การกำจัดสารประกอบฟีนอลโดยเปอร์ออกซิเดสที่ได้จากวัสดุเหลือทิ้งทางการเกษตร

นางชื่นสุมณ ยิ้มถิน

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REMOVAL OF PHENOLIC COMPOUNDS BY PEROXIDASE DERIVED FROM AGRICULTURAL WASTE

Mrs.Chuensumon Yimthin

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การศึกษานี้มุ่งเน้นการกำจัดสารประกอบฟีนอลโดยเปอร์ออกซิเดสที่สกัดได้จากวัสดุเหลือทิ้งทางการเกษตร สารประกอบฟีนอลที่ศึกษา ได้แก่ ฟีนอล, 2-คลอโรฟีนอล, 3-คลอโรฟีนอล, 4-คลอโรฟีนอล, ออร์โท-ครีซอล, เมทา-ครีซอล, พารา-ครีซอล, แคทิคอล, รีซอร์ซีนอล, ไฮโดรควินโนน, 1-แนฟทอล และ 2-แนฟทอล ผลการทดลองพบว่าเปอร์ออกซิเดสที่ได้จากการสกัดเปลือกมันเทศโดยใช้สารละลายฟอสเฟตบัฟเฟอร์มีปริมาณเปอร์ออกซิเดสสูงสุด และมีประสิทธิภาพในการกำจัดสูงสุด ภาวะที่เหมาะสมสำหรับปฏิกิริยาการกำจัดฟีนอล คือ 30 องศาเซลเซียส, 100 นาที, พีเอช 7, 20 พีพีเอ็มของฟีนอล, 0.5 เปอร์เซ็นต์โดยน้ำหนักของไฮโดรเจนเปอร์ออกไซด์, 10 ยูนิตต่อมิลลิลิตร, 20 พีพีเอ็มของ พีอีจี-6000 และสารละลายฟอสเฟตบัฟเฟอร์เป็นตัวทำละลาย อัตราเร็วในการเกิดปฏิกิริยาของฟีนอล คือ1.045 ไมโครโมลต่อชั่วโมง ส่วนภาวะที่เหมาะสมในการกำจัดสารประกอบฟีนอล คือ 30 องศาเซลเซียส, 100 นาที, พีเอช 7, 20 พีพีเอ็มของสารประกอบฟีนอล, 1.0 เปอร์เซ็นต์โดยน้ำหนักของไฮโดรเจนเปอร์ออกไซด์, 4 ยูนิตต่อมิลลิลิตร และสารละลายฟอสเฟตบัฟเฟอร์เป็นตัวทำละลาย ผลิตภัณฑ์ที่ได้จากการกำจัดฟีนอลเป็นพอลิเมอร์ที่ประกอบด้วย 2-ฟีนอกซี-ไบฟีนอล, 1,1′-ไบเฟนิล-2,2′ไดออล และ 4-ฟีนอกซี-ไบฟีนอล ส่วนผลิตภัณฑ์จากการกำจัดสารประกอบออร์โท-ครีซอลเป็น 2,2′อีทิลีนไดฟีนอล

สาขาวิชา..ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์..ลายมือชื่อนิสิต.....ชื่นสุมณ ยิ้มถิน.....

ปีการศึกษา.....2548.....ลายมือชื่ออาจารย์ที่ปรึกษา.....วรินทร ชวศิริ.....

4573421123 : MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEY WORD: PEROXIDASE / REMOVAL / PHENOLIC COMPOUND

CHUENSUMON YIMTHIN: REMOVAL OF PHENOLIC COMPOUNDS BY PEROXIDASE DERIVED FROM AGRICULTURAL WASTE. THESIS ADVISOR: ASSISTANT PROFESSOR WARINTHON CHAVASIRI, Ph.D.; 70 pp. ISBN 974-14-3350-6.

This study focuses on the removal of phenolic compounds by peroxidase derived from agricultural waste. The phenols studied are phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, catechcol, resorcinol, hydroquinone, 1-naphthol and 2-naphthol. The extraction of crude POD from sweet potato with phosphate buffer exhibited the highest activity and removal efficiency. The optimum conditions for removal of phenol was at 30^0C, 100 min, pH 7, 20 ppm of phenol, 0.5 % w/w of H_2O_2, 10 units/mL of crude POD, 20 ppm of PEG-6000 and phosphate buffer as solvent. Rate of conversion of phenol under this condition was 1.045 μmol per h. The optimum condition for removal of phenols was at 30^0C, 100 min, pH 7, 20 ppm of phenols, 1.0 % w/w of H_2O_2, 4 units/mL of crude POD and phosphate buffer as solvent. The product from the removal of phenol by POD is polymer consisted of 2-phenoxy-biphenyl, 1,1′-biphenyl-2,2′-diol and 4-phenoxy-biphenyl while that derived from the removal of *o*-cresol by POD is 2,2′-ethylenediphenol.

Field of study Petrochemirtry and Polymer Science.Student's signature .....

Academic year.....2005.....Advisor's signature.....

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Ultimately, the author would like to express her inmost gratitude to her family and her best friends for their friendship, understanding, encouragement and social support throughout her entire education. Thanks are to everyone who has contributed suggestions and support during this research.

CONTENTS

Pages

Pages

Pages

Pages

LIST OF FIGURES

Figures **Pages**

Figures **Pages**

LIST OF TABLES

Tables **Pages**

LIST OF SCHEMES

Schemes **Pages**

LIST OF ABBRAVIATIONS

Phenol	phenolic compounds
POD	peroxidase
BOD	biological oxygen demand
HRP	horseradish peroxidase
CCP	cytochrome *c* peroxidase
AP	ascorbate peroxidase
LiPs	lignin peroxidases
MnPs	manganese dependent peroxidases
ARP	*Arthromyces ramosus* peroxidase
PNP	peanut peroxidase
U/g	units per gram
^{0}C	degree celsius
4-AAP	4-aminoantipyrine
PEG	polyethylene glycol
2,4-DCP	2,4-dichlorophenol
SBP	soybean peroxidase
PEG-3350	polyethylene glycol molecular weight 3350
PEG-8000	polyethylene glycol molecular weight 8000
mg	milligram
L	liter
LP-SBP	low purity soybean peroxidase
TP	turnip peroxidase
Udm^{-3}	unit per decimeter
Con-A	concanavalin A
μL	microliter
mM	millimolarity
mL	milliliter
UV-Vis spectrometer	Ultraviolet Visible Spectrometer
FT-IR	Fourier Transform Infrared Spectrometer

NMR	Nuclear Magnetic Resonance Spectrometer
GC-MS	Gas Chromatography-Mass Spectrometer
nm	nanometer
UV-lam	Ultraviolet lamp
PEG-3000	polyethylene glycol molecular weight 3000
PEG-6000	polyethylene glycol molecular weight 6000
ppm	part per million
w/w	weight by weight

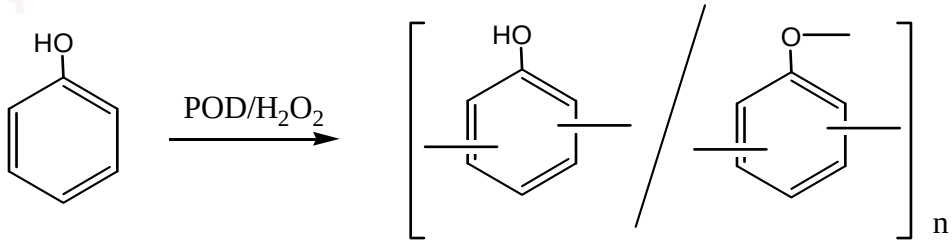
CHAPTER I

INTRODUCTION

Phenolic compounds (phenols) are present in waste waters which derived from a number of petrochemical industries such as plastics, coal conversion, resins, petroleum refining, textiles, dyes and organic chemicals, *etc.* phenols are known to be toxic and also hazardous carcinogens that can accumulate in the food chain. Due to their toxicity, must be removed from waste water before being discharged into the environment. [1]

Conventional processes for removal of phenols from petrochemical industrial wastes water include extraction, adsorption on activated carbon, steam distillation, bacterial and chemical oxidation, electrochemical techniques, *etc*. All of these methods, although certainly feasible and useful, suffer from some serious drawback *e.g*., high cost, incompleteness of purification or low efficiency. [2] Thus, efforts to develop new efficient methods to remove phenols from waste waters.

A recent method for dephenolization of waste waters is based on the use of peroxidase (POD), the enzyme that polymerizes phenols in plants. Enzyme treatment offers a high degree of specificity, [3-12] operation under mild conditions, and high reaction velocity. The enzyme oxidizes numberous phenols with hydrogen peroxide (H_2O_2), [13-16] forming free radicals which undergo spontaneous polymerized product precipitating out from the aqueous solution and can be readily separated by simple filtration. [17,18]



Polymerized products (HAAH)

Most intensive researches have been concerned with the purified POD. Nevertheless, this method was complex and expensive since many procedures for enzyme purification such as ammonium sulfate fractionation, gel filtration on Bio-gel P-60 and ion exchange on DEAE-Sephadex was required. [19-24] Thus, this research encourages to look for non expensive sources of enzyme. Various plants have been identified as a rich source of POD including soybean, sweet potato, pineapple, peanut and from agricultural waste of plants that could provide a cheaper and abundant source of POD.

Aim of this research.

The aim of this research was to study the feasibility of using a crude POD extract from agricultural waste, in the removal of phenols from synthetic waste waters.

In this research, the optimum conditions for removal of phenols used to crude POD extract from soybean, sweet potato, pineapple and peanut. Experimental parameters included species of plant, solvent, temperature, pH, concentration of substrates and activity of enzyme.

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Phenolic compounds (phenols)

2.1.1 Phenol

Phenol has been in production since the 1860. One of the early uses for phenol was as an antiseptic. Towards the end of the 19th century, industrial scientists found new uses for phenol in the synthesis of dyes, aspirin, and one of the first high explosives, picric acid. As early as 1872, it was found that phenol could be condensed with aldehydes (for example methanal) to make resinous compounds, a process still in use today.

