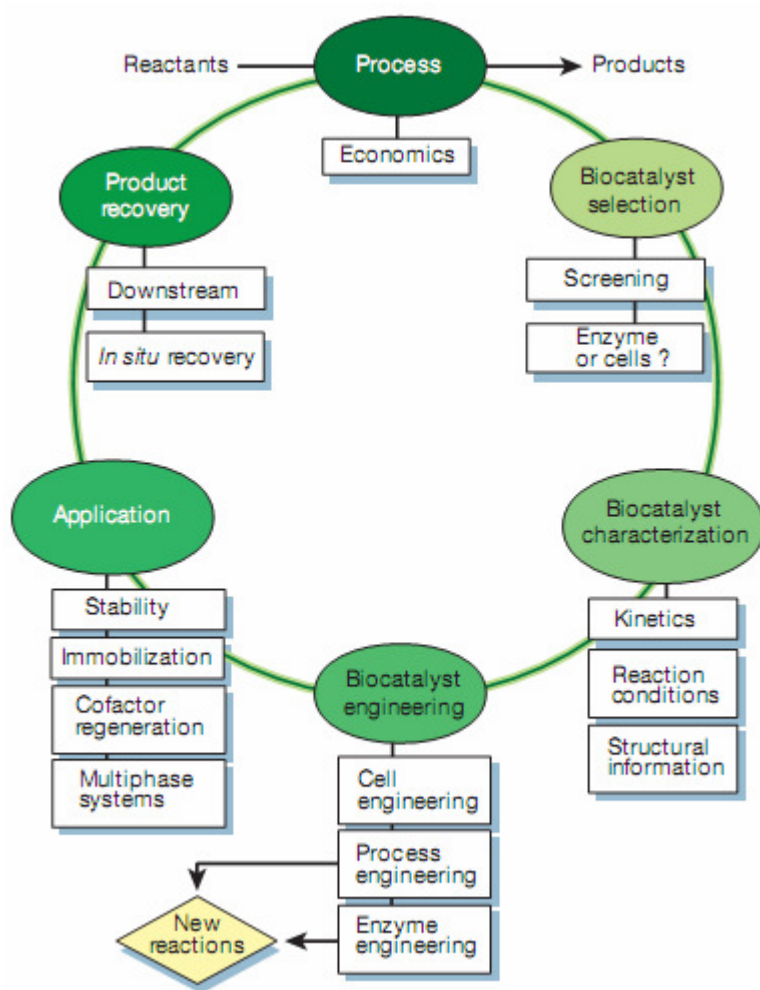


Figure 1. 2 The biocatalysis cycle [13]

1.2.3. Selective Biooxidation; Oxidative Biotransformation

Nowadays, biocatalyst technology in industry has been mainly limited to selective hydrolyses or ester/amide bond formation. Due to the need for expensive cofactors such as cofactor regeneration in biocatalytic system and low selectivity, stability and activities of biocatalyst, some important parts of synthetic chemical synthesis such as C-C bond formation and selective oxidation have still been remained inoperative by biocatalyst [15]. However, advance technologies in biotechnology promise great opportunities to overcome these problems. Nonetheless, there are some unsolved problems in oxidative synthetic chemistry such as uncontrolled reaction, predictability of the product structures and expensive oxidizers. Oxidative biocatalysts can also solve these unsolved problems. Oxidative enzymes can be classified according to the nature of the oxidizing substrate [16] (Figure 1. 3).

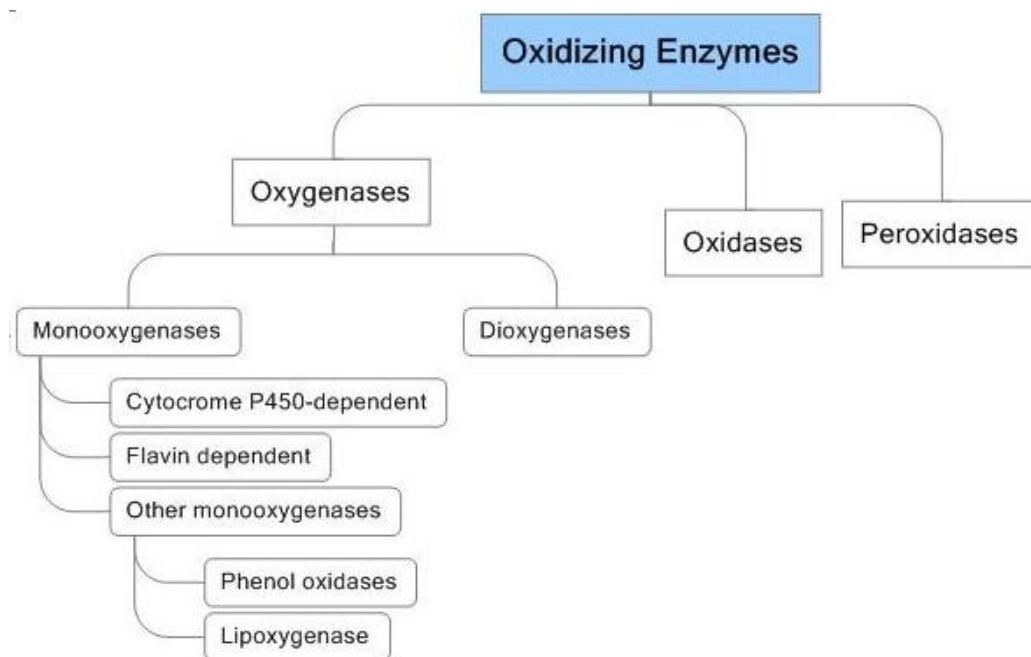


Figure 1. 3 Classification of oxidizing enzymes [16]

1.2.4. Current Trends and Future Prospects

There are some difficulties for the approval of biocatalyst in chemical synthesis such as;

- limited substrate specificity
- non-availability
- limited number of biocatalyst
- low stability
- requirement of cofactors

On the other hand, the impact of advances in technologies such as the recombinant DNA technology, bioprocess technology and biocatalyst screening techniques has played an important role to overcome mentioned difficulties and these technologies have resulted in the extension of the field of biocatalysis since the 1980's [12]. Aforementioned key technologies provide biocatalyst with altered structure, function, stability, availability and selectivity, and using the biocatalyst in non-aqueous environments. Application of enzymes in industry has been become widespread day by day [6] (Figure 1. 4). The use of the biocatalysts in organic and pharmaceutical chemistry for the synthesis purposes has been remarkable in recent years and it has gained much attention.

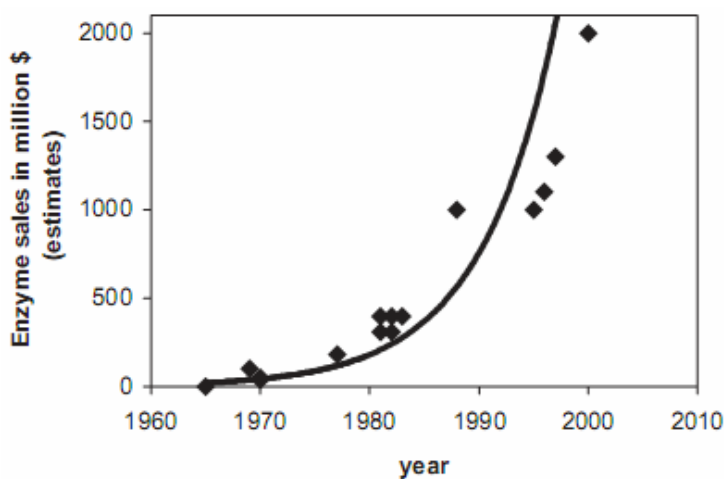


Figure 1. 4 The growth of biocatalysts market [6]

Table 1. 3 Some commercialized biocatalytic process [12], [17] and [13]

Product	Substrate	Reaction	Biocatalyst	Company
R-amide; S-amine	Rasemic Amines	Resolution	Lipase	BASF
(R)-2-(4'-Hydroxy-phenoxy) propionic acid	(R)-2-Phenoxy-propionic acid	Oxidation	Whole cells, Oxidase	BASF
Enantiopure D-amino acids	Racemic hydantoins	Dynamic resolution	In vico process; Hydantoinases, decarbamylases rasemase	Degussa
B-Lactam antibiotics	7-ADCA or &-APA and acid derivatives	Enzymatic hydrolysis/synthesis	Acylases	DSM
L-Aspartic acid	Fumaric acid	Enantio-selective synthesis	Ammonia lyase	DSM
5-Methylpyrazine-2-carboxylic acid	2,5-Dimethylpyrazine	Oxidation	Whole cells, xylene degradation pathway	Lonza
Niacinamide	Nicotinonitrile	Hydrolysis	Immobilized whole cells, nitrile hydratase	Lonza
7-ACA	Cephalosporin C	Fermentation	D-Amino acid oxidase and glutaryl amidase	Novartis
Various (S)-ester amides	Racemic aralactones	Dynamic resolution	Immobilized triacylglycerol acylhydrolase	Chiretech
Cacao Butter	Lipids	Hydratation	Lipase	Unilever
Acrylamide	Acrylonitrile	Hydratation	Nitrile hydratase	Nitto

Comparison of biological and chemical catalyst for novel process is an important issue. Jacobsen and Finney suggest the five criteria for comparison chemical catalysts

performance [18], these five criteria could be taken into account when comparison of biocatalyst and chemical catalyst;

1. Enantioselectivity of the product
2. Amount of product obtained per amount of catalyst consumed
3. Availability and costs of the catalyst
4. Substrate specificity (range of substrate)
5. Comparison of the method with alternative strategies

Biocatalysts present great opportunities and are likely to be the only solution for future economic sustainability. Academic and industrial efforts in biocatalyst discovery, design, development and implementation continue to decrease petroleum dependence and to eliminate environmentally destructive processes.

1.3. Laccase as a Green Chemistry Tool

1.3.1 Green Chemistry

Most process which concern the use of the chemicals and most substance which somehow contact with the people have the potential to cause a negative impact on the environment and human beings. Green chemistry philosophy was coined only 10 years ago to minimize risk by minimizing hazard [19]. The most widely accepted definition of green chemistry is “the design, development and implementation of chemical processes and products to reduce or eliminate substances hazardous to human health and the environment.”. This definition can be expanded into 12 principles [20];

1. It is better to prevent waste than to treat or clean up waste after it is formed.
2. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.

4. Chemical products should be designed to preserve efficiency of function while reducing toxicity.
5. The use of auxiliary substances (e.g. solvents, separation agents, etc) should be made unnecessary wherever possible and, innocuous when used.
6. Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
7. A raw material or feedstock should be renewable rather depleting wherever technically and economically practicable.
8. Unnecessary derivatisation (blocking group, protection/deprotection, and temporary modification of physical/chemical processes) should be avoided whenever possible.
9. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products.
11. Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents, including releases, explosions and fires.

1.3.2. Laccase

Laccases are oxidoreductases belong to the multinuclear copper-containing oxidase and contain four copper atoms per molecule. These can be secreted or intracellular and their physiological function is different in the various organisms but they all catalyze polymerization or depolymerization processes. Typical reaction of laccase is oxidation of a phenolic compound with the concurrent reduction of molecular oxygen to water.

After four cycle of single-electron oxidation forming free radicals, the enzyme reduces one molecule of oxygen, generating two molecules of water (Figure 1. 5). Three types of copper atom can be distinguished by their spectroscopic and paramagnetic properties: type 1 (T1), type 2 (T2) and type 3 (T3) (Figure) [21].

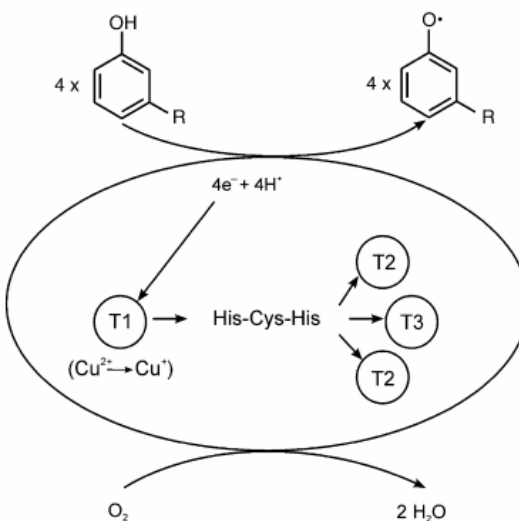


Figure 1. 5 An example of laccase action on substrate [22]

Laccases were detected in various bacteria, fungi, plants and insects and play an important role in many cellular and microbial activities such as radical-based mechanism of lignin formation in plants [23] and morphogenesis, fungal plant-pathogen/host interaction, stress defense and lignin degradation in fungi [24]. Extensive characterization of laccases has been carried out in the past decades. In general, a fungal laccase has a molecular mass of ~60-80 kDa and isoelectric point pI of ~4-7, depending on glycosylation [22]. The three-dimensional structure of a laccase from *Trametes versicolor* was elucidated by Choinowski *et al* (Figure 1. 6).

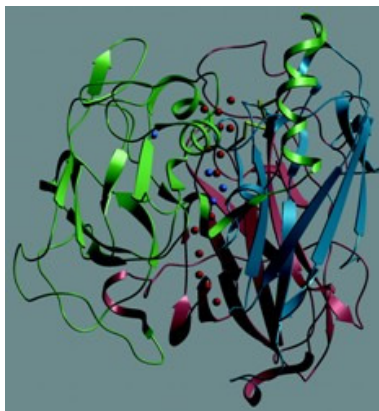


Figure 1. 6 Ribbon diagram of TvL showing the two channels leading to the T2/T3 cluster. Water molecules are depicted as red spheres, and copper ions are depicted as blue spheres [25].

A very wide range of substrate can be oxidized by laccases such as phenols, anilines, thiols, *N*-hydroxyls, *N*-oximes, phenazines, phenoxazines, phenothiazines and transition metal complex, etc [15]. However, the catalytic constant of laccase may be different for each type of substrate. Different laccase enzymes can also differ in their catalytic preferences [22].

1.3.2. Laccase Applications

The use of the enzymes in food, materials and chemical industries is major component of the green technology revolution. Nowadays, efficient and environmentally benign processes for industry has increased interest in laccase essentially ‘green’ catalysts, which work with air and produce water as the only by-product [26]. Laccases have a serious application potential in various industrial areas;

Food and Beverage Industry

- Enhancing and modifying the colour appearance of food or beverage
- Potential application of laccase; bioremediation, beverage processing, ascorbic acid determination, sugar beet pectin gelation, baking and as a biosensor [27]

Pulp and Paper Industry

- alternative to bleaching systems [28]
- modification of wood fibers and functionalizing lignocellulosic fibers [29]

Textile Industry

- Textile bleaching
- Removal of dyes from industrial effluents [30]

Diagnostics

- Enzyme linked immunoassays (EIA) [31]
- Biosensors and nanobiotechnology [32]

Other laccase applications

- Soil bioremediation [33]
- Synthetic chemistry [34] and cosmetics [35]

The major drawbacks of commercialization of laccases are the lack of enzyme stocks and the cost of redox mediators. To achieve overproduction of this biocatalyst and to obtain more robust and active enzymes, their modifications by chemical means and protein engineering have to be employed. Moreover, the development of an effective system for laccase immobilization is of great importance for the use of laccase in industry.

1.3.4. A Case Study of Biocatalysis

One of the most successful examples of biocatalytic production of a chemical is the conversion of acrylonitrile to acrylamide [36]. Acrylamide is an important monomer needed for the production of a range of economically useful polymeric materials and can be produced by the addition of water to acrylonitrile under the use of a reduced copper catalyst (Cu^+). However, the yield is poor, unwanted polymerization or conversion to acrylic acid may occur at the relatively high temperatures involved (80 -140°C) and the catalyst is difficult to regenerate. An alternative way to produce acrylamide by *Pseudomonas chloraphis* B 23 mutant strain as whole-cell biocatalysis was developed by Nitto Chemical Industries (now part of Mitsubishi Rayon Co., Ltd) [37]. Today, The company currently produces around 20 000 metric tons per year of acrylamide using a third-generation biocatalyst, *Rhodococcus rhodochrous* J1, which was first isolated by Kobayashi and Yamada [38]. Acrylamide is produced continuously from acrylonitrile at

10°C in a series of fixed-bed reactors using polyacrylamide-immobilized *Rhodococcus rhodochrous* J1 cells.

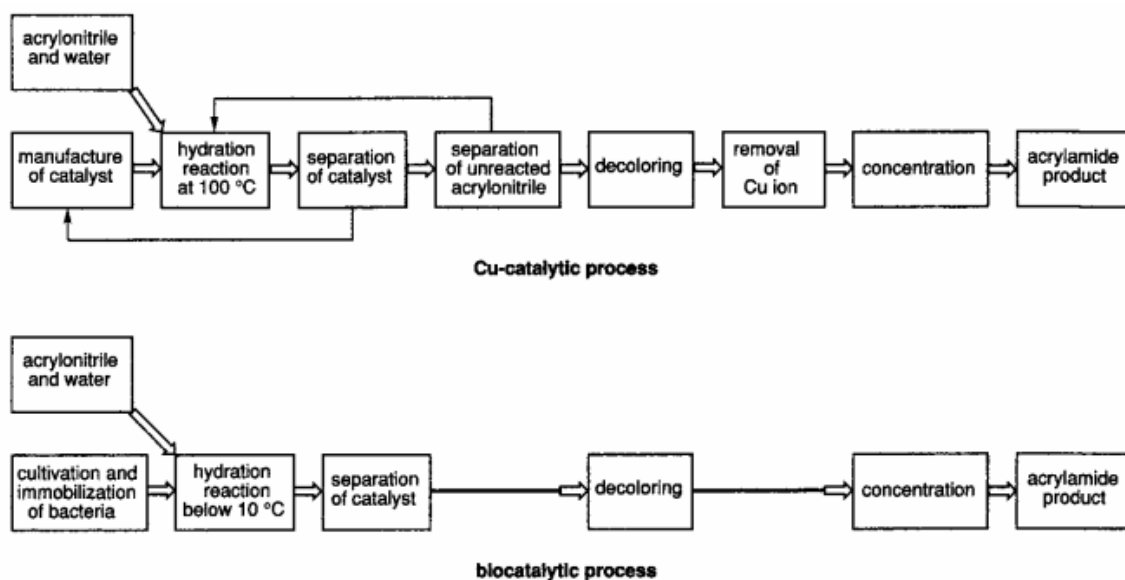
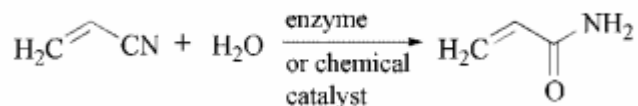


Figure 1. 7 Acrylamide production route by Cu-catalytic process and biocatalytic process [8]

The biocatalytic process may eliminate;

- heat and pressure
- heavy metal catalyst

The bioprocess also produces higher purity product and less wastewater than the chemical process [39]. The chemical and biocatalytic production processes for acrylamide are compared on Figure 1.7.

1.4. Production of Novel Textile Dyes by Laccase-catalyzed Oxidative Biocatalysis

The ability of laccases to catalyze the oxidation (by O_2) of various substances is remarkable for synthesis of novel or existing chemicals. The laccases store four electrons on coppers which directly involved at active site. Thus, it can be called as 'molecular battery'. In laccase-mediated oxidation, the substrate loses a single electron and forms a free radical. Substrates such as phenols form semi-quinone free radicals in this process. The unstable free radicals may undergo further laccase-catalyzed oxidation, coupling other phenolic structure or nonenzymatic reactions such as hydration and polymerization. One example of laccase-catalyzed reaction and possible products was showed Duc et al. (Figure 1.9) [40].

The redox potential of fungal laccase is independent of their species of origin and is in the range of 0.5–0.8 V. Nonetheless, horseradish peroxidase and lignin peroxidase are clearly stronger oxidant than the laccase. However, the laccase is relatively selective oxidant on phenolic substance in comparison to lignin peroxidase and horseradish peroxidase [41].

Production of novel textile dyes by laccase-catalyzed oxidative biocatalysis is a completely new approach in terms of biocatalysis technology. The idea of the use of laccase in oxidative biocatalysis and coupling of substitute phenolic substances by laccase is the basics of our studies. For this purpose, fifteen dye precursors which commonly used in textile dye production by means of chemical synthesis have been selected. Laccase-catalyzed micro-plate screening reactions were designed in order to find out color alteration in each micro-well which contains only two precursors of interest (Figure 1. 8). At this stage, we expected that the oxidative biocatalytic reactions in wells result in coupling between two precursors and give colored solutions such as green, brown, orange, red and black. Thin layer chromatography and spectrum analysis of extracts were used for determination of colors and products. To accurately determine formation of products, laccase oxidation effects on each precursor and understand the enzymatic reaction mechanism, HPLC analysis were employed. The positive selected reactions in micro-plates were scaled-up at bench-scale and then antimicrobial activity tests were carried out. Finally, industrial dye quality and toxicity of selected colored

extracts were tested by Setas Kimya San. AS. and Wetlands Engineering S.P.R.L. respectively. This thesis describes one of the micro-plate reactions catalyzed by laccase and aforementioned characterization studies.

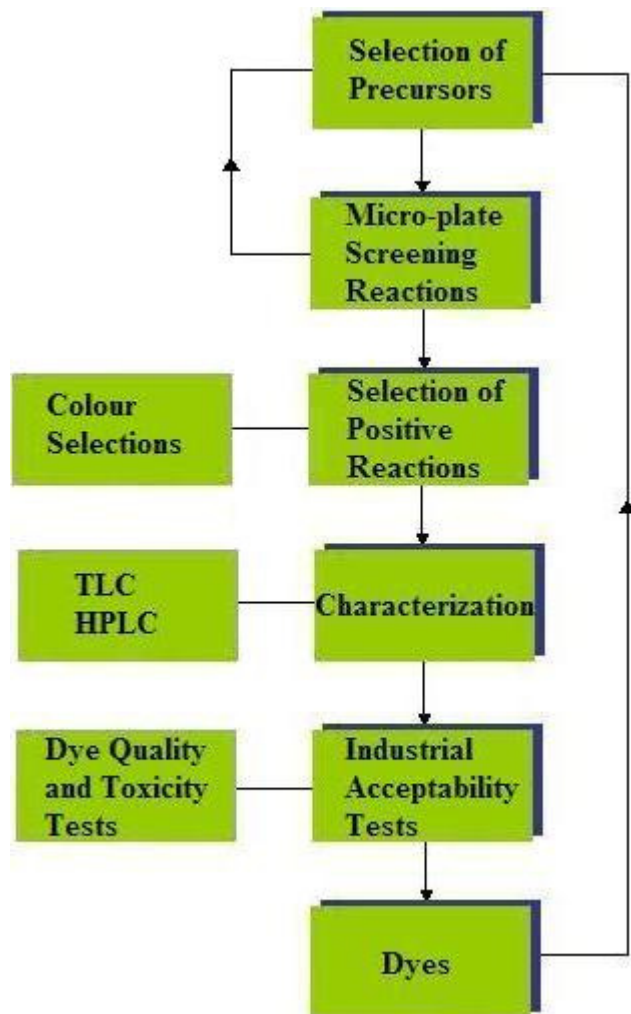


Figure 1. 8 The route for synthesis of novel textile dyes by laccase-catalyzed oxidation

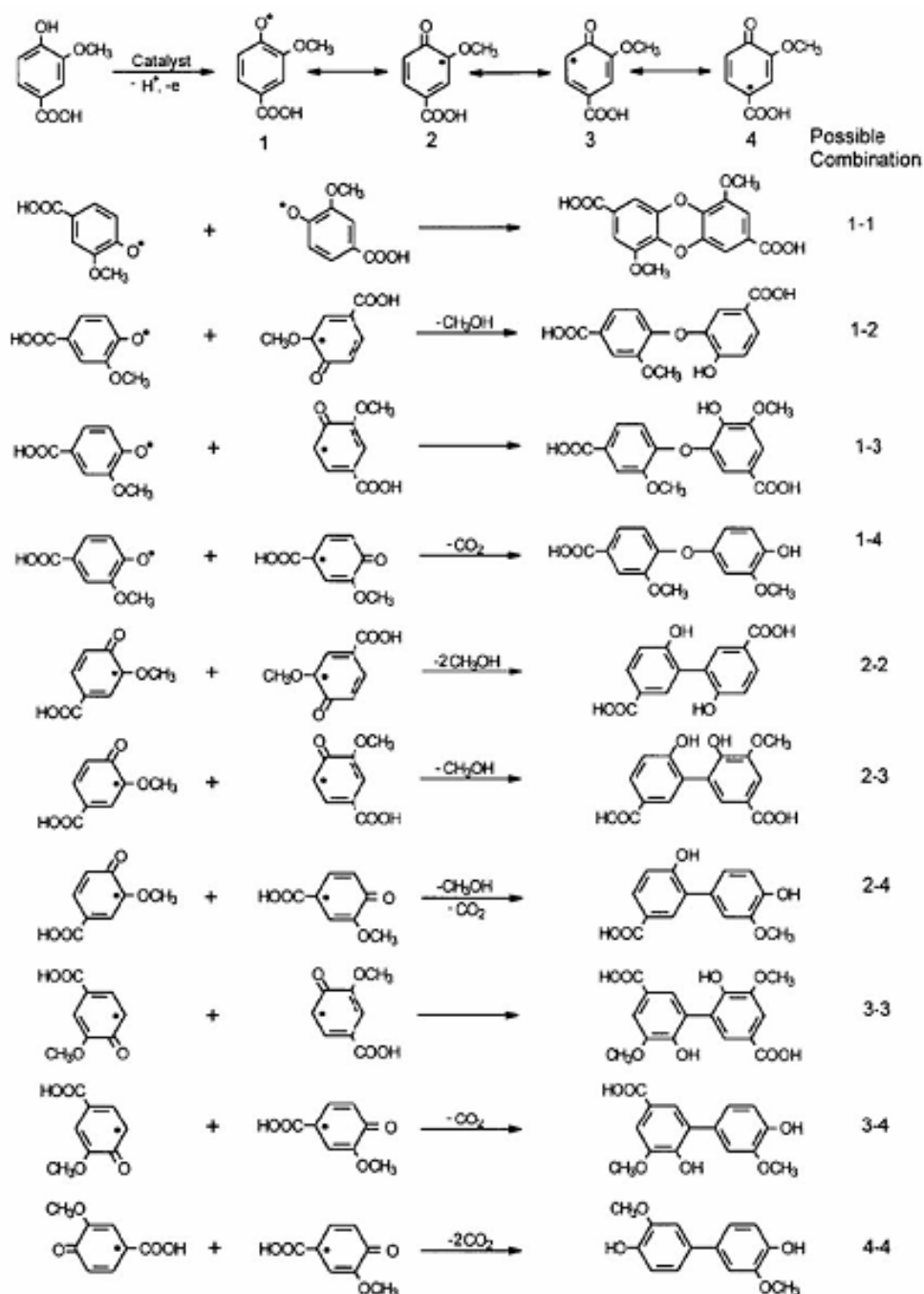


Figure 1. 9 Mechanism of oxidative coupling as referred to the four resonance forms of free radicals generated laccase-mediated oxidation of vanillic acid [40].