Phenol-methanal (formaldehyde) resins are the basis of the oldest plastics, and are still used to make low cost thermosetting plastics such as melamine and bakelite, used in electrical equipment. These resins are also used extensively as bonding agents in manufacturing wood products**.**

Phenol is also used to make chemical intermediates for a wide range of other applications, ranging from plastics to pharmaceuticals and agricultural chemicals. [25]

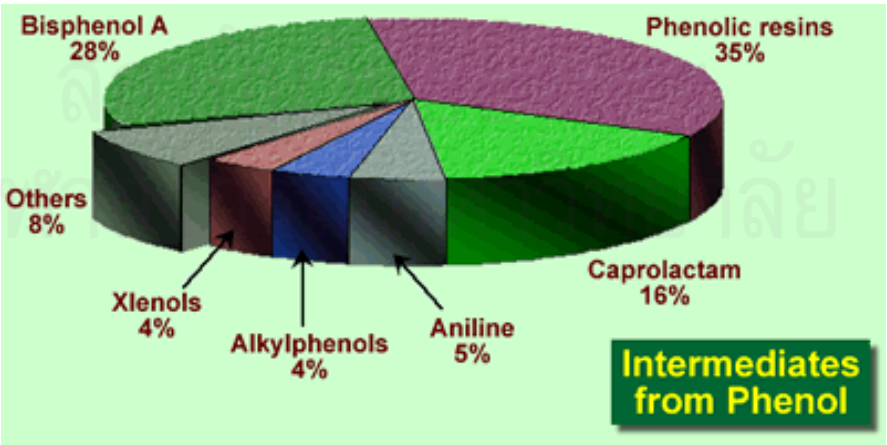


Figure 2.1 Intermediates from phenol [25]

2.1.2 Phenols from waste water

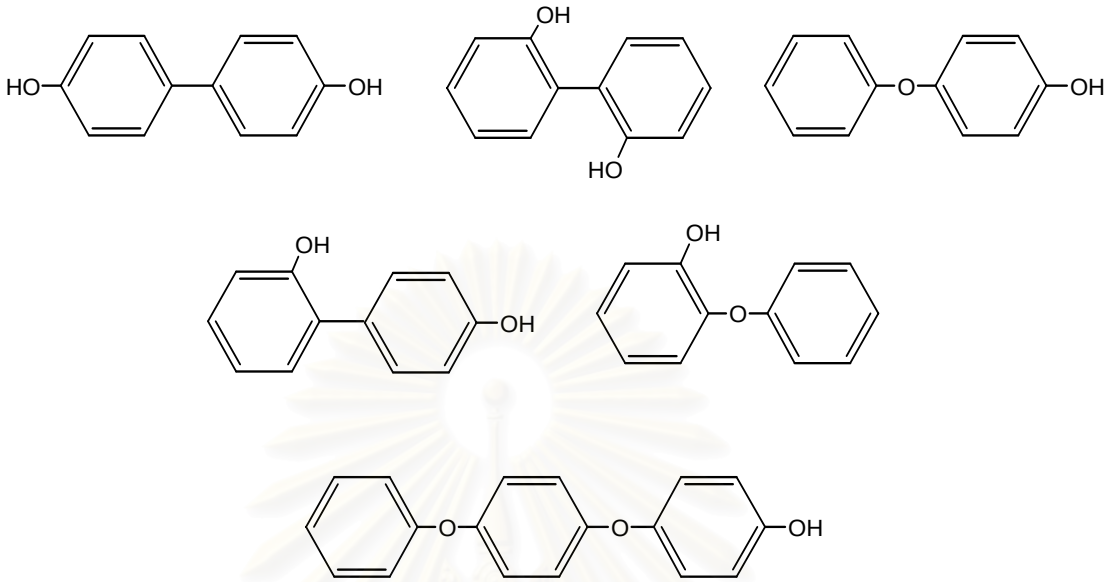
Phenols are pollutants contained in various industrial waste waters such as waste from petroleum refineries, resin manufacturing plants, and coal processing factories. The process-intensive petrochemical industry has demanding environmental management challenges to protect water, soil and atmosphere of the refinery pollution. The process petrochemical industry has different kinds of technologies and processes to the waste water and the process water from the refinery industries. Petroleum refineries use relatively large volumes of water, especially for cooling systems. In fact, waste water from the petrochemical industry usually contains hazardous chemicals, as hydrocarbons, phenol or ammonia nitrogen among others. Below we will see some petroleum refinery waste and process water issues. [26]

2.1.3 Toxic and Environmental Hazardous of phenols

Phenol can remain in the air, soil, and water for long periods of time if a large amount of it is released at one time, or if it is constantly released to the environment from a source. Small amounts of phenol do not remain in the air for longer than a day, in the soil for longer than 2 to 5 days, and in water for longer than 9 days.

Phenols as a particular case are highly toxic materials also have a relatively high biological oxygen demand (BOD) and hence, in sufficient concentration, can deplete the oxygen in receiving body of water. They are easily absorbed via the skin, by inhalation or by ingestion. Being soluble in both polar and non-polar solvents they are highly mobile in aquatic systems and are suspected endocrine disruptors. [27]

It has been found that these phenols can be removed by enzyme- catalyzed polymerization using horseradish peroxidase (HRP) which has little substrate specificity. The phenols are oxidized to phenoxy radicals which undergo further reactions yielding various polymers and oligomers that are less toxic. These products can be removed from water easily since they have low solubility properties in water. From a two-hour reaction involving HRP, hydrogen peroxide, and phenol, five stable dimeric products and one stable trimeric product were obtained:



Schemes 2.1 Product of phenol with POD [28]

Further oxidation of phenol dimers by HRP with hydrogen peroxide yields dimeric radicals that couple with each other to form trimers, tetramers, and longer polymers.

2.2 Peroxidase enzyme (POD)

2.2.1 POD enzyme

POD is heme containing enzymes that use hydrogen peroxide as the electron acceptor to catalyses a number of oxidative reactions. POD is heme containing enzymes that use hydrogen peroxide as the electron acceptor to catalyses a number of oxidative reactions. [29-33] POD is found in bacteria, fungi, plants and animals. On the basis of sequence similarity, fungal, plant and bacterial POD can be viewed as members of a superfamily consisting of three major classes. [34,35]

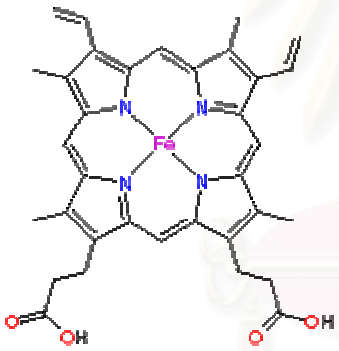
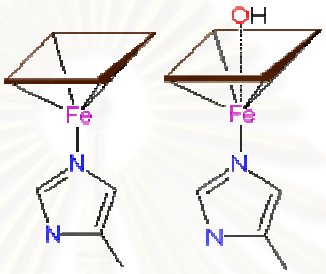
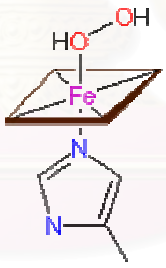
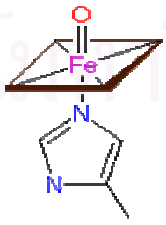
Class I, the intracellular POD, includes: yeast cytochrome c peroxidase (CCP), a soluble protein found in the mitochondrial electron transport chain, where it probably protects against toxic peroxides; ascorbate peroxidase (AP), the main enzyme responsible for hydrogen peroxide removal in chloroplasts and cytosol of higher plants and bacterial catalase- peroxidases, exhibiting both POD and catalase activities. It is thought that catalase-peroxidase provides protection to cells under oxidative stress

Class II consists of secretory fungal POD: ligninases, or lignin peroxidases (LiPs), and manganese dependent peroxidases (MnPs). These are monomeric glycoproteins involved in the degradation of lignin. In MnP, Mn^{2+} serves as the reducing substrate. Class II proteins contain four conserved disulphide bridges and two conserved calcium binding sites.

Class III consists of the secretory plant POD, which have multiple tissue specific functions: *e.g.*, removal of hydrogen peroxide from chloroplasts and cytosol; oxidation of toxic compounds; biosynthesis of the cell wall; defence responses towards wounding; indole3acetic acid catabolism; ethylene biosynthesis.Class III POD are also monomeric glycoproteins, containing four conserved disulphide bridges and two calcium ions, although the placement of the disulphides differs from class II enzymes.

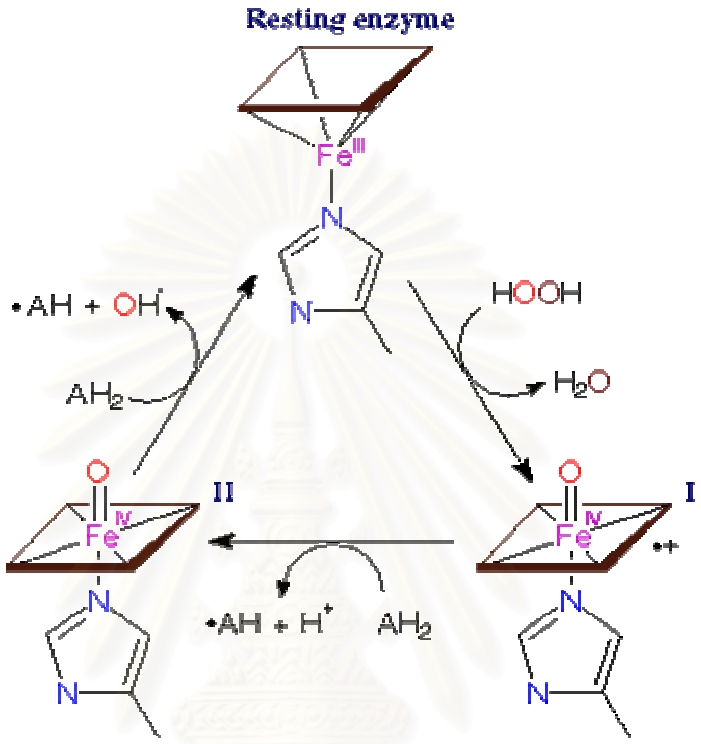
The structures of several POD have been determined, including yeast CCP, *Phanerochaete chrysosporium* LiP and MnP, *Arthromyces ramosus* peroxidase (ARP), pea cytosolic AP, peanut peroxidase (PNP) and horseradish peroxidase C (HRPC). All these proteins share the same architecture, consisting of two all-α domains; two antiparallel helices from two domains form a crevice in which the haem group is inserted. One helix, contributed by the C terminal domain, contains the fifth (proximal) haem iron ligand (His175 in CCP). The imidazole ring of the proximal His lies approximately perpendicular to the porphyrin plane with $N^{\varepsilon 2}$ bonded to haem iron and $N^{\delta 1}$ hydrogen bonded to the buried carboxylate group of an Asp residue (Asp235 in CCP). A number of POD (CCP, AP, LiP, MnP) also contain an aromatic residue (Trp or Phe) parallel to and in van der Waals contact with the imidazole ring of the proximal His.

Table 2.1 Heme peroxidase [36]

Heme type	Heme iron coordination	Axial iron ligand(s)	Formal iron oxidation/spin states
 Heme peroxidase	 Pentacoordinate / Hexacoordinate	N^{ε}_{His}; (H_2O or OH^-)	Fe^{II} (S=2); Fe^{III} (S=5/2)
	 Hexacoordinate	N^{ε}_{His}; H_2O, H_2O_2 or O_2	Fe^{II} (S=0); Fe^{III} (S=1/2)
	 Hexacoordinate	N^{ε}_{His}; O (O·)	Fe^{IV} (S=1)

2.2.2 Mechanism of POD

Most heme POD follow the reaction scheme:



Schemes 2.2 Mechanism of POD [37]

In this mechanism, the enzyme reacts with one equivalent of hydrogen peroxide to give compound I, a porphyrin π cation radical containing Fe^{IV}. This is a two electron oxidation/reduction reaction where hydrogen peroxide is reduced to water and the enzyme is oxidised. One oxidising equivalent resides on iron, giving the oxyferryl (Fe^{IV}=O) intermediate. Compound I then oxidises an organic substrate to give a substrate radical (·AH). Compound I undergoes a second one electron oxidation reaction yielding compound II, which contains an oxyferryl centre coordinated to a normal (dianionic) porphyrin ligand. Finally, compound II, is reduced back to the native ferric state with concomitant one electron substrate oxidation. The overall charge on the resting state and compound I is +1, while compound II is neutral.