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Dye Precursors

2.1.1.1. 2-Aminophenol-4-sulphonic acid

2-Aminophenol-4-sulphonic acid is a well-known dye precursor. This substance was kindly provided by Setas Kimya San. AS. Molecular weight of 2-Aminophenol-4-sulphonic acid is 189.10 g/mol. It is named as S₁ in our studies.

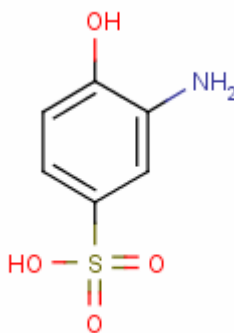


Figure 2. 1 Structure of 2-Aminophenol-4-sulphonic acid

2.1.1.2. 3,4,5-Trihydroxybenzoic acid (Gallic acid)

3,4,5-Trihydroxybenzoic acid is also well-known dye precursor. This substance was kindly provided by Setas Kimya San. AS. Molecular weight of gallic acid is 170.119 g/mol. It is named as S₂ in our studies.

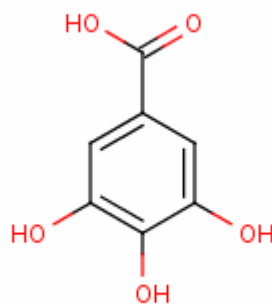


Figure 2. 2 Structure of Gallic acid

2.1.2. Enzymes

The laccase from *Trametes versicolor* was purchased from Fluka, Biochemika. It was used as an oxidative biocatalyst in our studies. The activity of *Trametes versicolor* laccase was about 27.5 U/mg. Optimum pH of TvL was 4.5.

2.1.3. Bacterial Culture Media

2.1.3.1. Mueller-Hinton Agar Medium

2 g meat infusion (Merck Co.), 17.5 g casein hydrolysate (Acumedia), 1.5 g starch (Merck Co.), and 13 g agar (Merck Co.) were dissolved in distilled water up to 1L and pH was adjusted to 7.0 with 10 M NaOH, then prepared medium was autoclaved at 121 °C throughout 15 minutes.

2.1.3.2. Luria-Bertani Media (LB Media)

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen) were dissolved in 1 L distilled water. Medium pH was adjusted to 7.0 with 10 M NaOH, then prepared medium was autoclaved at 121 °C for 15 minutes.

2.1.4. Silica Gel Plates

Thin layer chromatography plates (20 x 20 cm) were purchased from Merck (LuxPlate® Silica gel 60 F254). The plates have an UV indicator to monitor the substance.

2.1.5. Stock Solutions

2.1.5.1. Tartrate Buffer

100 mM 1 lt pH 4.5 tartrate buffer was prepared by dissolving 15.08 g tartaric acid (Merck Co.) in 1 L distilled water and pH was adjusted to 4.5 with NaOH.

2.1.6. Freshly Used Solution

2.1.6.1. TLC Eluent

For the TLC analysis, mixture of *n*-Butanol (Lab-Scan), acetone (Sigma), water, ammonia (Sigma) (5:5:1:2) was used as an eluent. 100 mL of eluent was freshly prepared for each TLC analysis.

2.1.6.2. Hydrogene Peroxide

Hydrogene peroxide (Sigma) was freshly prepared in each reaction.

2.1.6.3. Other Chemicals

Ethylene glycol (Merck) was used as a co-solvent in reaction.

2.1.7. Lab Equipments

Lab equipments are given in Appendix D.

2.2. Methods

2.2.1. Selection of Dye Precursors

Laccases are excellent oxidants of aromatic cycles substituted by electrodonating groups like diphenols (ortho and para), polyphenols, phenols substituted by methoxyl group. To achieve successfully biotransformation of precursors with laccase, selection of precursor is of great importance. Hence, the candidate precursors were selected considering the following limitations;

- easy to oxidation
- contain desired organic groups

- cheap and accessible
- existing textile dyes precursors

2.2.2. Laccase Activity Assay

The activity of laccase was determined by measuring the oxidation of 25 mM ABTS (2,2-azino-bis-(3-ethylbenzo thiazoline-6-sulfonic acid) at 414 nm ($\epsilon_{\max}=34219 \text{ M}^{-1} \text{ cm}^{-1}$) in 100 mM tartrate pH 4.5 at 25 °C into a stable cationic radical $\text{ABTS}^{\cdot+}$. The unit enzyme (U/L) activity was calculated by the formula given below using the slope of the spectrogram (Table 2.1.). One unit of laccase activity was defined as the amount of enzyme that oxidizes 1 μmol of ABTS per minute.

Table 2. 1 Formula for laccase activity measurement

$\text{Laccase (U/L)} = ((\Delta A/t) / \epsilon . d) . (1 \times 10^6 \mu\text{mol/mol}) . (V/v)$
ΔA = absorbance change at 414 nm – (dA/dt)
ϵ = extinction coefficient of ABTs at 414 nm – $3600 \text{ M}^{-1} . \text{cm}^{-1}$
d = light path of the cuvette container cell (cm) – 1 cm
V = total reaction volume (ml) – $1200 \mu\text{l}$
v = enzyme volume (ml) – <i>varies according to the dilution rate</i>

2.2.3. Improvement of Precursor Solubility

Solubility of precursors in the reaction medium was an important issue which has to be enhanced. Etylene glycol was added to the reaction mediums to increase the solubility. To determine ethylene glycol effect on laccase activity, medium A and medium B without ethylene glycol was prepared (Table 2.2). Both of the medium were incubated throughout 24 hours, 150 rpm, and at 25 C°. Enzymatic activity of both medium was detected throughout 24 hours. Laccase activity assays were performed by addition of 100 μL 5 mM ABTS solution into 1100 μL of medium A and B which incubated.

Table 2. 2 Medium A and B composition

	Laccase (mg)	Ethylene Glycol (mL)	Buffer (pH 4.5 Tartrate, mL)	Total Volume (mL)
Medium A	10	10	100	110
Medium B	10	-	110	110

2.2.4. Laccase-catalyzed Oxidative Biocatalysis in Micro-plate

S₁ was reacted at an equimolar ratio with S₂ in tartrate buffer (pH 4.5; 100mM) in the presence of laccase for 24 hours at room temperature in the volume of 200µl micro plate well. 96-wells plastic plate was used for this purpose. Reaction was carried out triplet in micro plate. To enhance the conversion rate, the plate was shaken at 100 rpm at 28° C. In addition to this main reaction (Medium I), the following reaction was carried out as control groups in the same plate;

Medium I : S₁ and S₂ in the presence of laccase

Medium II : control 1, S₁ and S₂ in the absence of laccase

Medium III : control 2, S₁ and S₂ in the presence of 1 M H₂O₂ as an oxidizer agent

Medium IV : control 3, S₁ and S₂ in the presence of 200 mM H₂O₂ as an oxidizer agent

Medium V : control 4, S₁ in the presence of laccase

Medium VI : control 5, S₂ in the presence of laccase

Medium VII : control 6, S₁ in the presence of 500 mM H₂O₂ as a oxidizer agent

Medium VIII : control 7, S₂ in the presence of 500 mM H₂O₂ as a oxidizer agent

Medium IX : control 8: S₁ in the absence of catalyst

Medium X : control 9: S₂ in the absence of catalyst

For enzymatic coupling reaction sets, reaction medium are shown below (Table 2.3).

Table 2. 3 Medium composition of coupling reaction sets

	Volumes for Enzymatic Reaction (μl)	Volumes for Blank Reaction (μl)
Dye Precursor 1 (25 mM)	16.6	16.6
Dye precursor 2 (25 mM)	16.6	16.6
Tartrate Buffer (100 mM pH 4.5)	158.4	166.8
Laccase (10 U/mL)	8.4	-
Total Volume	200	200

2.2.5. Bench Scale Production

ITU22, the product of laccase-catalyzed biooxidation reaction, was produced at bench scale in 100 ml of 500 ml shake flask. The flasks was shaken with 200 rpm, at 28 °C throughout 24 hours. The same reaction medium composition was used (Table 2. 4)

Table 2. 4 Laccase-catalyzed biooxidation medium

	Volume of Enzymatic Reaction (mL)	Volume of Blank Reaction (mL)
Dye Precursor 1, S ₁ (52.17 mM P1 in 47.92 mL 100 mM pH 4.5 tartrate buffer)	47.92	47.92
Dye precursor 2, S ₂ (52.17 mM P2 in 47.92 mL 100 mM pH 4.5 tartrate buffer)	47.92	47.92
100 mM pH 4.5 tartrate buffer	-	4.16
Laccase (10 U/mL)	4.16	-
Total Volume	100	100

2.2.6. Antibacterial Activity Test by Kirby-Bauer Method

One method that is used to determine antimicrobial susceptibility is the sensitivity disk method of Kirby-Bauer. The principle of the method is to determine effect of the substances on microbial growth by means of a rich agar.

1. *Preparation of Plates*; Mueller-Hinton agar and paper disks was prepared.
2. *Inoculation*; 10 µL of the dye extract was diffused over paper disks, same procedure was employed on the other precursors and dye extract without laccase. Then the papers were placed on a seeded Mueller-Hinton agar plate using a mechanical dispenser or sterile forceps.
3. *Incubation*; The plate is then incubated for 16 to 18 hours, and the diameter of the zone of inhibition around the disk is measured to the nearest millimeter.
4. *Measurement*; The inhibition zone diameter that is produced will indicate the susceptibility or resistance of a bacterium to the substance [42]

2.2.7. Spectrum Analysis of ITU22 by Visible Micro-Plate Reader

In order to determine color content, product formation and substrate consumption in laccase-catalyzed biooxidation reaction, visible spectra of the extracts in micro-plate were monitored by scanning at 340-800 nm.

2.2.8. Thin Layer Chromatography

Thin layer chromatography (TLC) is a chromatography technique used to separate chemical compounds. It involves a stationary phase consisting of a thin layer of adsorbent materials, usually silica gel, aluminum oxide, or cellulose immobilized onto a flat, inert carrier sheet. The principle of the methods is that the substances are separated by their differential migration caused by a mobile phase flowing through a porous, adsorptive medium. The following procedure was used to analysis of dye extracts;

1. *Preparation of Plates*; aluminum baked silica gel plate was heated at 50 C° for 5 minutes.
2. *Choosing Appropriate Eluent*; for the acid and basic dye classes, following eluent was recommended [43].
Eluent: n-Butanol, acetone, water, ammonia (5:5:1:2) [44].
3. *Sample Spotting*; the extracts included resulting extract, resulting extract without laccase, precursor I and precursor II were spotted onto the plate about 1 cm from the lower edge by capillary tubes.
4. *Development Chambers*; the eluent was added to the chamber and allowed to stand in the closed container for a few minutes before development, then the chromatograms were developed vertically in a glass chamber.
5. *Resolution*; the plate was eluted until good resolution was achieved.

2.2.9. HPLC Analysis of ITU22

To clearly understand formation of products and laccase-catalyzed oxidation effects on precursors, HPLC analysis was performed. Six samples analyzed by HPLC;

1. S₁ and S₂ in the presence of laccase
2. S₁ and S₂ in the absence of laccase
3. S₁ in the presence of laccase
4. S₂ in the presence of laccase
5. S₁ in the absence of laccase
6. S₂ in the absence of laccase

Preparation of sample;

- 5 mL sample dissolved in 50ml distilled hot water in 100 ml vol. flask
- Add 20 mL of Acetonitrile
- Adjust the volume to 100 ml with dH₂O

Column; Hicrom Nucleosil 100-7C18

Flow: 1,0 mL/dk

Mobil phase: Buffer:Acetonitrile (1:1)

2.2.10. Industrial Dye Quality Tests of ITU22

To find out industrial acceptability of ITU22, industrial dye quality tests of ITU22 were carried out by Setas Kimya San AS which is a dye producer company from Turkey. The dried ITU22 dissolved in appropriate solvents and concentration was adjusted at 5 % (w/v).

Multifibre dyeing test

Material: Nylon/EI

Dyeing : 120 °C, 40 min.

Chlorinated water fastness test

Material : Nylon/EI.

Dyeing : 120 °C

Test method: ISO 105 E03 (20 ppm active chlorine, 20 ±2 °C, 1 hours)

Determination of remaining dyestuff in the bath

Material: Nylon/EI

Dyeing : 120 °C, 40 min.

Washing fastness test

Material: Nylon/EI

Dyeing : 120 °C, 40 min.

Test method: ISO 105 C06 :A2S (40 ±2 °C, 30 min.)

Water fastness test

Material: Nylon/EI

Dyeing : 120 °C, 40 min.

Test method: ISO 105 E01 (37 ±2 °C, 4 hours)

Light fastness control test

Equipment: Megasol V2.00 light fastness machine

Method: ISO-105 B02 (normal conditions, 20 Hours)

2.2.11. Dye Cytotoxicity Tests of ITU22

The adverse effects resulting from interference with structure and/or processes essential for cell survival, proliferation, and/or function are referred to cytotoxicity [45]. A number of basal cytotoxicity endpoints can be used for this purpose. Neutral red is a weakly cationic water-soluble supravital dye which stains living cells. NR can readily diffuse through the plasma membrane and binds lysosomal matrix. When toxicants alter the cell surface or the lysosomal membrane, the NR retains. Therefore, the cytotoxicity can be quantified by means of neutral red uptake (NRU) [46]. Several types of cell cultures can be also used for this purpose. Caco-2 and RTG-2 cells were used for cytotoxicity of ITU22 by Wetlands Engineering S.P.R.L, a Belgian biotech company.

3. RESULTS AND DISCUSSION

3.1 Enhancement of Precursor Solubility

S₁ and S₂ up to 50 mM are well soluble in 100 mM of tartrate buffer (at pH 4.5). High soluble precursors are favorable, because of high product yield. There was need to improve substrates solubility. For this purposes, ethylene glycol was used as co-solvent in laccase activity assay to determine effects on laccase activity.

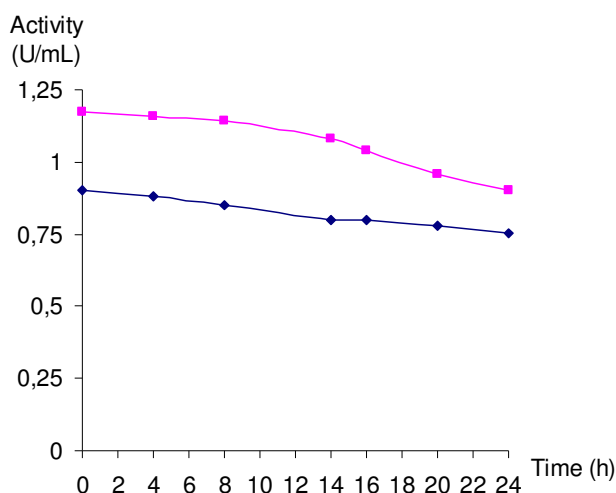


Figure 3. 1 Laccase activity throughout 24 hours in two different medium (-■- indicates medium B without ethylene glycol, and -◆- indicates medium A with ethylene glycol)

Incubation of laccase with medium A and medium B throughout 24 hours have resulted 23 % and 17 % activity losses, respectively (Figure 3.1). However, average laccase activity of medium A and medium B throughout 24 hours were 1.06 and 0.82, respectively. It is clear from this study that the use of ethylene glycol as co-solvent in the laccase mediated reaction is not favorable.

3.2. Production of Novel Textile Dyes by Laccase-catalyzed Oxidative Biocatalysis

3.2.1. Enzymatic Oxidative Micro-plate Reactions

Enzymatic oxidation and control reactions were carried out in micro-plate. Since each triplet wells contained different reaction medium, the wells on micro-plates were differently named as “I” to “X”. Enzymatic reactions resulted in highly dark colored extracts. To visually monitor the extracts, the extracts in the wells were diluted with distilled water (4, 16 and 160 fold) (Figure 3. 2). The red color which obtained from oxidation of S_1 and S_2 in well I by laccase-catalyzed oxidative biocatalysis was monitored at 4 fold dilution ratio. These extract was called as ITU22.

According to the results of micro-plate reaction, there was no visually monitored color difference in medium II micro wells, containing S_1 and S_2 (Figure 3. 2). On the contrary, the medium I, which contains S_1 and S_2 with laccase, had highly dark colored extract. The color alteration in the medium I resulted from laccase-catalyzed oxidation. Furthermore, 1 M of H_2O_2 in medium III resulted in a little color difference (Figure 3. 2). Therefore, laccase effect on S_1 and S_2 was clearly monitored. Medium V and VI which contain respectively S_1 and S_2 in the presence of laccase resulted in dark colored extracts (Figure 3. 2). These results indicate that individually S_1 and S_2 could be oxidized by laccase. Therefore, there are some possible products in these wells. Oxidation effect of laccase and possible products would be discussed after the thin layer chromatography, visible spectral analysis and HPLC results.

Medium numbers and their contents;

Medium I : S_1 and S_2 in the presence of laccase

Medium II : S_1 and S_2 in the absence of laccase

Medium III : S_1 and S_2 in the presence of 1 M H_2O_2 as a oxidizer agent

Medium IV : S_1 and S_2 in the presence of 200 mM H_2O_2 as a oxidizer agent

Medium V : S_1 in the presence of laccase

Medium VI : S_2 in the presence of laccase

Medium VII : S_1 in the presence of 500 mM H_2O_2 as a oxidizer agent

Medium VIII : S₂ in the presence of 500 mM H₂O₂ as a oxidizer agent

Medium IX : S₁ in the absence of catalyst

Medium X : S₂ in the absence of catalyst

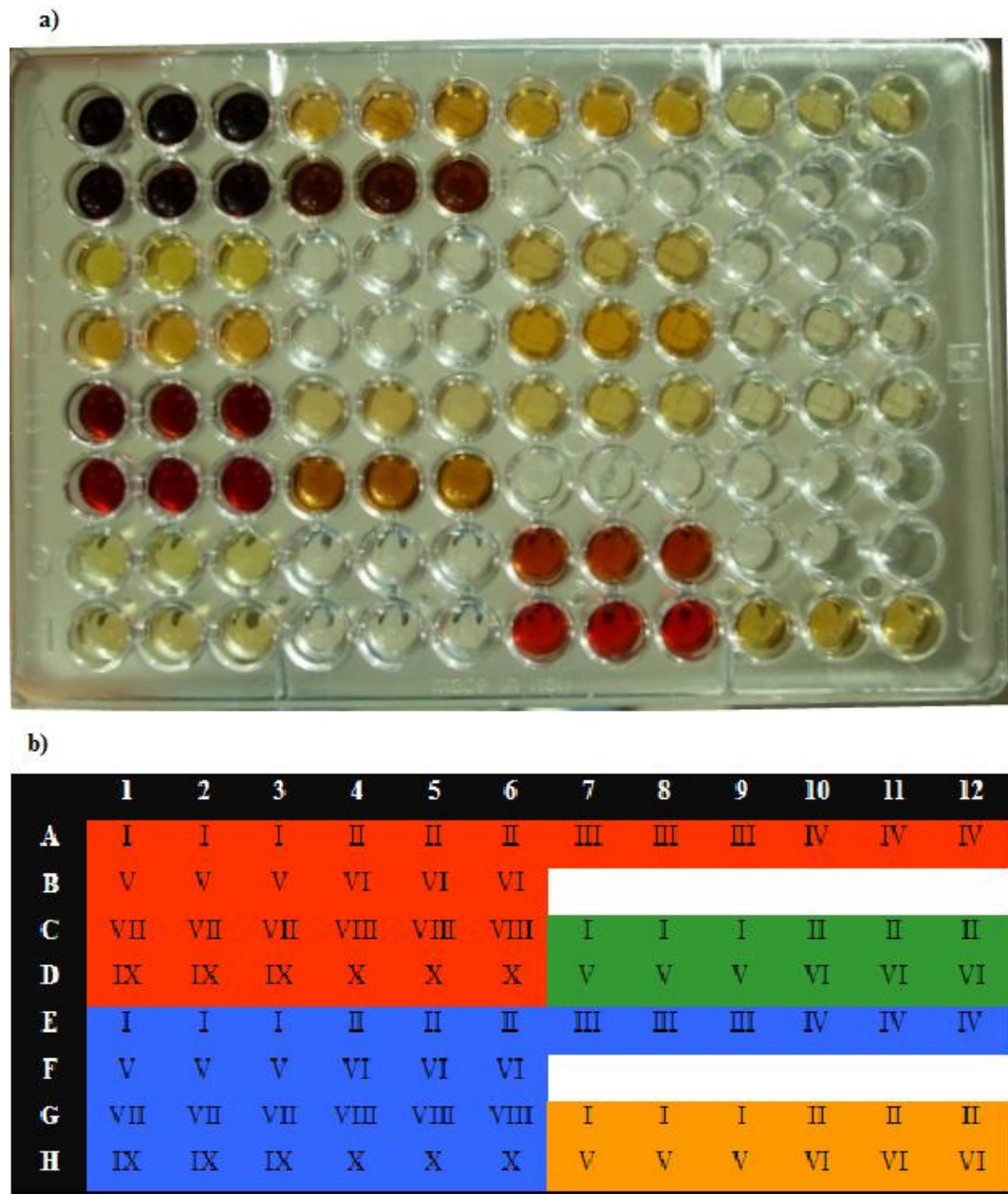


Figure 3. 2 a) shows the laccase catalyzed micro-plate reaction, b) indicates well numbers and colors, the red painted boxes indicate; no dilution, the blue painted boxes; 4 fold dilution, the orange painted boxes; 16 fold dilution, the green painted boxes; 160 fold dilution.

Visible spectrums of micro-plate reaction extracts were monitored. Medium I (S_1 and S_2 in the presence of laccase) has one peak at 410 nm and interference came from infrared section (Figure 3. 3 (b)). Medium II (S_1 and S_2 in the absence of laccase) has also one peak at 370 nm. These results indicate that the laccase is able to oxidize the medium I.

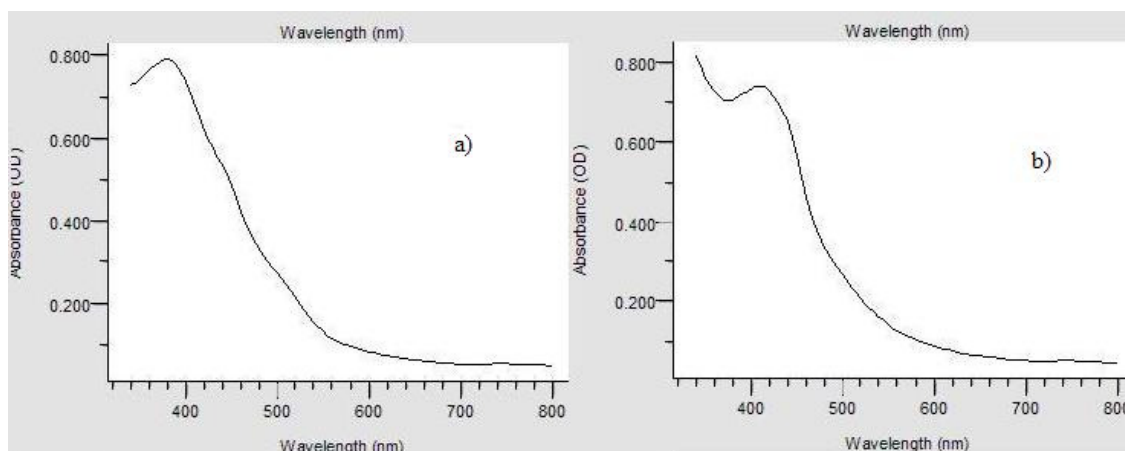


Figure 3. 3 (a) four fold diluted Medium II (b) 160 fold diluted Medium I

In the absence of catalyst, spectrums of individually S_1 and S_2 were measured (Figure 3.4).

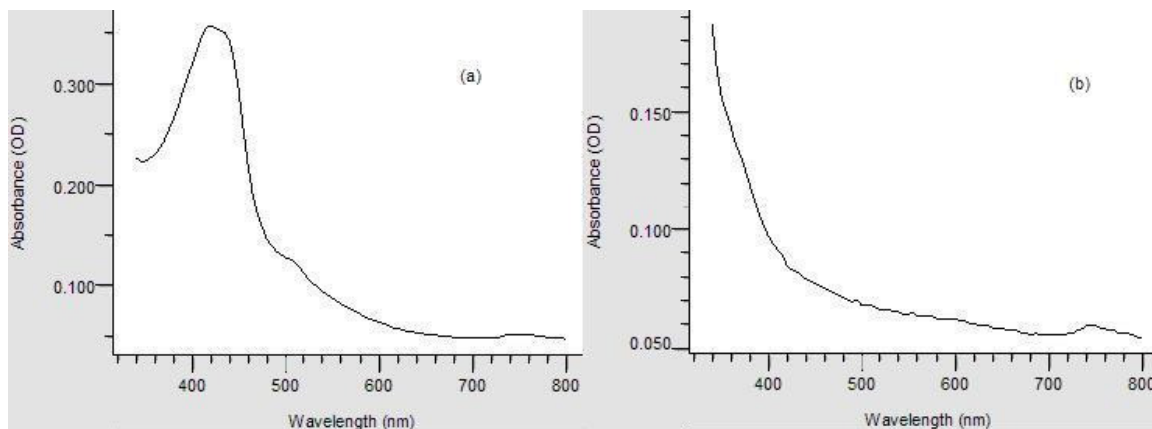


Figure 3. 4 spectrums of (a) four fold diluted Medium IX (b) four fold diluted Medium X

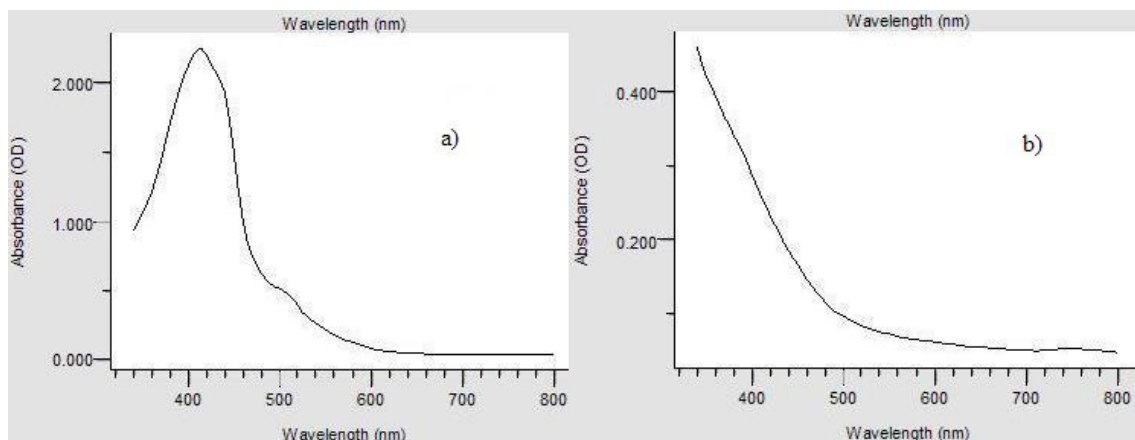


Figure 3. 5 (a) 160 fold diluted Medium V (b) four fold diluted Medium VI

There are small amount of spectrum differences when compared to Figure 3. 4 and Figure 3. 5. However, S_1 in the presence of laccase (medium V) was 40 fold diluted than the S_1 in the absence of laccase (medium IX). It indicates that laccase somehow has an oxidation effect on S_1 .