2.2.3 Activity of POD

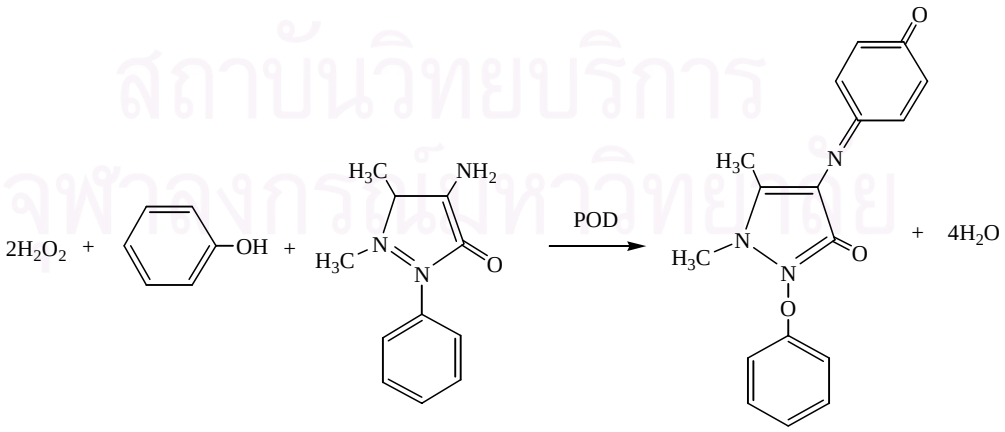
Total POD activity was reported in units (U/g of plant). One unit is the mount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minutes at 25^0C and pH 7.0. [38,39]

The activity of an enzyme is reduced by the extent of thermal denaturation which depends on both the temperature and incubation time. The productivity of an enzyme depends on how the rate of reaction varies with incubation time. The extent of any thermal denaturation will therefore have an important bearing on the enzyme's productivity.

Before an enzyme can be used for an industrial process, it is essential to characterise it in terms of its kinetic properties. One of these is its thermal stability. POD, a heme-containing oxidoreductase, is a commercially important enzyme which catalyses the reductive cleavage of hydrogen peroxide by an electron donor. POD is often used in radical polymerization and coupling reactions.

The reaction used in this practical involves hydrogen peroxide rapidly reacting with phenol and 4-aminoantipyrine (4-AAP) in the presence of HRP to produce a quinoneimine chromogen which is intensely colored with a maximum absorbance at 510 nm.

The amount of active POD present will directly influence the amount of quinoneimine product formed. There is a direct relationship between the quantity of active POD present and the color produced.



4-AAP quinoneimine

Schemes 2.3 Reaction of phenol with POD [40]

The absorbance of a 1.0 M quinoneimine chromogen assay product in a $1cm^3$ path-length cell is 6,580 (ΔA_{510} of 1.0 is equivalent to 1/6580 mol L^{-1} or 1/6.58 mmol mL^{-1}). Therefore the enzyme activity is given by:

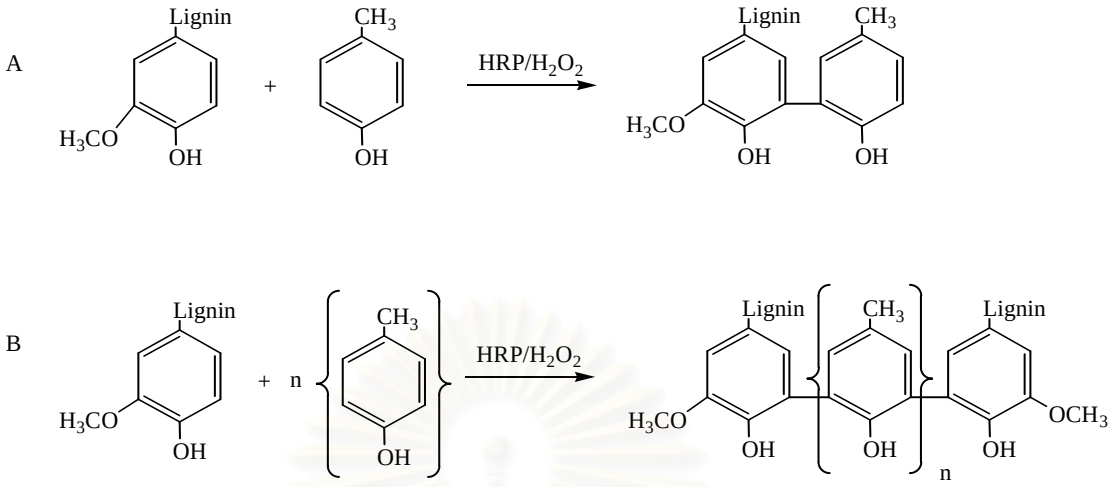
$$\text{Enzyme activity } (\mu mol\ min^{-1} mL^{-1} \text{of plant}) = \frac{\Delta A_{510} / min}{\mathbf{6.58}\ mL\,\mu mol^{-1} \times mg\ enzyme\ mL^{-1}\ reaction\ mixture}$$

2.3 Literature Reviews

In 1980, Klibanov *et al*. [2] reported removal of phenols and anilines from industrial waste waters. The treatment of aqueous solution containing the pollutants with HRP and hydrogen peroxide, results in precipitation of phenols and aromatic amines from water. This approach was used to remove over 30 different phenols and aromatic amines from water. The efficiency of the enzymatic removal is very high (exceeding 99%).

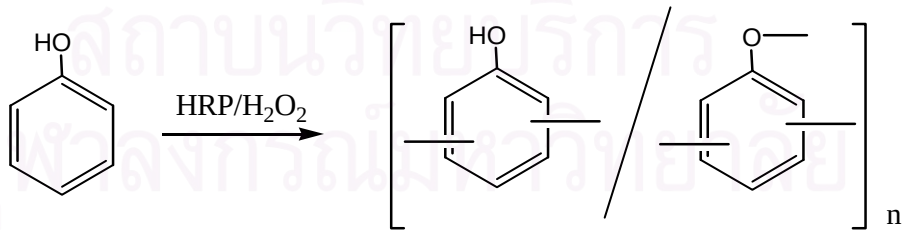
In 1983, Klibanov *et al.* [41] studied POD-catalyzed removal of phenols from coal-conversion waste waters. Treatment with HRP and hydrogen peroxide precipitates 97 to 99 percent of the phenols in a wide range of pH (3-12) and phenol concentration (0.01-5 g/L) have been treated successfully. Other pollutants, such as polychlorinated biphenyls, can be enzymatically coprecipitated with the phonols.

In 1999, Liu *et al.* [42] reported copolymerization of lignin with cresol catalyzed by POD in reversed micellar systems. POD catalysed copolymerization of strawpulp-lignin with cresol in a reversed micellar system was performed successfully. The molecular weight of the copolymer was controlled by adjusting the surfactant concentration.

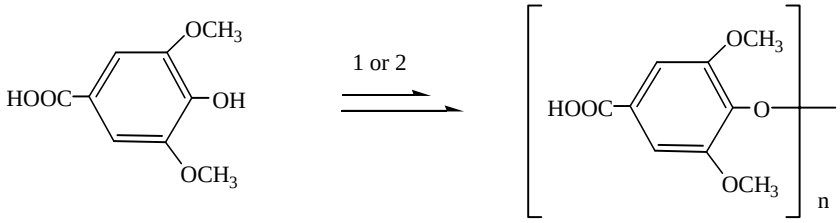


Schemes 2.4 Reaction for HRP catalyzed copolymization of lignin with cresol [42]

In 1999, Uyama *et al*. [43] reported that the preparation of polyphenol particles had been achieved by POD catalyzed dispersion polymerization. The polymerization using a water-soluble polymer as stabilizer in a mixture of 1,4-dioxane and phosphate buffer (pH 7) (80:20 vol%) afforded a powdery polymer. Similar particles were also obtained from *m*-cresols and *p*-cresols and *p*-phenylphenol. The thermal treatment of the polyphenol particles at 1000^0C under nitrogen produced the particles of carbonized polymer. The polymer was found to be composed of a mixture of phenylene and oxyphenylene units.



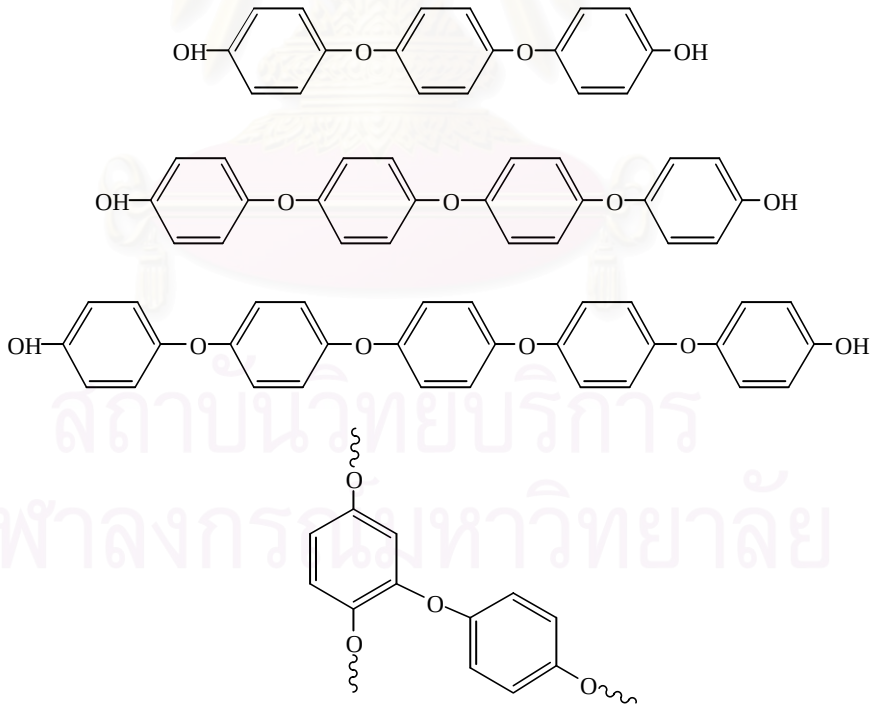
Schemes 2.5 The polymer of a mixture of phenylene and oxyphenylene units [43]



1 : POD + H_2O_2, -H_2O, -CO_2
2 : Laccase + O_2, -H_2O, -CO_2

Schemes 2.6 Enzymatic synthesis of poly(phenylene oxide) from 3,5-dimethoxy-4-hydroxybenzoic acid [43]

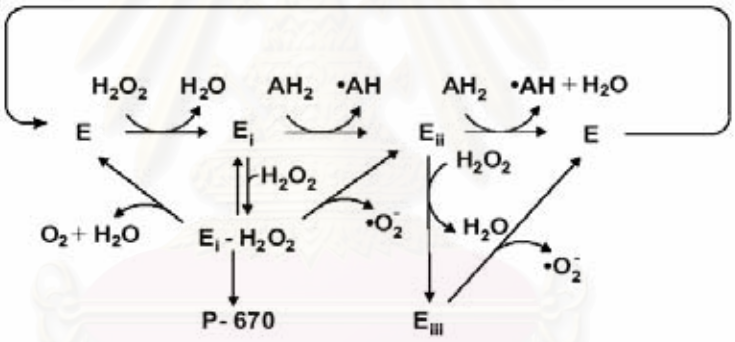
In 2000, Fukuoka *et al.* [44] studied POD-catalyzed oxidative polymerization of 4,4′-dihydroxydiphenyl either. The HRP and hydrogen peroxide were used as catalyst and oxidizing agent, respectively. The product was achieved (93%). The product was identified as trimer, tetramer, pentamer and hexamer of oligo (1,4-oxyphenylene), respectively.