200 mM and 1 M of hydrogen peroxide were used as an oxidizing agent in control reactions (medium III and IV). 500 mM of hydrogen peroxide was also used as an oxidizing agent for individually S_1 and S_2 . In the visible spectrum results, there is no evidence for oxidation on precursors (Figure 3. 6).

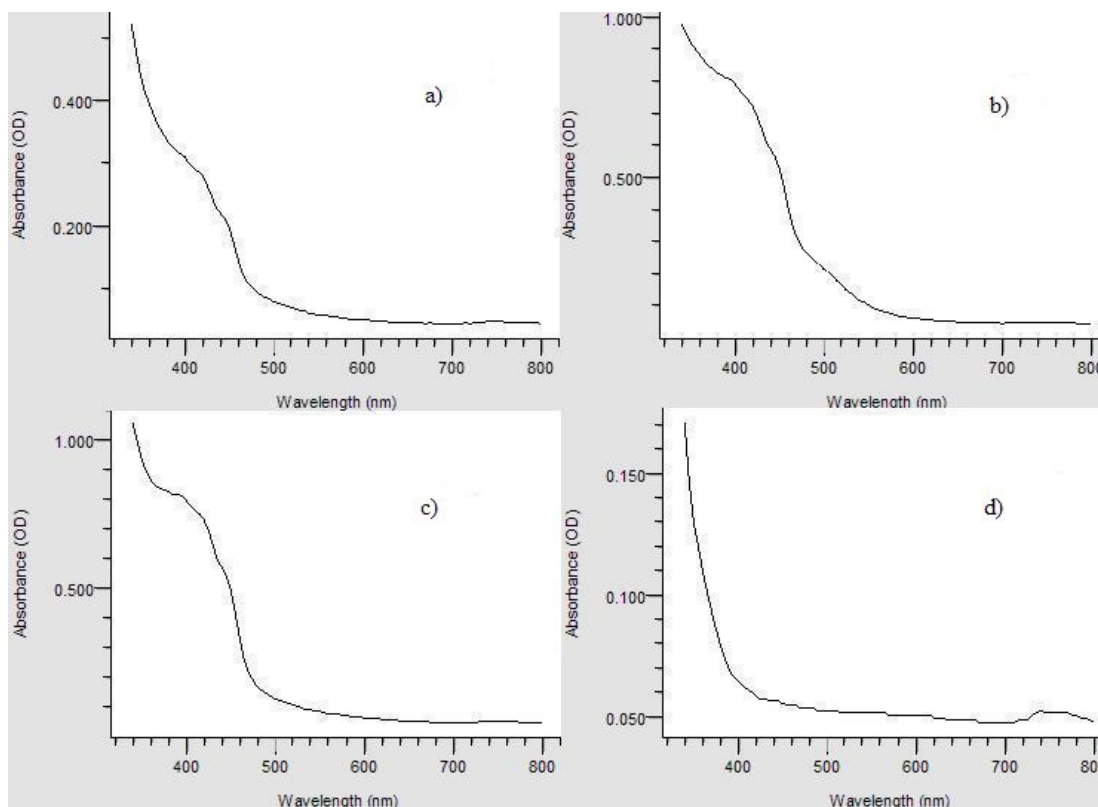


Figure 3. 6 (Continued) (g) four fold diluted Medium IV (h) four fold diluted Medium III (i) four fold diluted Medium VII (k) four fold diluted Medium VIII

In order to separate micro-plate reaction products, thin layer chromatography (TLC) with UV indicator probe was used. Appropriate eluent system was used for this purpose and the extracts were greatly separated by TLC. According to the result of TLC for Medium I (S_1 and S_2 in the presence of laccase), there were five substance in Medium I (Figure 3. 7) at least. However, there was interestingly no coupling between S_1 and S_2 by oxidation. The five substances were probably resulted from individual oxidation of S_1 and S_2 .

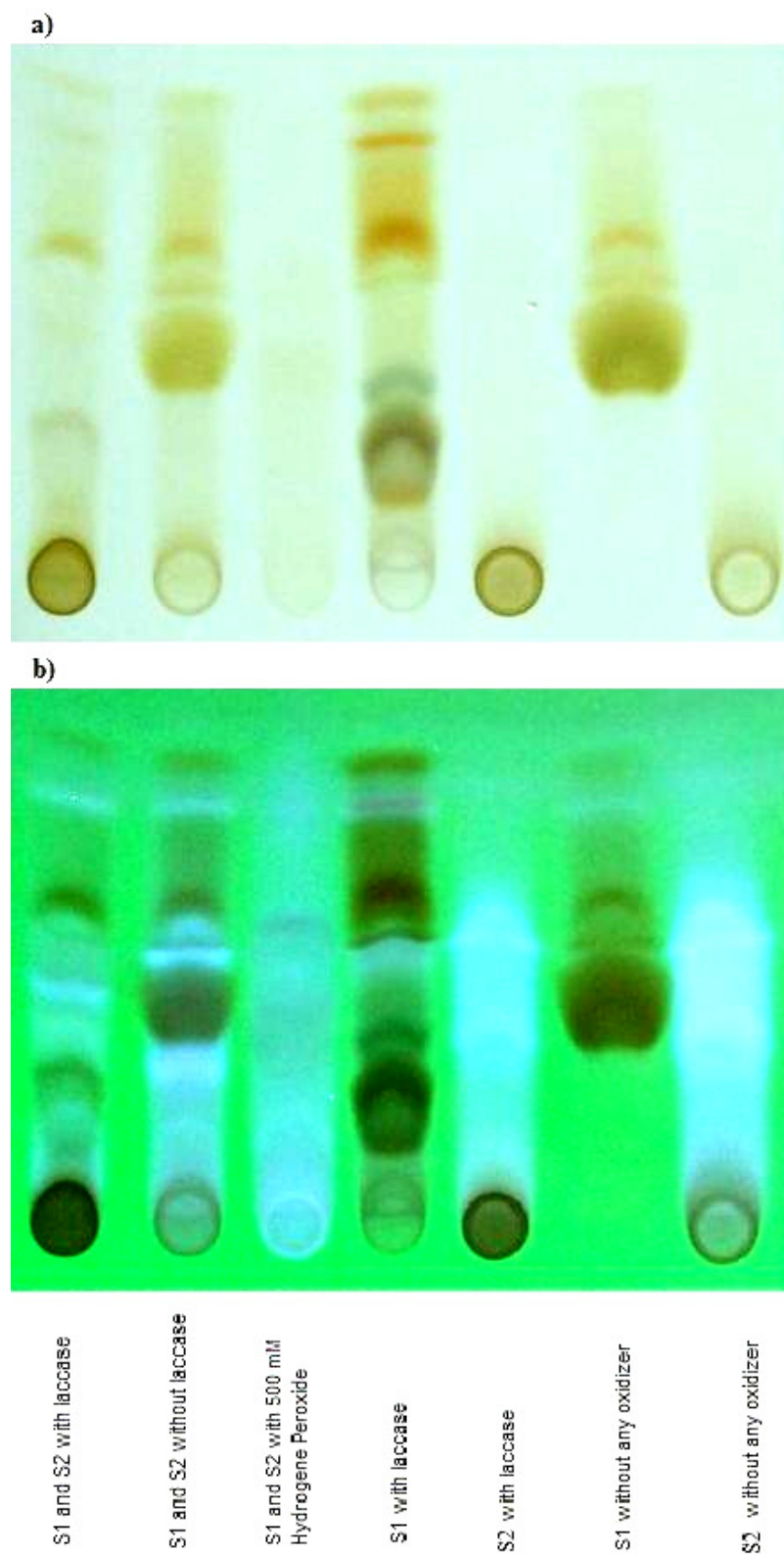


Figure 3. 7 TLC results of micro-plate reactions (a) photograph taken under day light, (b) taken under 254 nm UV light

The dark spots located starting points on TLC indicate the polymerization. Laccase-catalyzed oxidation of individual S_1 resulted in eight products at least. However, there is polymerization which came from laccase-catalyzed oxidation of individual S_2 (Figure 3. 7). To understand the wide range of product which arise from laccase-catalyzed oxidation of individual S_1 , oxidation model and possible products were studied (Figure 3. 8). There are likely to be theoretically nine possible coupling according to the resonance structure of S_1 , and eight substances at least were experimentally occurred in the micro-plate reaction (Figure 3. 7).

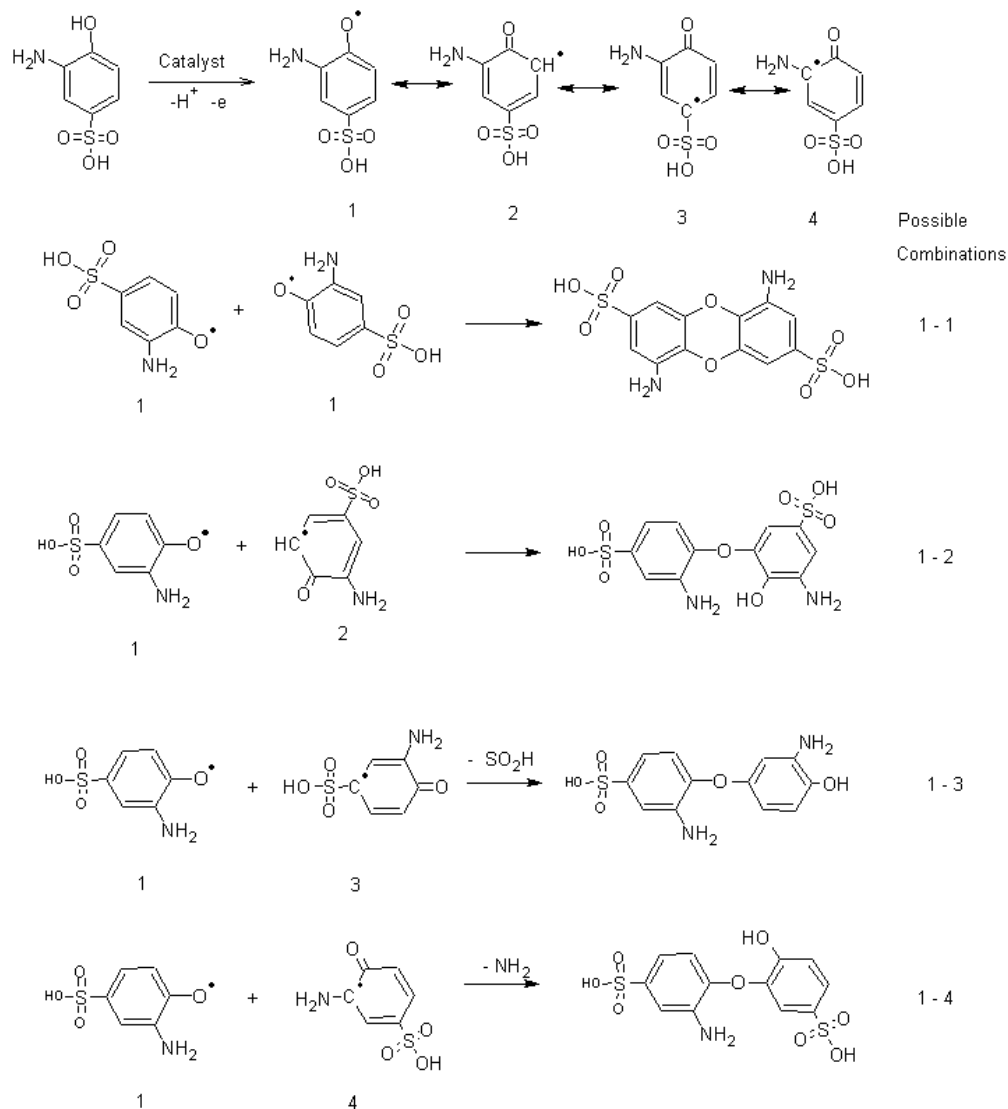


Figure 3. 8 Four resonance forms of free radicals and possible coupling by laccase mediated oxidation

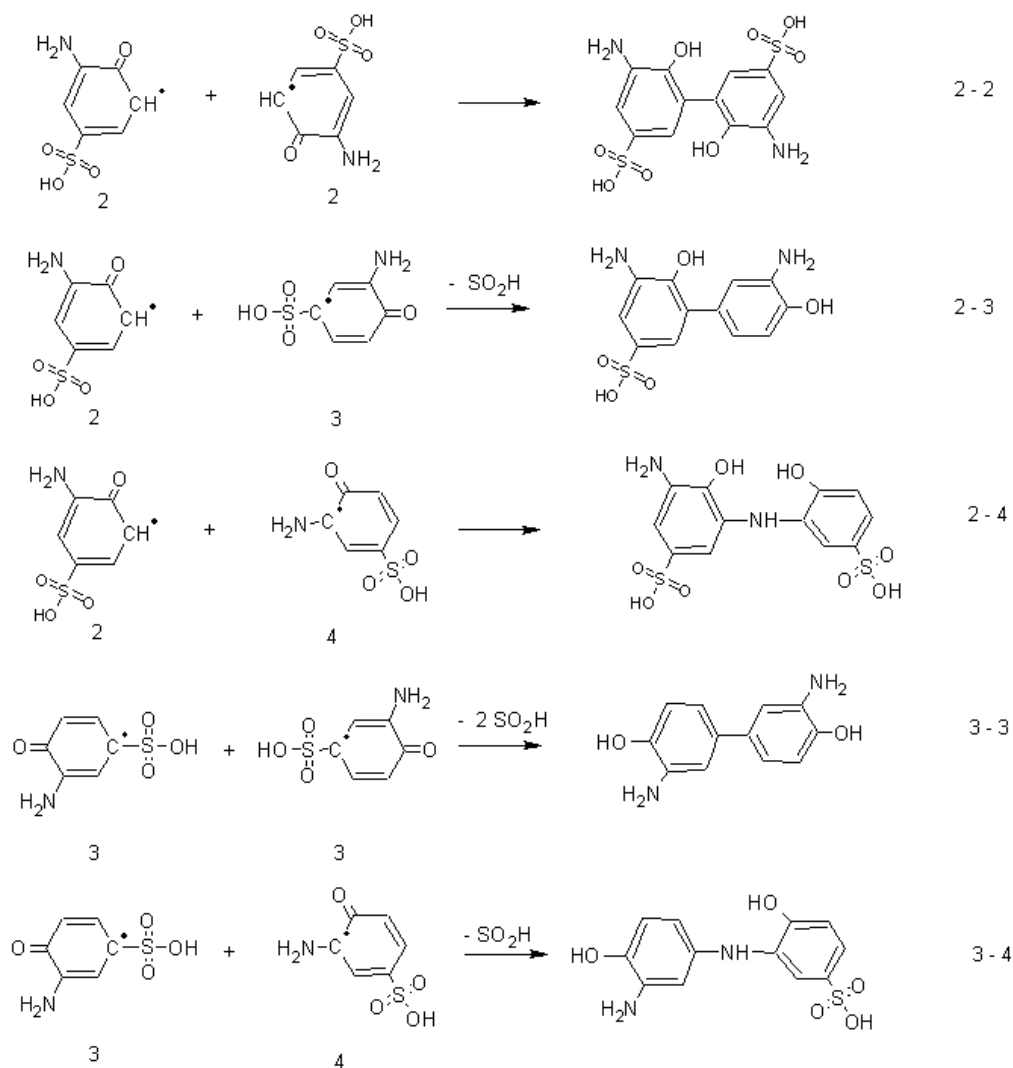


Figure 3. 9 (continued) four resonance forms of free radicals and possible coupling by laccase mediated oxidation

To analyze product formation and precursor consumption in laccase mediated and blank mediums, HPLC was carried out. On the HPLC chromatogram of S_1 and S_2 reaction in the presences of laccase mediated oxidation, one sharp peak at 1.66 min. was observed (Figure 3. 10). Nonetheless, there was no sharp peak at same retention time on blank reaction medium chromatogram (Figure 3. 12). On the chromatogram of blank reaction medium, one sharp peak was observed at 2.18 min. It is clearly understood that these are different substances when compared to the spectrogram of these peaks (Figure 3. 11 and 3. 13).

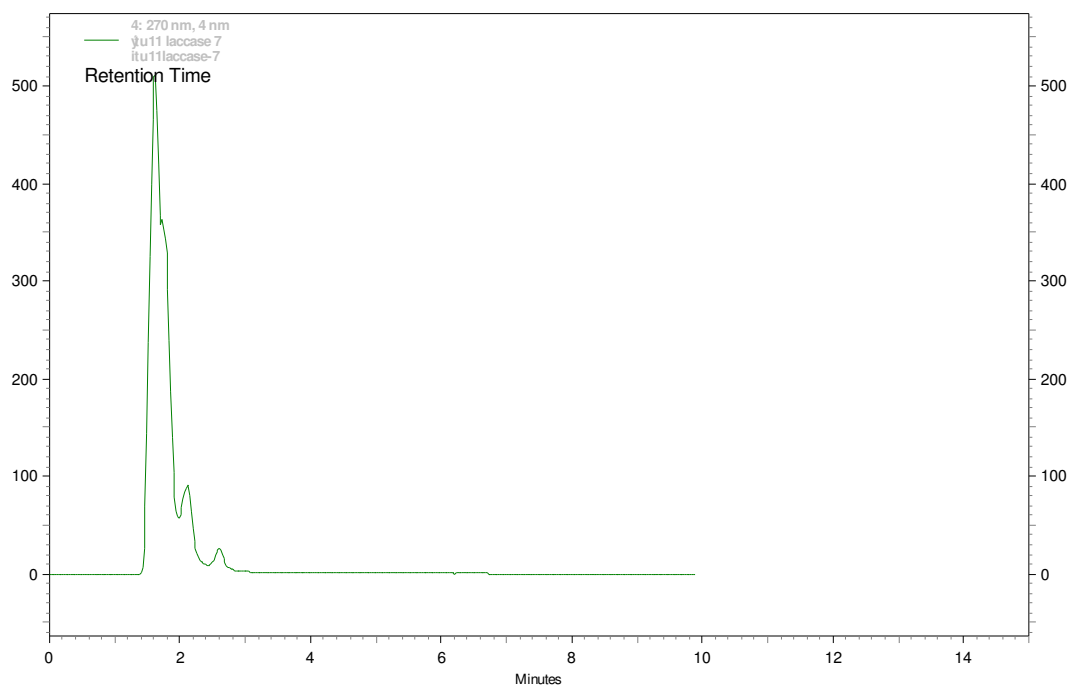


Figure 3. 10 HPLC chromatogram of S₁ and S₂ in laccase mediated oxidation
Spectrum at time 1,66 min.

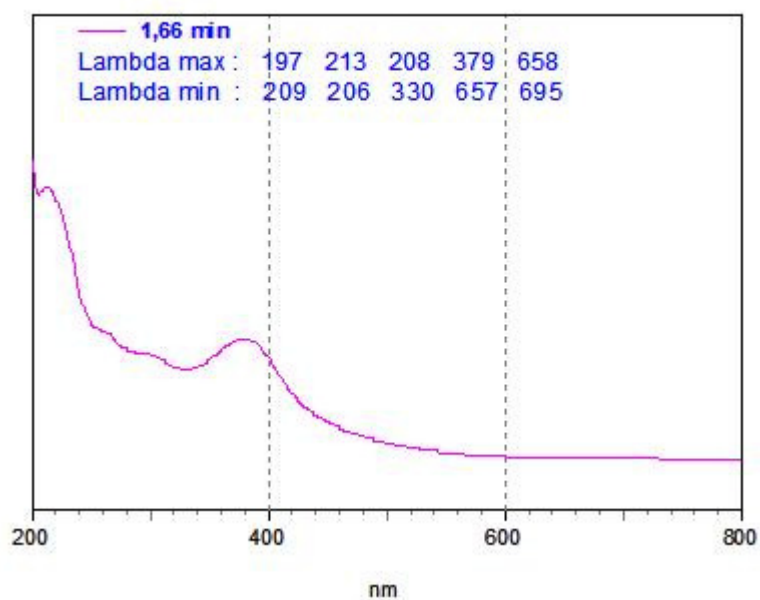


Figure 3. 11 HPLC spectrogram of S₁ and S₂ at 1.66 min. in laccase mediated oxidation

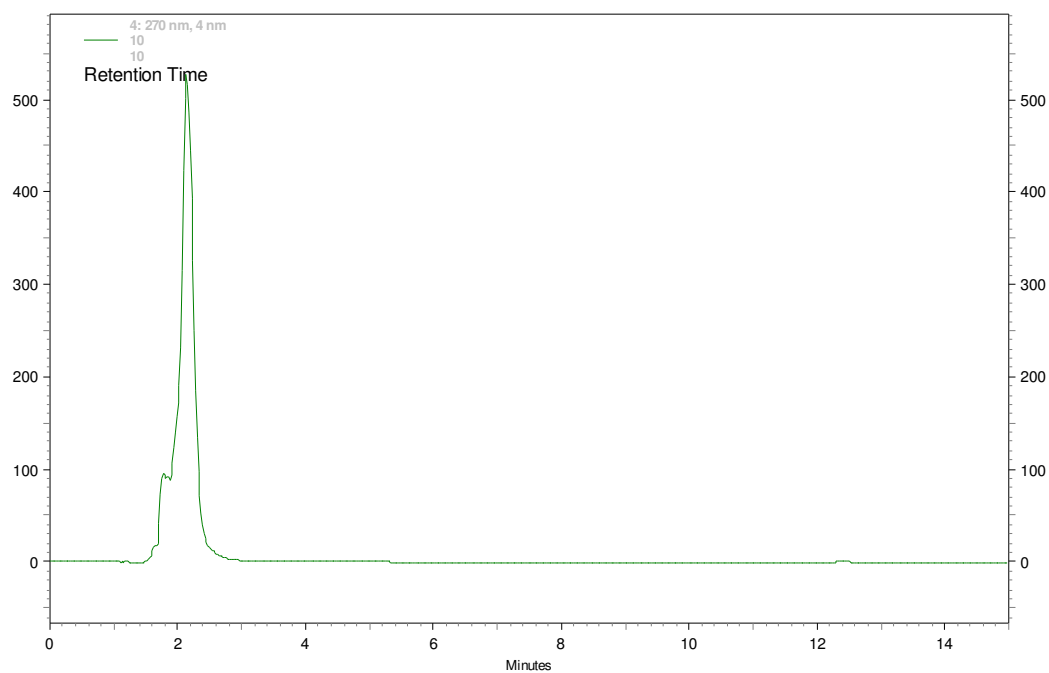


Figure 3. 12 HPLC chromatogram of S_1 and S_2 in the absence of any oxidant

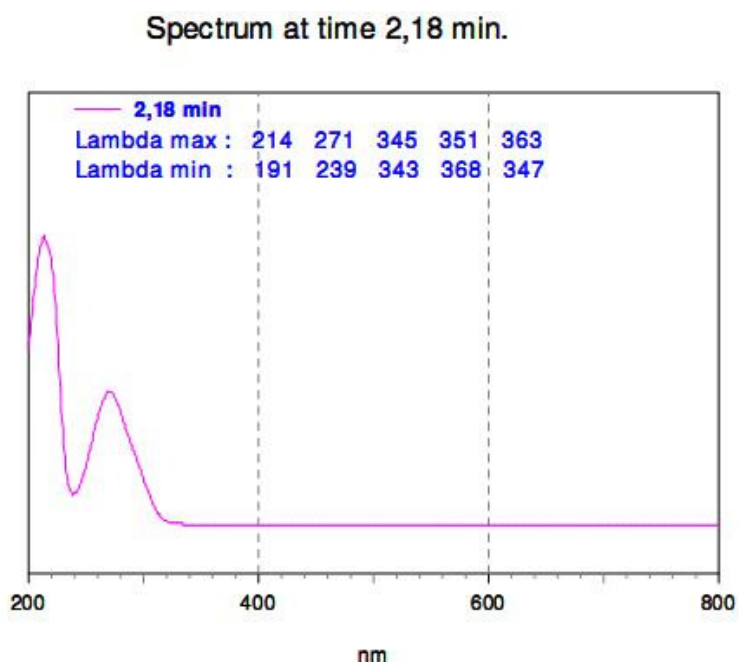


Figure 3. 13 HPLC spectrogram of S_1 and S_2 at 2.18 min.

To understand effect of laccase mediated oxidation on S_1 , the medium which include only S_1 in the presence of laccase was analyzed by HPLC (Figure 3. 14). One sharp peak at 1.66 min. was observed. However, there is also a small interference. It is likely to come from the unreacted S_1 , because the same peak was observed on HPLC chromatogram of S_1 in the absence of any oxidant (Figure 3. 18). Meanwhile, the same retention time on HPLC chromatogram of S_1 and S_2 in laccase mediated oxidation was noticed (Figure 3. 11). Both of the peaks have nearly similar spectral characteristics and retention time (Figure 3. 11 and Figure 3. 15).