∿∿∿ : Oligo (1,4-oxyphenylene) or H

Schemes 2.7 Trimer, tetramer, pentamer and hexamer of oligo(1,4-oxyphenylene)[44]

In 2002, Villalobos *et al.* [45] studied the removal of aqueous phenol by ARP, in the presence of high molecular weight polyethylene glycol (PEG) as a protective additive, and in the presence of excess hydrogen peroxide as oxidizing agent. The stoichiometry was observed to increase with increasing phenol conversion, and reached a maximum value of 0.9 mol phenol/mol peroxide when nearly complete phenol conversion was achieved. Spectral scans indicated that P-670 did not form with ARP in the presence of high concentrations of peroxide and in the absence of a reducing substrate. As long as phenol concentration is not limited, the turnover capacity varied by approximately 3% of average value. This indicates that only marginal improvement in the efficiency of enzyme would be achieved by adding enzyme. Similarly, sequential addition of hydrogen peroxide over an extended period of time yielded only a slight improvement in phenol removal from the reaction mixture.



Schemes 2.8 Complete peroxidase catalytic cycle with proposed side reactions [45]

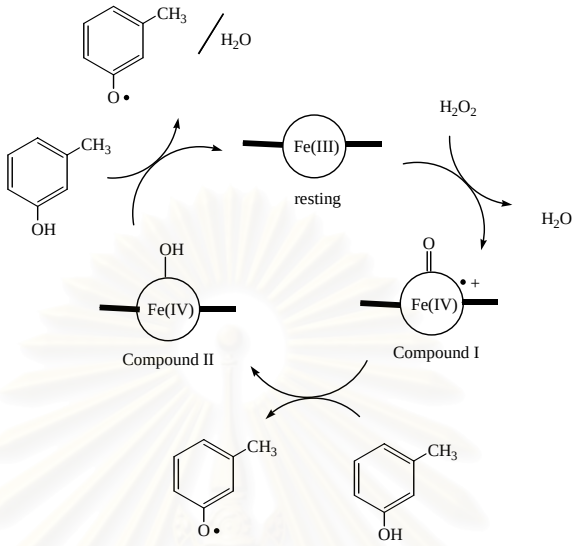
In 2002, Kennedy *et al.* [46] studied optimisation of soybean peroxidase (SBP) treatment of 2,4-dichlorophenol (2,4-DCP). Optimum pH for removal of 2,4-DCP using SBP as an oxidizing catalyst without PEG was pH 8.2. The pH operating range of SBP was from 2.5 to 9.4 which were wider than reported for HRP. Addition of PEG increased the effectiveness of SBP by factors of 10 and 50 for PEG-3350 and PEG-8000 respectively. The optimum pH of 6.2 was also found when SBP was used with PEG. Average 2,4-DCP removals achieved were 83.5%, 75.5% and 71.5% for 100, 200 and 300 mg/L 2,4-DCP concentrations respectively compared to 62%, 52% and 58% for the single batch addition control.

In 2002, Wilberg *et al.* [47] studied removal of aqueous phenol catalysed by a low purity soybean peroxidase (LP-SBP). LP-SBP obtained from waste seed hulls, as catalyst for phenol polymerization in aqueous solution in the presence of hydrogen peroxide is oxidizing agent. The product formed precipitate out from solution. The minimum LP-SBP required to 95 % precipitate of product. A retention time of 100 minutes was sufficient to achieve yields of 95 %.

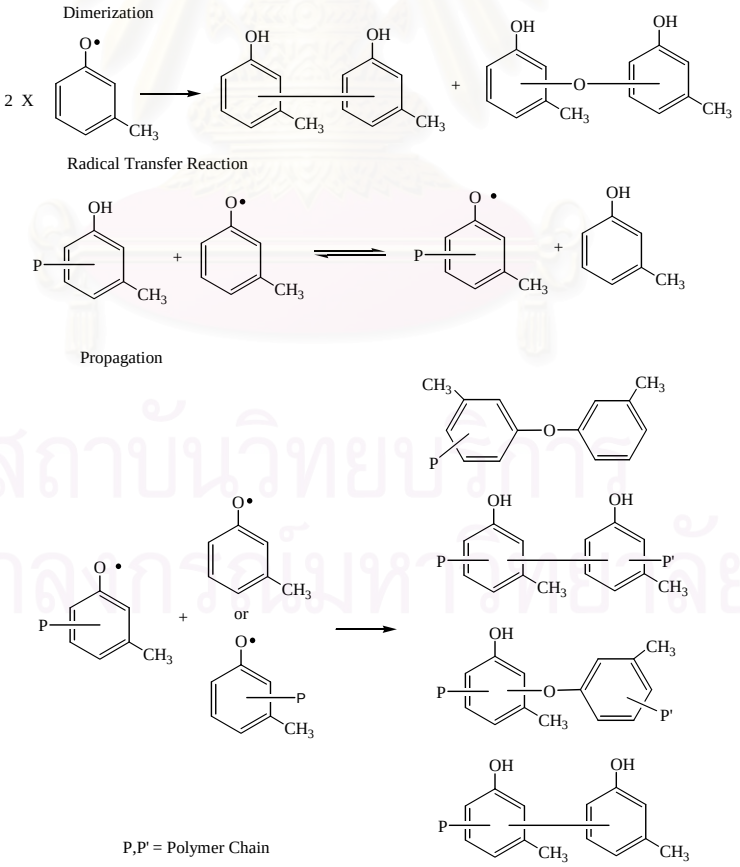
In 2002, Duarte-Vázquez *et al.* [1] studied the efficiency of using turnip peroxidase (TP) to remove several different phenols (phenol, 2-chlorophenol, 3-chlorophenol, *o*-cresol, *m*-cresol, 2,4-DCP and bisphenol-A). The remove efficiency $\geq 85\%$ was achieved at pH values between 4 and 8, after a contact time of 3 hours at 25^0C with 1.28Udm^{-3} of TP and 0.8 mmoldm^{-3} of hydrogen peroxide. Addition of PEG (100–200 mgdm^{-3}) significantly reduced the reaction time required (10 min) to obtain >95% removal efficiency and up to 230% increase in remaining TP activity. A relatively low enzyme activity (0.228 Udm^{-3}) was required to remove >95% of three phenols solutions in the presence of 100–200 mgdm^{-3} PEG. TP showed efficient and fast removal of aromatic compounds from synthetic wastewaters in the presence of hydrogen peroxide and PEG.

In 2002, Oguchi *et al.* [30] studied self-association of *m*-cresol in aqueous organic solvents: relation to enzymatic polymerization reaction. Oxidative polymerization of phenols using HRP in aqueous organic solvents (1,4-dioxane-water and methanol-water), which is an attractive pathway for synthesis of a new class of polyphenols without use of toxic formaldehyde, was remarkably influenced by the water content. The present study is focused on the self-association of *m*-cresol in such a solvent to understand the effect of solvent on its polymerization. The change of the molecular weight of the polymer with varying the water content was found to be related to the molecular clustering in the solution. On the other hand, the *m*-cresol clusters were formed favorably in methanol and were readily hydrated with increase of the water content in the solution. These effects of the water content on the clustering are related to the enzymatic polymerization. The polymer would be

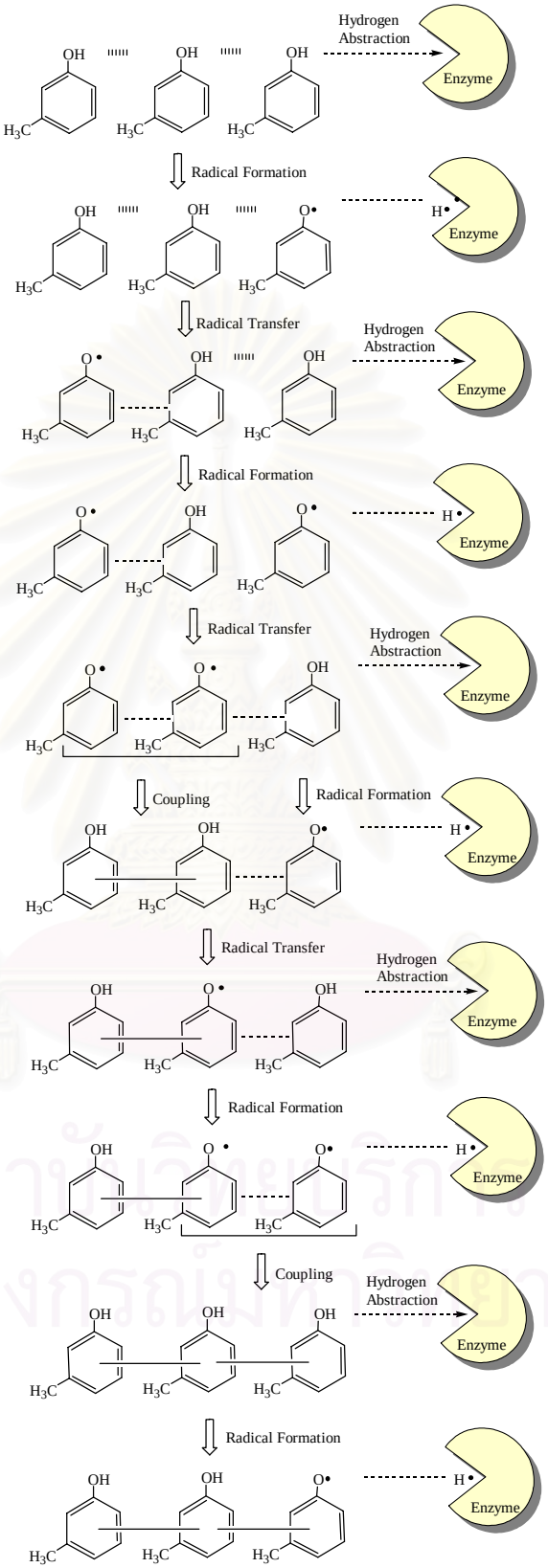
obtained in high yields when the *m*-cresol clusters were formed in the solution; however, the hydration of the *m*-cresol cluster would restrain the polymerization.