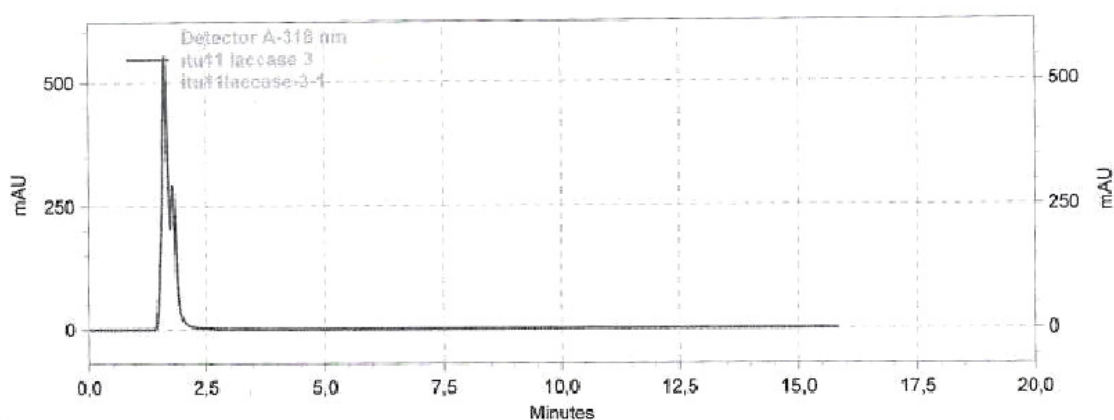


Figure 3. 14 HPLC chromatogram of S_1 in laccase mediated oxidation

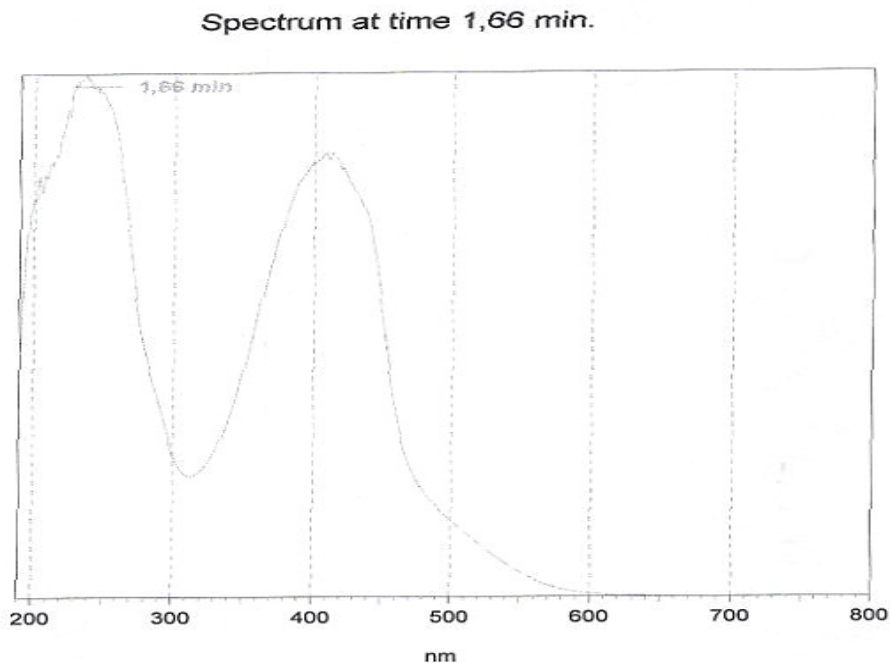


Figure 3. 15 HPLC spectrogram of S_1 at 1.66 min.

At a glance, differences between the HPLC chromatogram and spectrogram of S_2 in laccase mediated oxidation (Figure 3.16 and 3. 17) and the HPLC chromatogram and spectrogram of S_2 in the absence of oxidant (Figure 3. 19 and 3. 20) could be determined. At this point, quantity of chromatogram difference is important. Previously mentioned at TLC result section (Figure 3. 8), S_2 is likely to polymerized. Therefore, HPLC column can not able to separate it. The small peak on HPLC chromatogram of S_2 in laccase mediated oxidation probably based on the unpolimerized coupling products.

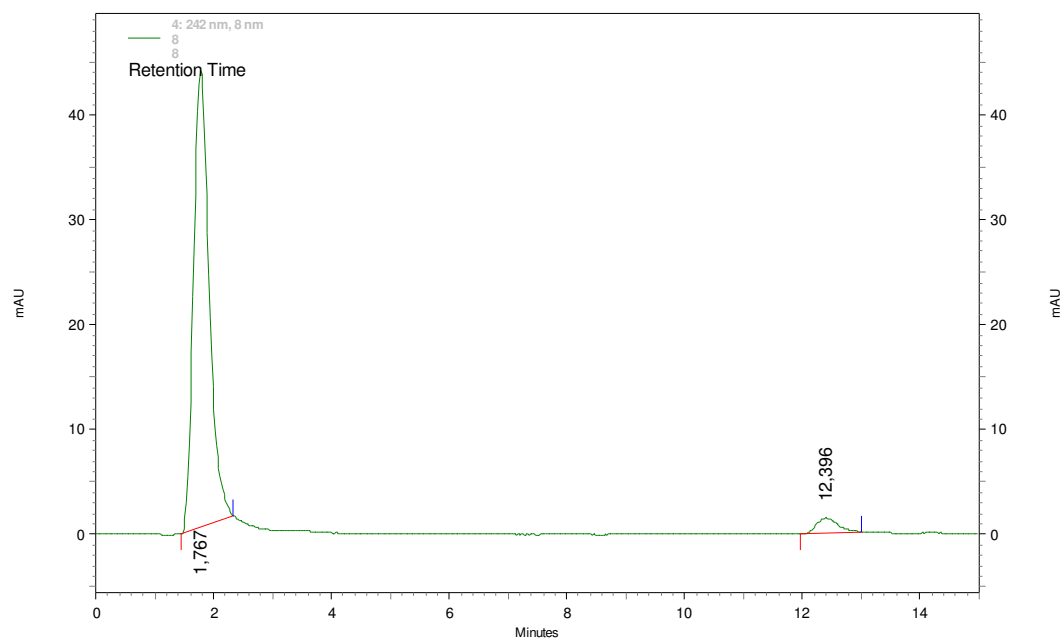


Figure 3. 16 HPLC chromatogram of S_2 in laccase mediated oxidation

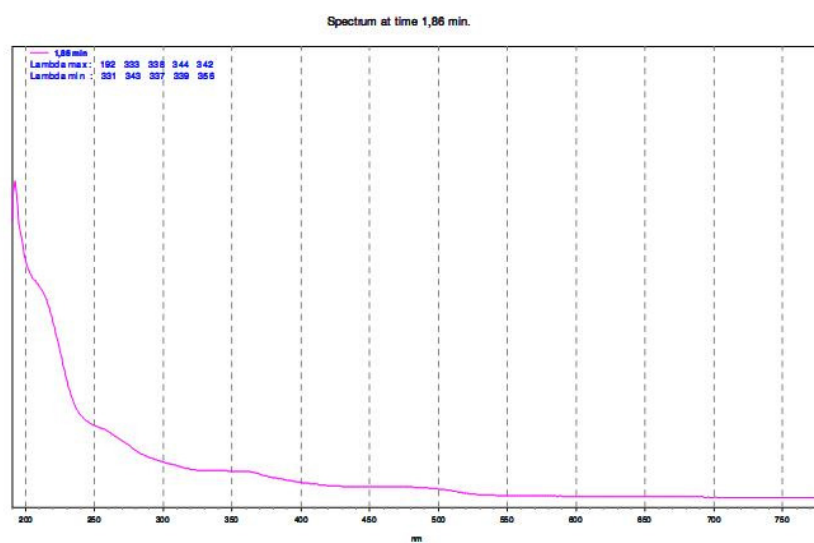


Figure 3. 17 HPLC spectrogram of S_2 at 1.86 min.

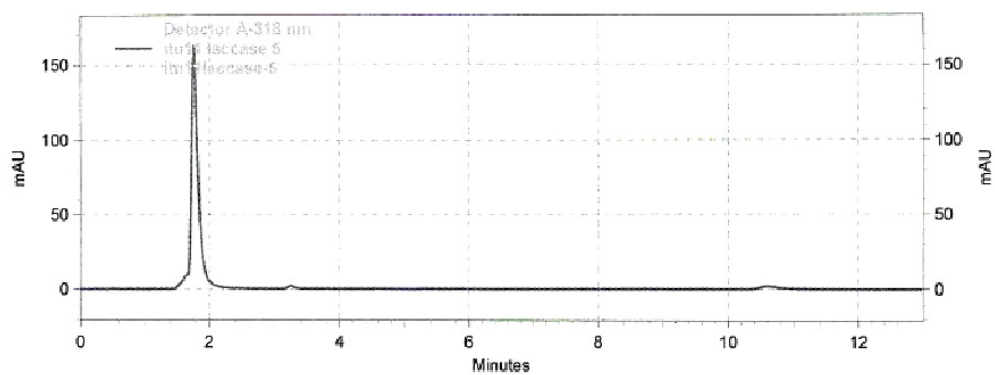


Figure 3. 18 HPLC chromatogram of S_1 in the absence of oxidant

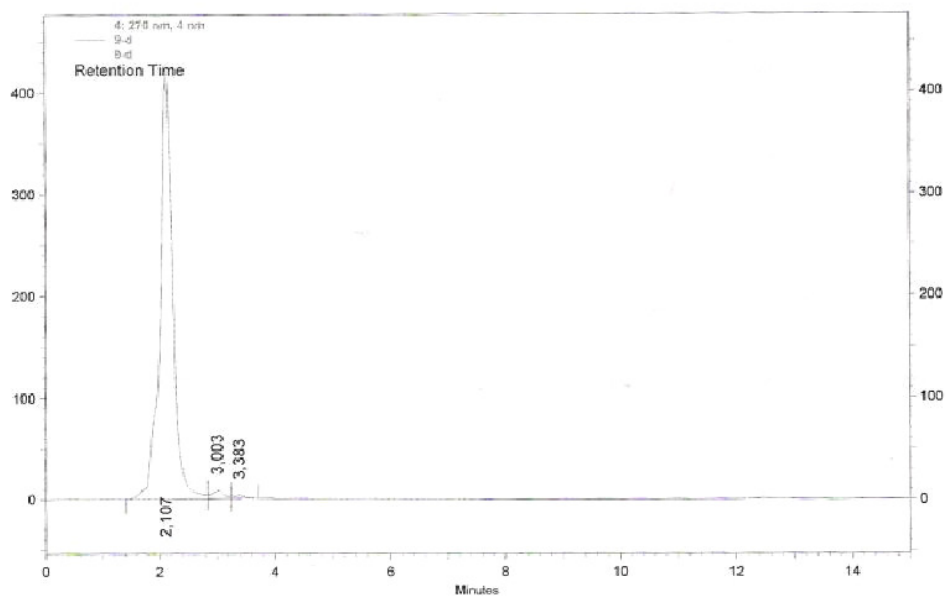


Figure 3. 19 HPLC chromatogram of S_2 in the absence of oxidant

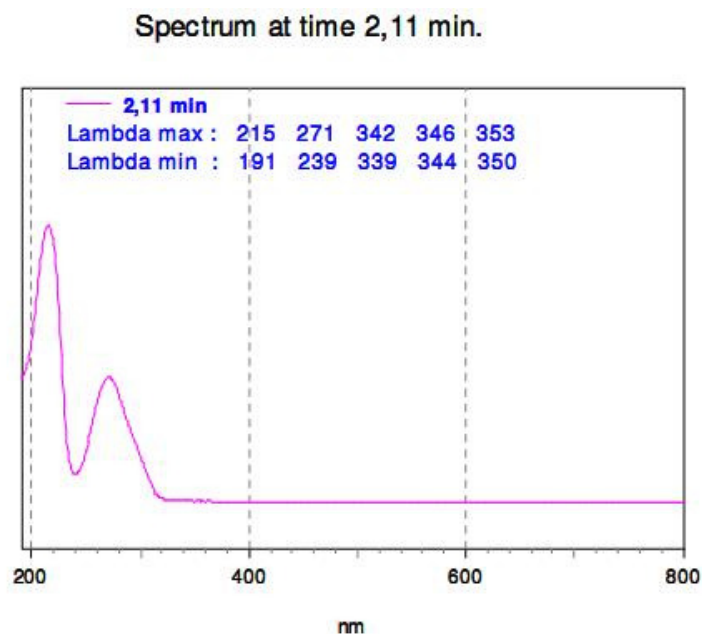


Figure 3. 20 HPLC spectrogram of S₂ at 2.11 min.

To sum up the characterization studies related to the ITU22, there is no clue for coupling between S₁ and S₂ in the presence of laccase-catalyzed oxidation. On the contrary, each precursor could be coupled itself by laccase oxidation. Furthermore, polymerization between S₂ substances was likely to occurred by laccase. By means of HPLC, TLC and spectral analysis regarding ITU22, we have proposed possible oxidation and coupling mechanism for phenolic substitute substances (Figure 3. 21).

Radical Formation

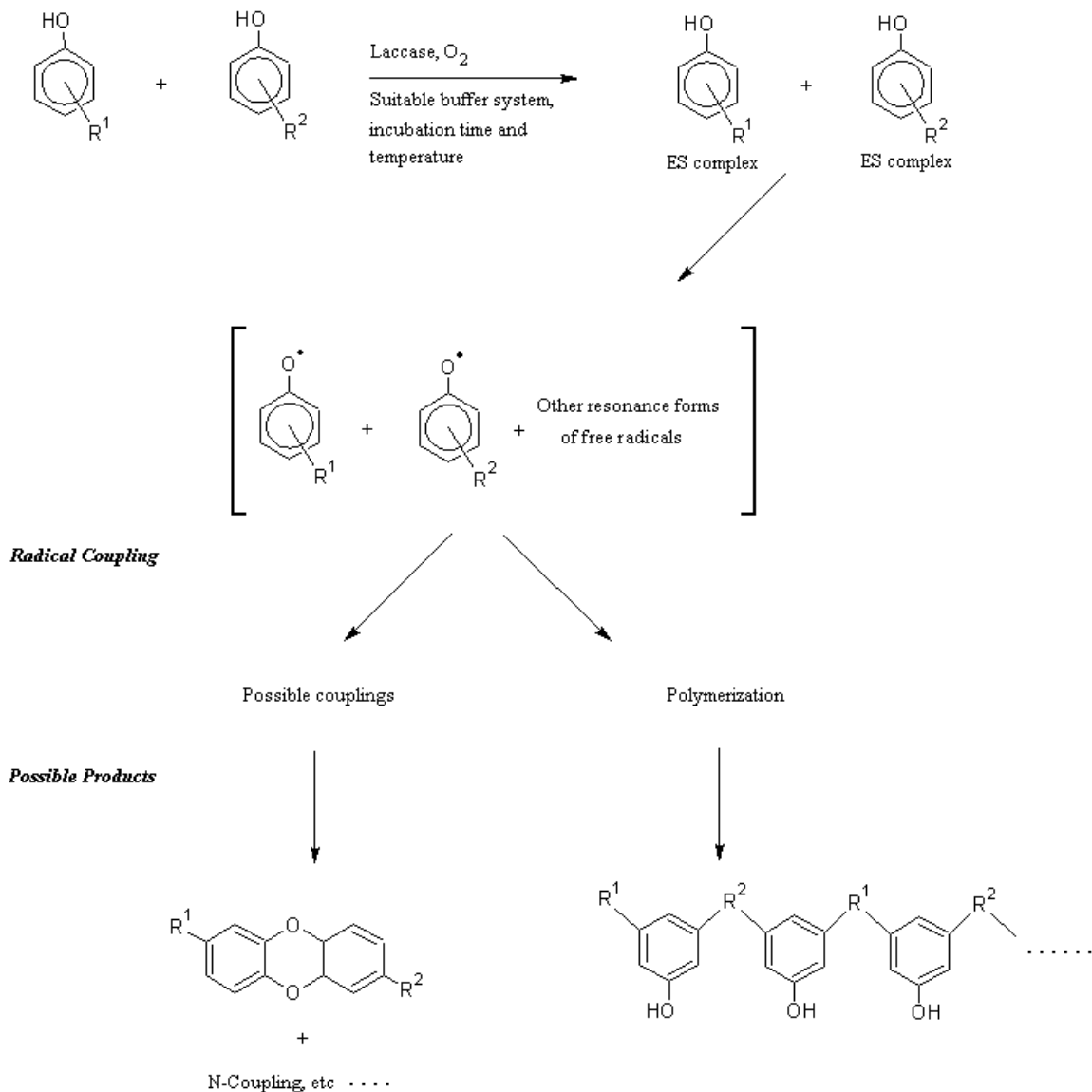


Figure 3. 21 Proposed reaction mechanism for laccase-catalyzed oxidation of phenolic substitute substances

3.2.2. Scale-up

ITU22 was produced at bench scale in shake flask by laccase mediated oxidation up to 100 mL. Since maximum precursor solubility is 25 mM, concentration of precursors was adjusted at 25 mM. The product was used for industrial dye quality tests by Setas Kimya San. AS.

3.3. Industrial Dye Quality of ITU22

Industrial quality tests which include multifibre dyeing, chlorinated water fastness, remaining dye stuff in the batch, washing fastness, water fastness and light fastness were performed by Setas Kimya San AS. (Figure 3. 22-27). According to the result of the multifibre dyeing test, ITU22 dyed light brown on nylon fabric and brown on wool fabric (Figure 3. 22). There were no dye effects on the acetate, cotton, PES and acrylic fabrics.



Figure 3. 22 Multifibre dyeing

According to the results of washing and water fastness quality of ITU22, small amount of remain dye was found on wool fabric (Figure 3. 23-24). These results indicate that ITU22 have good washing and water fastness. The fastness properties of ITU22 were evaluated by using gray scale of ISO (Table. 3.2). The ISO scale for dyes can be described in the following context.

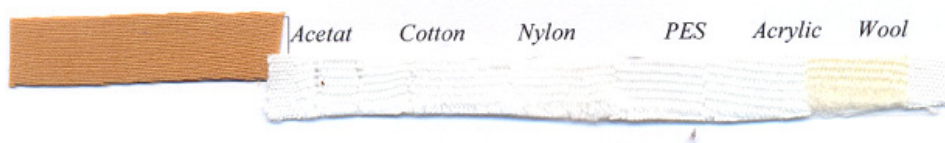


Figure 3. 23 Washing Fastness

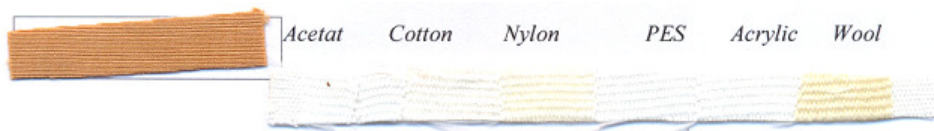


Figure 3. 24 Water Fastness

Chlorinated water fastness results showed that ITU22 had a good level fastness (Figure 3. 25), although light fastness was under the critical value (Figure 3.27). These may decrease quality of ITU22.



Figure 3. 25 Chlorinated water fastness

After dyeing process, remaining dyestuff in the bath was determined by the dyeing of new fabric. It is clear from this result that ITU22 has a good affinity for fabrics (Figure 3. 26).

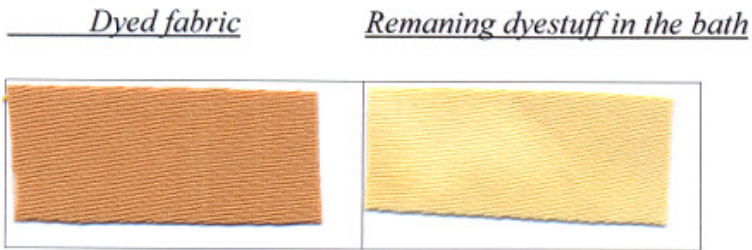


Figure 3. 26 Determination of remaining dyestuff in the bath

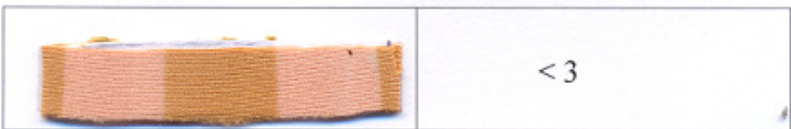


Figure 3. 27 Light fastness control

Consequently, according to the results of industrial dye quality of ITU22, it can be called as a moderate level acid dye. It has a potential for dyeing of nylon and wool fabrics.

Table 3. 1 Results of the chlorinated water fastness and light fastness

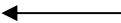
	Results
Chlorinated water fastness	4
Light Fastness	<3

Table 3. 2 Results of the washing fastness and water fastness

		Results					
		Acetat	Coton	Nylon	PES	Acrylic	Wool
Washing	Staining	4/5	4	4/5	4/5	4/5	4/5
Fastness	Color Change			4/5			
Water	Staining	4/5	3/4	3	4/5	4/5	4
Fastness	Color Change			4/5			

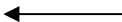
According to Blue scale of ISO, the value of light fastness is between 1-6;

- 1 is bad ,
- 5 is very good and
- 3 is the limit value

good 5 > 4 > 3 > 2 > 1 bad


And also according to Gray scale, the value of water ,washing and chlorinated water is between 1-5

- 1 is bad ,
- 5 is very good and
- 3 is the limit value

good 5 > 4 > 3 > 2 > 1 bad


3.4. Cytotoxicity Test of ITU22

Two different cell cultures were used for cytotoxicity of ITU 22. Although the test performed with RTG-2 gave promising results for ITU22, the results obtained from Caco-2 cells showed that ITU 22 has a high toxicity level for human health. Also ITU22 had no antimicrobial activity according to the Kirby-Bauer Method.

Table 3. 3 Cytotoxicity results of ITU22

	NRU Caco-2 cells (WET) IC50s in g/L	NRU RTG-2 cells (WET) IC50s in g/L
ITU22	0,079	> 1

Interpretation of IC50 values:

> 1 g/L	no toxicity detected or really low toxicity
0,01 g/L ≤ IC50 ≤ 1 g/L	average toxicity
> 0,01 g/L	high toxicity

4. CONCLUSION

In this study, fifteen selected precursors were screened to find out coupling reactions in micro-plate scale in the presence of laccase and some of the positive reactions which have appropriate colors were scaled up. As the results of these studies, several valuable novel textile dyes from these screening reactions were obtained. One of these novel textile dyes was named as ITU22. In the context of this thesis, the novel screening technique for textile dye synthesis and ITU22 synthesis by laccase-catalyzed oxidation have been studied. Our all experimental route was shown in Figure 1.8, in detail.

Applicability tests of ITU22 have revealed that ITU22 is a light brown dye on fiber. Multifiber test which shows the affinity of the dye on specific fabrics indicated that ITU22 dyes nylon and wool fibers and has properties of acid dyes. Industrial dye quality tests such as washing fastness, chlorinated water fastness, water fastness, light fastness and remaining dye in batch were performed and these tests results showed that it has an average quality, although the light fastness of ITU22 was under the critical level.

Most of the existing dyes which synthesized via conventional chemical processes are generally toxic for human health. It is an important issue for chemical companies, workers and end-users. Today's, European legislation implements a new system called REACH for Registration, Evaluation, and Authorization of Chemicals in Europe to ensure the protection of human health and environment. There are needs for new non-toxic, eco- and environmentally- friendly dyes. In our studies, several non-toxic dyes were obtained by laccase catalyzed biooxidation method developed by our group; it is a promising alternative method to produce safe dyes in this area. However, for ITU22 dye, the cytotoxicity tests performed by NRU tests indicated that ITU22 was not toxic for fish cell line (RTG-2), although it was highly toxic for human intestinal cell line (RTG-2). On the contrary of ITU22, several of other dyes had good industrial dye quality and

non-toxicity. To protect proprietary rights of novel textile dyes, patent request of these dyes is pending and large scale production studies are ongoing.

As a result of ITU22 characterization studies such as HPLC and TLC, we have concluded that ITU22 results from S_1 coupling by laccase-catalyzed biooxidation, not coupling between S_1 and S_2 . Great deals of S_2 were polymerized by laccase in this reaction. For phenolic substitute substances, we have proposed possible synthesis pathway (Figure 3. 21).

In the future, to obtain coupling products from different precursors, more screening reactions by the use of more precursors are required. We have also proposed a modified experimental route for this purpose (Figure 4. 1). To enhance selectivity of laccase on phenolic substitute substances, laccase can be modified and/or engineered by recombinant DNA technology. In the subsequent synthesis studies, engineered and/or modified laccase can be used. In addition to characterization studies, MS and NMR can be implemented in the experimental route to determine structure of products.

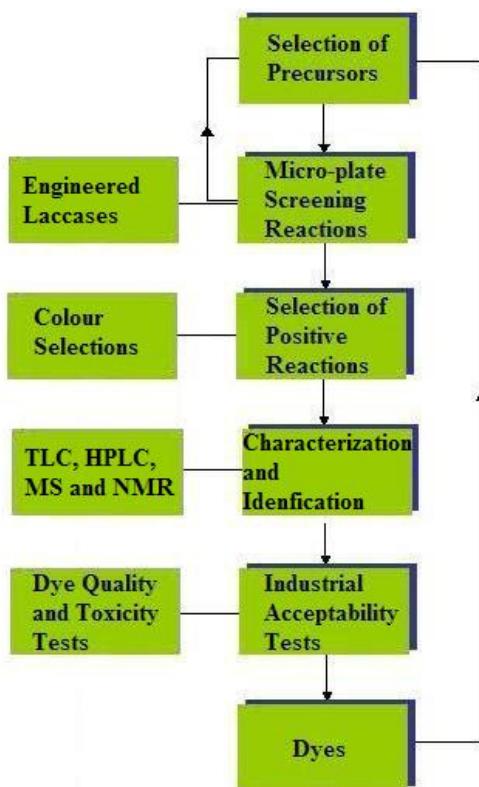


Figure 4. 1 Modified experimental route

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APPENDIX

Lab Equipment

Autoclaves	: 2540 ML benchtop autoclave, Systec GmbH : NuveOT 4060 vertical steam sterilizer, Nuve
Deep freezes and refrigerators	: Heto Polar Bear 4410 ultra freezer, JOUAN : 2021 D refrigerator, Arcelik
HPLC	: Shimadzu (DAD dedector)
Inbubators	: EN400, Nuve
Orbital shaker	: Certomat S II, Product# 886 252 4, B. Braun Biotech Internationnal GmbH
Magnetic stirrer	: AGE 10.0164, VELP Scientifica srl.
Micro-plate reader	: Bio-rad Benchmark Microplate Reader
Pipettes	: Pipetteman P10, P100, P1000, P5000, Eppendorf
Ph meter	: MP 220, Metter Toledo International Inc. Wissenschaftlich-Technische Werstätten
Pure water systems	: USF Elga UHQ-PS-MK3, Elga Labwater
Spectrophotometer	: DU530 Life Science UV/Vis, Beckman : UV-1601, Shimadzu Cor.
Sterilizer	: FN 500, Nuve
Vortexing machine	: Reax Top, product# 541-10000, Heidolph2.2

CV

Mustafa Kahraman was born in Kırşehir in 1981. He graduated from Kırşehir High School in 1999 and in 2000 he started to study in Ege University Biochemistry Department. He had his Bachelor degree from the same department in 2005 and consequently, he was accepted to Molecular Biology - Genetics and Biotechnology Graduate Program, which is a part of Advanced Technologies Department. Biotechnology at Istanbul Technical University, biocatalysts, biotransformation, fermentation and protein engineering are among his professional interest topics.

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