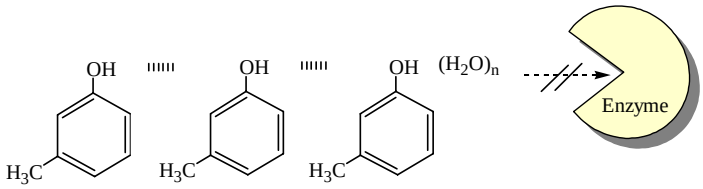
Schemes 2.9 POD-catalyzed reaction of *m*-cresol, a phenoxy type radical [30]



Schemes 2.10 Coupling of two radicals involving a radical transfer reaction [30]



Schemes 2.11 Polymerization proceeds the continuous radical formation process[30]



Schemes 2.12 Interaction between the *m*-cresol clusters and enzyme [30]

In 2003, Naghibi *et al.* [29] studied decontamination of water polluted with phenol using *Raphanus sativus* Root. Plant materials have been found useful in decontamination of water polluted with phenols. The detoxification effect is due to POD contained in plant tissue. Enzyme mediated oxidative coupling of phenol is followed by precipitation of the formed polymer and its removal from the aqueous phase. A synthetic wastewater buffered at pH = 7.4 containing 0.9 mM phenol was treated in this research with cut *Raphanus sativus* root and its juice. Cut Raphanus or Raphanus juice were added separately as enzyme source to phenol solution in buffer and in tap water in two series of experiments. The reaction was initiated by the addition of hydrogen peroxide. After three hours stirring the phenol content of the mixtures was determined. More than 90% of phenol was removed in both cases.

In 2003, Agostini *et al.* [31] studied Phytoremediation of 2,4-DCP by *Brassica napus* hairy root cultures. Hairy root cultures of *Brassica napus* with high biomass and genetic stability which produce POD, enzymes involved in biodegradation processes. Hairy root cultures were used to study the removal of 2,4-DCP, a common contaminant in industrial. The optimum conditions to obtain high efficiency in the removal process were established. Roots were able to remove 2,4-DCP from aqueous solutions containing 100–1000 mg/L, in the presence of hydrogen peroxide concentrations ranging from 5 to 10 mM. After a short period of incubation (15 min), high removal efficiencies were achieved (91–94%) and maximal removal, of approx. 97–98%, was obtained with 1 h of reaction. High removal efficiencies (93–95%) were observed in a broad pH range (pH 3–9), reaching 98–99% in the range pH 4–8. The roots could be re-used, almost for six consecutive cycles, to remove 2,4-DCP. The oxidation catalysed by POD would be the main mechanism involved in this process.

CHAPTER III

EXPERIMENTAL

In the present study of removal of phenols by POD derived from agricultural waste, the experimental procedure was divided into five steps.

1. General procedure for the extraction of crude enzyme under the optimum conditions.
2. Assay the activity of crude enzyme from agricultural waste.
3. Searching for the optimum conditions for removal of phenols
4. Characterization of product of removal phenol.

3.1 Analytical measurements.

3.1.1 UV-Vis Spectrophotometer.

UV-Vis Spectra were carried out on Perkin Elemer UV-lamp at Department of Chemistry, Rajamangala University of Technology Suvarnabhum.

3.1.2 Nuclear Magnetic Resonance Spectrometer (NMR)

^{1}H-NMR spectra were obtained on Bruker ACF 200 MHz and Varian model Mercury 400 spectrometers at Department of Chemistry, Chulalongkorn University. The NMR spectra were measured in dimethyl sulfoxide-d_6 (DMSO-d_6) at room temperature.

3.1.3 Fourier Transform Infrared spectrometer (FT-IR)

Fourier Transform Infrared spectra were recorded on Nicolet FT-IR Impact 410 Spectrophotometer at Department of Chemistry, Chulalongkorn University. The liquid samples were spread on sodium chloride (NaCl) cells. The solid samples were prepared by pressing the sample with potassium bromide (KBr). Infrared spectra were recorded between 400-4000 cm^{-1} in transmittance mode.

3.1.4 Gas chromatography -Mass spectrometer (GC-MS)

Gas chromatography was carried out on Varian CP-3800 and mass spectrometry was carried out on Varian, Saturn 2200 at Department of Chemical Technology, Chulalongkorn University.

3.2 Reagents and solvents.

The reagents and solvents used in this experiment were analytical grade.

Table 3.1 Reagents and solvents

Reagents and solvents	suppliers
1. Phenol	Carlo Erba Reagent
2. *o*-Cresol (*o*-methylphenol)	Asia Pacificialty Chemicals Limited
3. m-Cresol (*m*-methylphenol)	Fluka ChemieA.G.,Switzerland
3. *p*-Cresol (*p*-methylphenol)	Fluka Chemie A.G.,Switzerland
4. 2-Chlorophenol	Fluka Chemie A.G.,Switzerland
5. 3-Chlorophenol	Fluka Chemie A.G.,Switzerland
6. 4-Chlorophenol	Fluka Chemie A.G.,Switzerland
7. Pyrocatechcol (1,3-Benzenediol)	Asia Pacificialty Chemicals Limited
8. Hydroquinone (1,4-Benzenediol)	Asia Pacificialty Chemicals Limited
9. 1-Naphthol (α-Naphthol)	Fluka ChemieA.G.,Switzerland
10. 2-Naphthol (β-Naphthol)	Fluka Chemie A.G.,Switzerland
11. 4-Aminoantipyrine	Fluka Chemie A.G.,Switzerland
12. Citric acid	Carlo Erba Reagent

Table 3.1 Reagents and solvents

Reagents and solvents	**suppliers**
13. Hydrogen peroxide	Carlo Erba Reagent
14. Sodium citrate	Mallinckrodt Baker, Inc. Phillipsburg
15. Acetic acid	Mallinckrodt Baker, Inc. Phillipsburg
16. Sodium acetate	Mallinckrodt Baker, Inc. Phillipsburg
17. Monobasic sodium phosphate	Carlo Erba Reagent
18. Dibasic sodium phosphate	Carlo Erba Reagent
19. Boric acid	Carlo Erba Reagent
20. Borax	Carlo Erba Reagent
21. Sodium carbonate	Asia Pacificialty Chemicals Limited
22. Sodium bicarbonate	Asia Pacificialty Chemicals Limited
23. Acetone	Ajax Fine chem
24. Chloroform	VWR International Limited
25. Ammonium hydroxide	Carlo Erba Reagent
26. Potassium ferricyanide	Asia Pacificialty Chemicals Limited
27. Sodium chloride	Ajax Chemicals PTY Limited

3.3 General procedure for the extraction of crude enzyme

3.3.1 Extraction of crude enzyme (peroxidase enzyme)

This procedure of crude enzyme extract: wash, peel a fresh plant and cut the tissue into small cubes. A fresh plant was homogenized with solvents, and incubated at temperature (30^0C, 60^0C) for 1 hr. Extracts were filtered through nylon cloth and centrifuged at 2000 rpm for 10 minutes. Pour supernatant (contain POD) into a clean and labeled test tube and store on ice.

3.3.2 Effect of plant species

Plant species were varied: 10 g seed coat of soybean, peel of sweet potato, pulp of sweet potato, peel of pineapple, pulp of pineapple, axis of pineapple and peanut.

3.3.3 Effect of solvent type

The crude enzyme was extracted by three different solvents: 100 mL of distilled water, 5 mM NaCl and 5 mM phosphate buffer.

3.3.4 Effect of temperature

The crude enzyme was extracted at two different temperatures: 60^0C and 60^0C.

3.3.5 Effect of pH

The crude enzyme was extracted differ pH: 3.0, 5.0, 7.0, 9.0 and 11.0. A rang of buffers, citrate, acetate, phosphate, boric acid-borax and carbonate-bicarbonate were used at pH 3.0, 5.0, 7.0, 9.0 and 11.0 respectively.

3.4 Assay the activity of crude enzyme

3.4.1 Enzyme activity assays

The activity was measured using a reaction mixture consisting of 1.4 mL of 0.0025 M. 4-AAP with 0.17 M phenol and 1.5 mL of 0.0017 M of H_2O_2. The reaction was initiated by adding 0.10 mL of the crude enzyme. The total volume of the assay mixture was 3 mL and the light path of the cuvette used was 1 cm. The crude enzyme active concentration is proportional to the color development rate measured at 510 nm, during a period of time in which the substrate concentration is significantly induced. The color development rate during this period was converted to activity using an extinction coefficient of 6580 $M^{-1}cm^{-1}$ based on hydrogen peroxide. One unit of enzymatic activity is defined as the amount of enzyme which transforms 1.0 micromole of H_2O_2 per minute at 25^0C and pH 7.

3.4.2 Effect of plant species

Plant species were varied: 10 g seed coat of soybean, peel of sweet potato, pulp of sweet potato, peel of pineapple, pulp of pineapple, axis of pineapple and peanut.

3.4.3 Effect of solvent type

Solvent type was varied: 100 mL of distilled water, 5 mM NaCl and 5 mM phosphate buffer.

3.4.4 Effect of temperature

The temperature was varied: room temperature (30^0C) and 60^0C. The stabilization of crude enzyme were kept at different temperatures, such as -5^0C (freezing), 7^0C (cool), UV-lamp (30^0C) and room temperature (30^0C) for 1 hr. The crude enzyme should be brought to the measuring activity before they are kept.

3.4.5 Effect of pH

The pH was varied: 3.0, 5.0, 7.0, 9.0 and 11.0. A rang of buffers, citrate, acetate, phosphate, boric acid-borax and carbonate-bicarbonate were used at pH 3.0, 5.0, 7.0, 9.0 and 11.0 respectively.

3.5 Searching for the optimum condition for removal of phenol

3.5.1 Determination of phenol

The method was modified as follows: to 5 mL of 20 ppm phenol solution, 0.5 M ammonium hydroxide (NH_4OH) and 0.2 M. phosphate buffer were added until pH 7. After stirring, the mixture was supplemented with 3 mL of 2% w/w 4-aminoantipyrine (4-AAP) in water and then with 3 mL of 8% w/w potassium ferricyanide ($K_3Fe(CN)_6$). After standing for several minutes required for the development of full color, the dye formed was extracted with 25 mL chloroform. The samples absorbance was measured the maximum absorbance (460 nm).

3.5.2 Removal of phenols

The method was modified as follows: to 5 mL of 20 ppm. phenols solution, 0.5 M. ammonium hydroxide (NH_4OH) and 0.2 M phosphate buffer were added until pH 7 and added 5 mL of H_2O_2, 5 mL of crude enzyme. After stirring, the mixture was supplemented with 3 mL of 2% w/w 4-AAP in water and then with 3 mL of 8% w/w potassium ferricyanide ($K_3Fe(CN)_6$). After standing for several minutes required for the development of full color, the dye formed was extracted with 25 mL chloroform. The samples absorbance was measured the maximum absorbance.

3.5.3 Effect of plant species

The effect of plant species on removal of phenol was investigated.

3.5.4 Effect of solvent type

The effect of solvent type on removal of phenol was investigated.

3.5.5 Effect of temperatures

The effect of temperature on removal of phenol was investigated.

3.5.6 Effect of pH

The effect of pH on removal of phenol was investigated

3.5.7 Effect of time

The removal of phenol by POD was studied by varying time: 5, 10, 20, 40, 60, 80, 100 and 120 minutes respectively.

3.5.8 Effect of phenol concentration

The phenol concentration was varied: 5 mL of 20, 40, 60, 80 and 100 ppm of phenol.

3.5.9 Effect of hydrogen peroxide concentration

The hydrogen peroxide concentration was varied: 5 mL of 0.1, 0.5, 1, 3 and 5 % w/w H_2O_2.

3.5.10 Effect of POD activity

The POD activity was varied: 5 mL of 1, 2, 4, 6, 8 and 10 units/mL POD.

3.5.11 Effect of PEG

The effect of PEG molecular weight on removal of phenol and comparing the molecular weight of PEG: PEG-3000, PEG-6000.

3.6 Kinetic study of phenol removal

The kinetic study for phenol removal was performed by POD as biocatalyst under optimum conditions using initial rate method. The reaction was monitored by spectrophotometer.

3.7 Searching for the removal of phenol

3.7.1 Effect of hydrogen peroxide concentration

The hydrogen peroxide concentration was varied: 5 mL of 0.1, 0.5, 1, 3 and of 5 % w/w H_2O_2.

3.7.2 Effect of pH

The pH was varied: 3.0, 5.0, 7.0, 9.0 and 11.0. A rang of buffers, citrate, acetate, phosphate, boric acid-borax and carbonate-bicarbonate were used at pH 3.0, 5.0, 7.0, 9.0 and 11.0 respectively

3.8 General procedure for removal of phenols

The reaction mixture consisting of 5 mL of 20 ppm substrate solution, 0.5 M ammonium hydroxide (NH_4OH), 0.2 M phosphate buffer were added until pH 7 and added 5 mL of H_2O_2, 5 mL of crude enzyme. The reaction was stirred and incubated at room temperature for 1 hr, the product was extracted with 25 mL chloroform and the precipitate appeared upon evaporation of all solvent. Accordingly, the precipitate was easily recovered by centrifugation or filtration, washed with water to remove the traces of enzyme.

For products analysis by FT-IR, NMR, GC-MS the internal standard method was used. FT-IR reveals the types of functional groups present in a molecule, NMR gives information about the number of magnetically distinct atoms of the type being studied. When (protons) are studied, for instance, one can determine the number of each of the distinct types of hydrogen nuclei as well as to obtain information regarding the nature of the immediate environment of each type. The molecular mass of sample was determined by GC-MS.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The sample selection and general procedure for extraction

Four agricultural wastes including peel of sweet potato, seedcoat of soybean, peel of pineapple and axis of pineapple, and peanut were selected as material sources. The general extraction for enzyme activity assay consisted of three steps: the homogenizing of samples with a phosphate buffer (pH 7), filtrating through nylon and centrifuging to gain the crude extracts.

4.2 Enzyme activity assay

POD is ubiquitous and has been found in a wide range of plant species, in which it has multiple molecular forms and a broad subcellular distribution. It is found not only in cytoplasm, and cell organelles, but is also associated with cell walls.

The reaction used in this practical involves H_2O_2 rapidly reacting with phenol and 4-AAP in the presence of POD to produce a quinoneimine chromogen which is intensely colored with a maximum absorbance at 510 nm. The amount of active POD present will directly influence the amount of quinoneimine product formed. There is a direct relationship between the quantity of active POD present and the color produced. POD activity was expressed as $\mu mol\ min^{-1}\ g^{-1}$ of plant (unit/g of plant)

The reaction rate is determined by measuring an increase in absorbance at 510 nm in a spectrophotometer at 25^0C, and calculated the ΔA_{510} per minute using the quantity of active. The absorbance of a 1.0 m quinoneimine chromogen assay product in a 1-cm-path-length cell, and the crude POD activity was calculated using the extinction coefficient of 6,580 (ΔA_{510} of 1.0 is equivalent to 1/6,580 mol L^{-1} or 1/6.58 $\mu mol\ mL^{-1}$).

All the extracts obtained above were assayed according to the mentioned general procedure. The results of ΔA_{510} per minute derived from 7 samples including seed coat soybean, peel of sweet potato, pulp of sweet potato, peel of pineapple, pulp of pineapple, axis of pineapple and peanut are collected in Table 4.1.

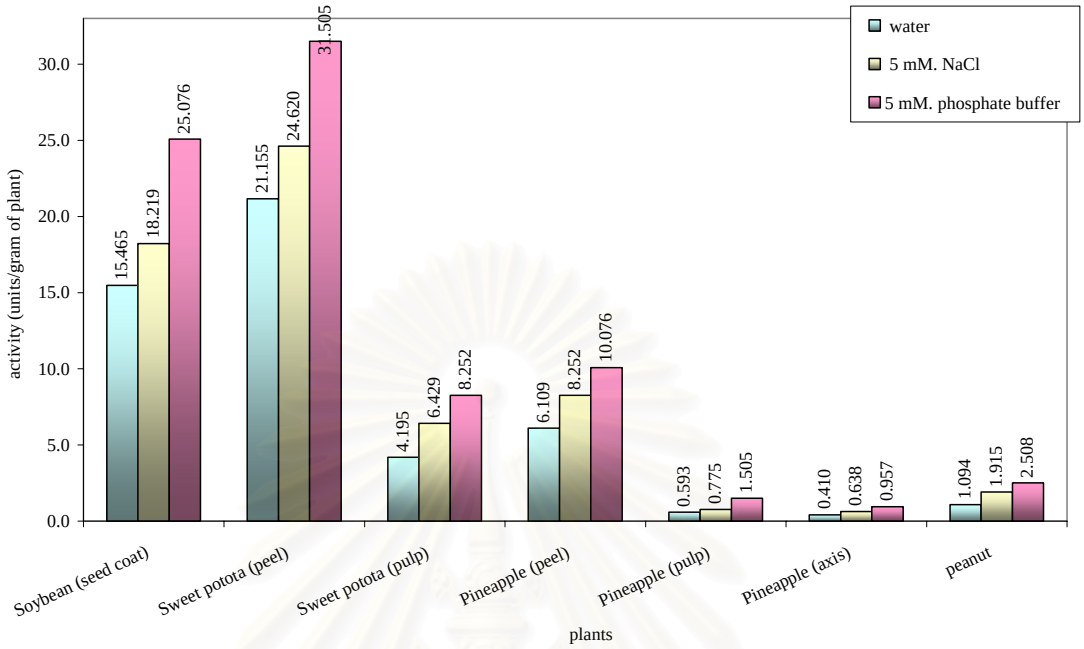
Table 4.1 ΔA_{510} per minute derived from 7 samples

sample	ΔA_{510} per minute
seed coat soybean	0.339
peel of sweet potato	0.691
pulp of sweet potato	0.181
peel of pineapple	0.221
pulp of pineapple	0.033
axis of pineapple	0.021
peanut	0.055

The results clearly revealed that the increasing of absorbance at 510 nm of crude POD from the peel of sweet potato was the highest (31.505 unit/g of plant). The lower activities were obtained from the seed coat of soybean, the pulp of sweet potato and the peel of pineapple (15.465, 10.076 and 8.252 unit/g of plant, respectively).

4.3 Effects of solvent types

The isoenzyme composition of free and bound POD appears to differ. Cationic POD may not be extracted by a low ionic strength medium, while anionic and neutral POD can readily be eluted by this medium. Various factors are evaluated to find conditions for the crude POD and the activity of crude POD. The next parameter studied in this reaction is solvent types: water, 5 mM NaCl and 5 mM phosphate buffer. The results are collected in Figure 4.1



Reaction conditions: 1.4 mL of 0.0025 M with 0.17 M phenol, 1.5 mL of 0.0017 M H_2O_2 and 0.1 mL of crude POD, at 25^0C and 510 nm

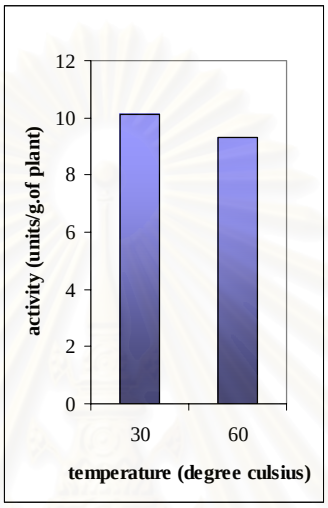
Figure 4.1 Activity of crude POD extracted from plants with three different solvents

The results exhibited that the crude POD activity was different depended on plant species and type of solvent. The activity of crude POD from peel of sweet potato is higher than those of seed coat soybean, peel of pineapple, pulp of sweet potato, peanut, pulp of pineapple, axis of pineapple, respectively. In case of solvent type, the order of the activity of crude POD gained from the highest to the lowest was achieved from 5 mM phosphate buffer, 5mM NaCl and water, respectively.

4.4 Effect of temperature

The activity of an enzyme is reduced by the extent of thermal denaturation which depends on both the temperature and incubation time. The productivity of an enzyme depends on how the rate of reaction varies with incubation time. The extent of any thermal denaturation will therefore have an important bearing on the enzyme's productivity.

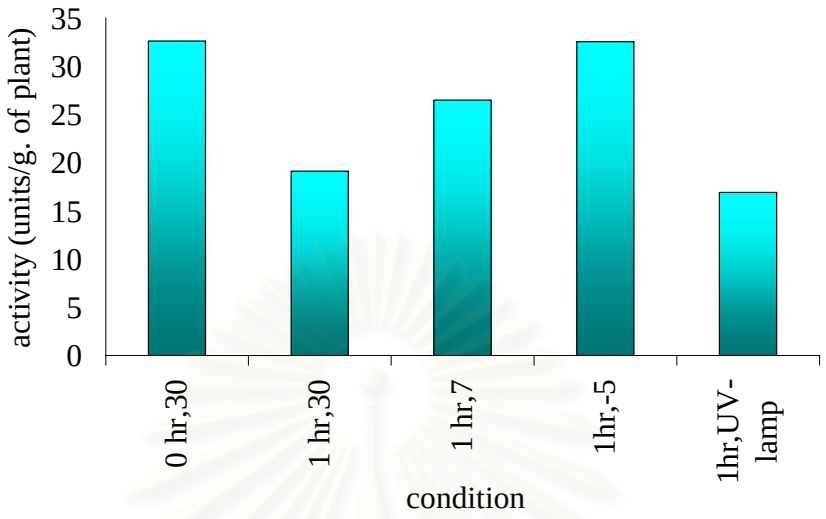
The temperature effect on the activity of crude POD was investigated. It was found that the activity of crude POD from the peel of sweet potato extracted by 5 mM phosphate buffer was enhanced with decreasing temperature. The activity of crude POD at 30^0C gives better activity than 60^0C about 8.1 %. The results are collected in Figure 4.2.



Reaction conditions: 1.4 mL of 0.0025 M with 0.17 M phenol, 1.5 mL of 0.0017 M H_2O_2 and 0.1 mL of crude POD, at 25^0C and 510 nm

Figure 4.2 Temperature effect on the activity of crude POD from the peel of sweet potato extracted with 5 mM of phosphate buffer

The stability of crude POD was another important point to be seriously considered. The crude extracts of the peel of sweet potato were then experimented by keeping in various conditions: -5 ^{0}C, 7^0C, 30^0C and 30^0C under UV-lamp for 1 hr. The results are presented in Figure 4.3.



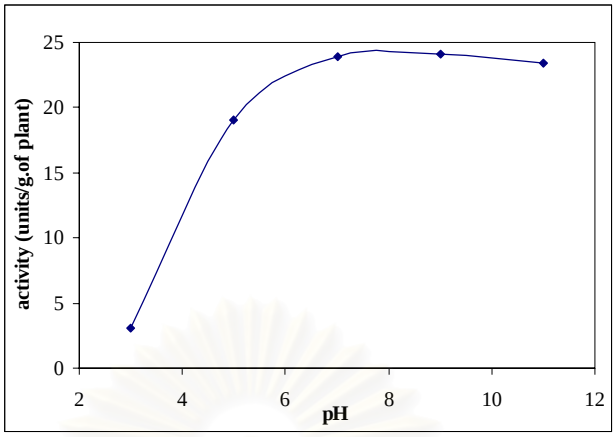
Reaction conditions: 1.4 mL of 0.0025 M with 0.17 M phenol, 1.5 mL of 0.0017 M H_2O_2 and 0.1 mL of crude POD, at 25^0C and 510 nm

Figure 4.3 Stability of crude POD under various conditions

Figure 4.3 reveals that temperature and UV light are two essential parameters affecting the stability of crude enzymes. At room temperature, the activity of the POD markedly decreased about 41.37%. Keeping the enzyme in refrigerator or in the freezer at 7^0C and -5^0C may assist to prolong the enzyme activity: the activity lost was 24.55% and only 0.25%, respectively. In addition, under UV-light, the enzyme activity lost was comparable to that observed in the first case. Thus, UV-light had additional effect to destroy enzyme activity, *i.e.*, the activity lost was 11.53% compared with the first case that exposed to normal light at room temperature.

4.5 Effect of pH

The pH is another important factor for condition optimization on the activity of crude POD. The pH was varied from 3,5,7,9 and 11 and the results are presented in Fig 4.4.



Reaction conditions: 1.4 mL of 0.0025 M. with 0.17 M. phenol, 1.5 mL of 0.0017 M. H_2O_2 and 0.1 mL of crude POD, at 25^0C and 510 nm.

Figure 4.4 Effect of pH on the activity of crude POD from peel of sweet potato

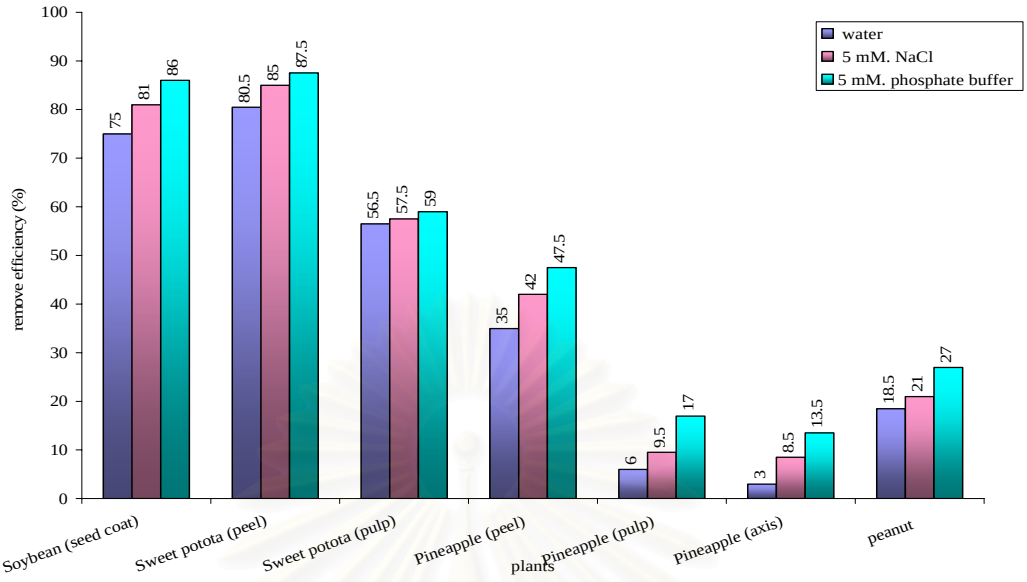
The effect of pH was clearly observed in Figure 4.4. The activity of enzyme can be maintained in the pH range 7-9. In acidic solution (pH < 7), the activity of enzyme was tremendously decreased. The main reason was possibly due to the pH dependence of enzyme. At lower pH, there was a gradual lost of POD efficiency compared with the activity at pH 7. Therefore, in an acidic environment it can be speculated that the POD enzyme is denaturing. On the contrary, the enzyme activity in basic solutions (pH 9-11) seemed to be comparable to that at pH 7.

4.6 Searching for the optimum conditions for removal of phenol

This section involves with systematically examination on the removal of phenol using crude POD as an oxidizing catalyst. The effects of species of plant, solvent type, temperature, pH, time, phenol concentration, H_2O_2 concentration, activity of crude POD and additive (PEG) were thoroughly investigated.

4.6.1 Effect of species of plants and effect of solvent type

Various factors including plant species and solvent type are evaluated to find suitable conditions for removal of phenols. Plant species are seed coat soybean, peel of sweet potato, pulp of sweet potato, peel of pineapple, pulp of pineapple, axis of pineapple and peanut. The next parameter studied in this reaction is solvent types: water, 5 mM NaCl and 5 mM phosphate buffer. The results are collected in Figure 4.5



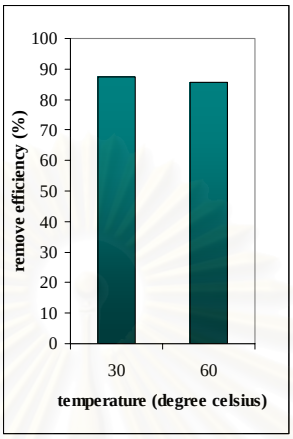
Reaction conditions: 5 mL of 20 ppm phenol, 5 mL of 0.1% w/w H_2O_2, 5 mL of 6 units/g of plant crude POD, 1-h treatment, pH 7 at room temperature.

Figure 4.5 Remove efficiency of phenol by crude POD was extracted from plants and solvent types.

The removal efficiency of crude POD derived from different plant sources and from different solvents was found not to be the same. The best results of the phenol removal above 85% could be observed from the treatment of phenol either with the crude enzyme from peel of sweet potato extracted by 5 mM phosphate buffer or with that gained from the seed coat of soybean by the same buffer solution. Other crude enzymes obtained from different plant species did not exhibit good results for phenol removal. Each plant species contained, in fact different enzymes, for instance in soybean composed of SBP enzyme, while the other plant species contained HRP enzyme instead of SBP. These obtained results strongly suggest that both enzymes could be utilized for phenol removal. In addition, SBP enzyme was reported to be able to tolerate at higher temperature than HRP. The result of extraction of enzyme by various solvents confirmed previous results that the extraction with buffer provided better results in terms of enzyme activity than the extractions with NaCl solution and water, [34]

4.6.2 Effect of temperatures

The temperature is another important factor for condition optimization on the removal of phenol by crude POD. The data showed in Figure 4.6



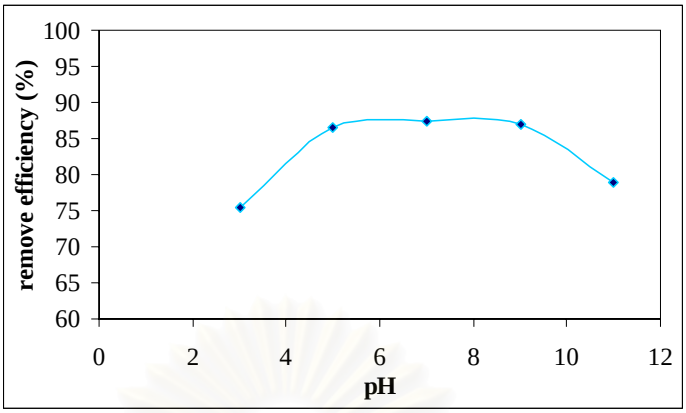
Reaction conditions: 5 mL of 20 ppm. phenol, 5 mL of 0.1% w/w H_2O_2, 5 mL of 6 units/g of plant crude POD, 1-h treatment and at pH 7

Figure 4.6 Effect of temperature on removal of phenol at 30^0C and 60^0C

The temperature in the reaction was varied from 30^0C to 60^0C**.** The results of the effect of temperature exhibited that the removal of phenol by crude POD from peel of sweet potato at 30^0C was of comparable efficient with that carried out at 60^0C. It may have an argument that the temperature above 30^0C would destroy the enzyme. However, certain literatures addressed that at higher temperature the rate of reaction enhanced by increasing the activity of enzyme. [46]

4.6.3 Effect of pH

The effect of pH on the efficiency of phenol removal was investigated. The results are collected in Figure 4.7



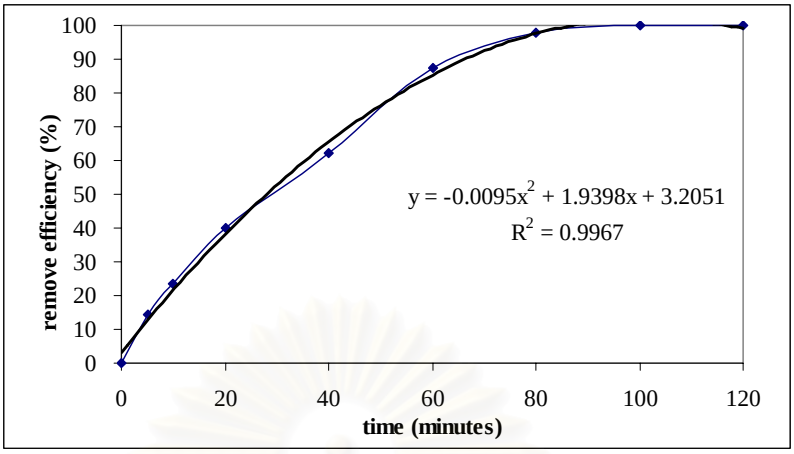
Reaction conditions: 5 mL of 20 ppm. phenol, 5 mL of 0.1% w/w H_2O_2, 5 mL of 6 units/g of plant crude POD, 1-h treatment and at room temperature.

Figure 4.7 Effect of pH for removal of phenol by crude POD

The pH in the reaction was varied from 3 to 11. The results showed that the removal efficiency of enzyme can be maintained within the pH range of 5-9. In acidic solution (pH <5), the removal efficiency of enzyme was decreased. The reason was possibly due to the pH dependence of enzyme, while the ferriheme group (essential for enzyme activity) may be dissocated from the protein, thereby inactivating. [1] At lower pH, there was a gradual lost of POD efficiency compared with that at pH 7. The removal efficiency in solutions (pH 9-11) was comparable to that at pH 7. The basic had been attributed to product instability and formed ligand complex of hydroxide to ferriheme group. [8]

4.6.4 Effect of time

The effect of time is another important factor for condition optimization. The efficiency of the reaction was justified from the complete removal of phenol 20 ppm. The time was varied from 5, 10, 20, 60, 80, 100 and 120 min. The data is collected in Figure 4.8.



Reaction conditions: 5 mL of 20 ppm phenol, 5 mL of 0.1% w/w H_2O_2, 5 mL of 6 units/g of plant crude POD, pH 7 and at room temperature

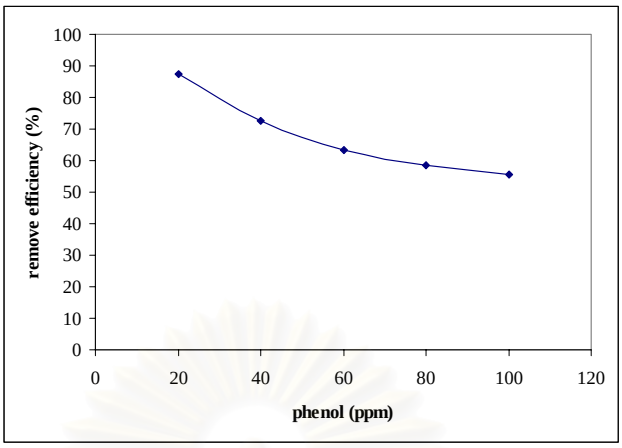
Figure 4.8 Effect of time for removal of phenol by crude POD

The removal of phenol using POD enzyme from the peel of sweet potato revealed interesting results. To illustrate this, the removal of phenol was completed within 80 min with the 50% efficiency of the reaction about 24 min.

The removal of phenol found to increase with the progress of the reaction. The fast initial reaction followed by a very slow removal process has been attributed to the chemical coupling of peroxidase-generated free radicals. The drastic slow down was mainly attributed to the simultaneous decrease of all reacting species or enzyme inactivation. The short times needed for POD treatment make this application attractive for the removal of phenol from solution, since physical and chemical treatment involves longer periods of time. [1]

4.6.5 Effect of phenol concentration

The effect of phenol concentration was investigated the condition suitable of removal efficiency phenol highest removal efficiency. The phenol concentration was varied from 20, 40, 60, 80 and 100 ppm. The results are collected in Figure 4.9



Reaction conditions: 5 mL of phenol, 5 mL of 0.1% w/w H_2O_2, 5 mL of 6 units/g of plant crude POD, pH 7 and at room temperature

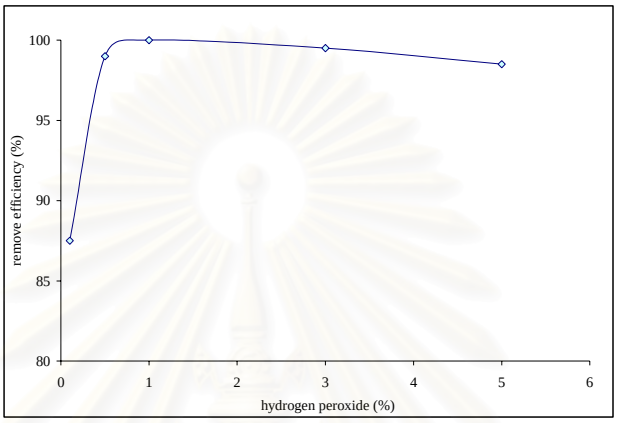
Figure 4.9 Effect of phenol concentration for removal of phenol by crude POD

In order to detect the maximum of phenol concentration suitable for this removal condition, a series of experiment was conducted. 20 ppm of phenol was still found to be applicable under the standard conditions examined. For the phenol tested, the highest phenol removal efficiencies were observed at the lowest concentration used (20 ppm). At the maximum phenol concentrations tested (100 ppm), the removal efficiency decreased to a low of 55.4%. However, the lowest phenol removal efficiency could conversion of phenol was achieved 55.4 ppm. Thus, the lowest phenol removal efficiency could conversion of phenol was achieved 17.5 ppm.

Lower removal efficiency at higher phenol concentration indicates enzyme inhibition by the substrate. Therefore, the amount of enzyme should be increased proportionally to the phenol compound' concentrations to achieve adequate removal efficiencies, when the concentration in waste water exceeds that used here. For the phenol compound tested, lower removal efficiency was observed when their concentration increased. The highest phenol removal efficiencies were observed at the lowest concentrations used. [46]

4.6.6 Effect of H_2O_2 concentration

The concentration of H_2O_2 in this reaction was important. The hydrogen peroxide concentration was varied: 0.1, 0.5, 1.0, 3.0 and 5.0% w/w. The results show in Figure 4.10



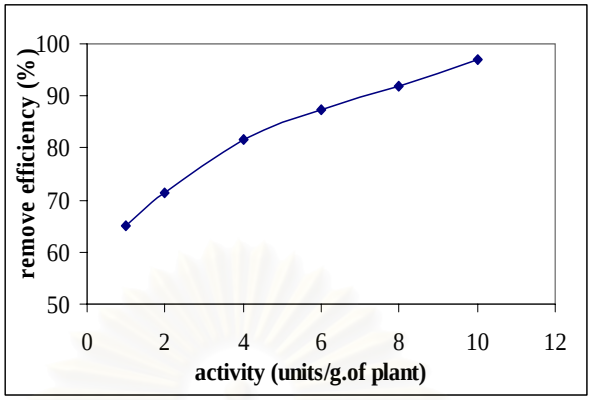
Reaction conditions: 5 mL of 20 ppm. phenol, 5 mL of H_2O_2, 5 mL of 6 units/g of plant crude POD, pH 7 and at room temperature

Figure 4.10 Effect of hydrogen peroxide concentration for removal of phenol by crude POD

The removal efficiency of phenol increased with the increment of hydrogen peroxide at concentration of 0.1-1.0 % w/w. Whereas, the removal efficiency of phenol decreased at the hydrogen peroxide concentration of 3.0 and 5.0 % w/w. In addition, high hydrogen peroxide concentration could act as an inhibitor of peroxidase catalytic activity by irreversible oxidation of the enzyme ferriheme group essential for peroxidase activity. [8]

4.6.7 Effect on activity of crude POD

The relationship between the rate of reaction and the activity of crude POD was studied. The activity of enzyme was varied from 1, 2, 4, 6, 8 and 10 units/mL. The data show in Figure 4.9**.**



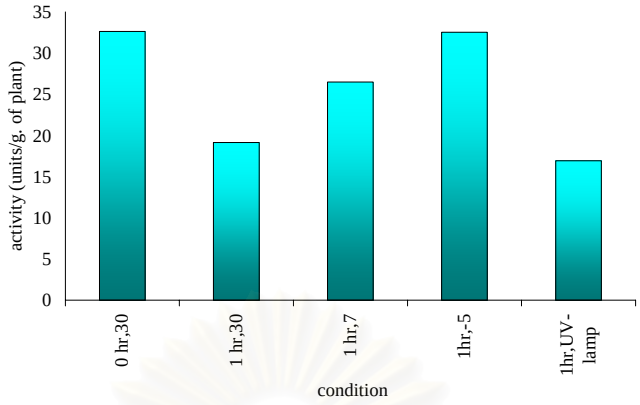
Reaction conditions: 5 mL of 20 ppm phenol, 5 mL of 0.1 % w/w H_2O_2, 5 mL of crude POD, pH 7 and at room temperature

Figure 4.11 Effect on activity of POD for removal of phenol by crude POD

The results displayed the activity of crude POD for removal of phenol in the reaction. The maximum conversion of phenol after 60 min was 10 units/mL and minimum conversion of phenol after 60 min was 1 unit/mL of crude POD. However, the minimum quantity of crude POD to convert phenol was 13 μg/unit. Thus, the activity of crude POD affected the removal efficiency of phenol. The highest removal efficiencies of phenol were achieved at the highest activity of crude POD used in reaction.

4.6.8 Effect of PEG

The optimum condition for removal of phenol on activity was in the presence of additive such as 20 ppm of PEG-3000, 20 ppm of PEG-6000, 20 ppm of PEG-6000 and 100 ppm PEG-6000 of conversion of phenol. The result are collected in Figure 4.12



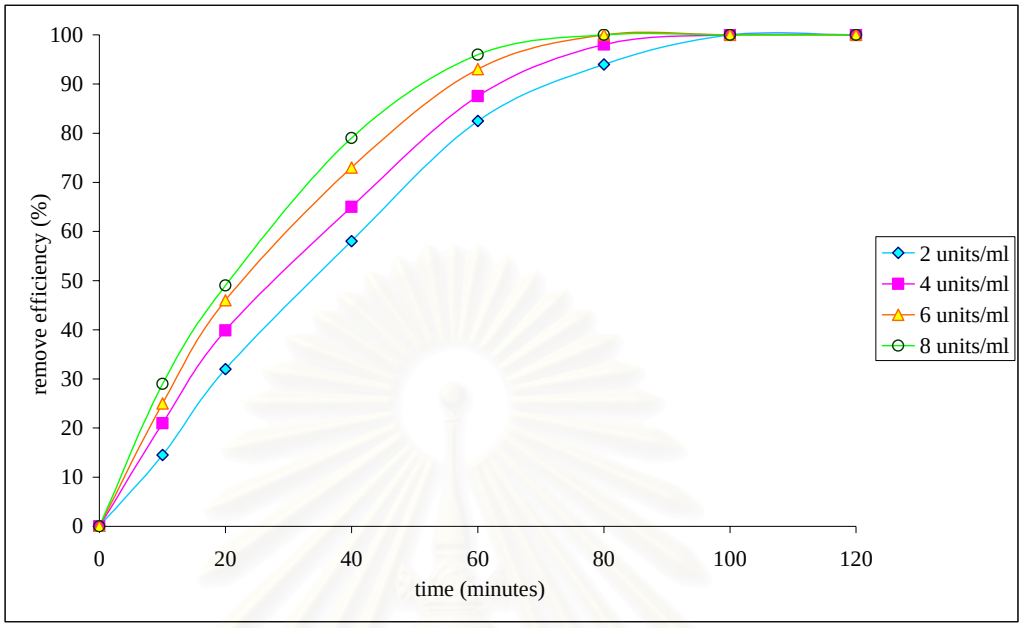
Reaction conditions: 5 mL of 20 ppm phenol, 5 mL of 0.1 % w/w H_2O_2, 5 mL of crude POD, pH 7 and at room temperature

Figure 4.12 Effect of PEG for removal of phenol by crude POD

When 20 ppm of PEG-3000 and 20 ppm of PEG-6000 were added, a significant increase in enzyme efficiency compared with the control non-PEG was observed from 87.5 as 88 and 92.5%. Thus, 100 ppm of PEG-3000, 100 ppm of PEG-6000 showed a significant decreasing efficiency compared with the control non-PEG from 87.5 as 85 and 86%. Nevertheless, for all experiments examined, added PEG exerted a significant protective effect on crude POD residual activity. The over PEG concentration could combine with activity of crude enzyme. The enzyme could not react with phenol. PEG offers free radicals that form a binding site to attach to another free radical. Such as PEG can be used to bind with the forming polymers and prevent the enzyme from becoming entrapped. Therefore multiplying the molecular mass from 3000 to 6000, the removal efficiency was increased by 4.5% at concentration of 20 ppm. Increasing the molecular mass achieves the marginal better enzyme efficiency. [46]

4.6.9 Effect of time and activity of crude POD

The effect of activity of crude POD in the reaction removal of phenol was varied from 2 units/mL, 4 units/mL, 6 units/mL and 8 units/mL And the effect of reaction time was varied from 10, 20, 40, 60, 80, 100 and 120. From data in Figure 4.13



Reaction conditions: 5 mL of 20 ppm phenol, 5 mL of 0.1 % w/w H_2O_2, 5 mL of crude POD, pH 7 and at room temperature

Figure 4.13 Effect of time and activity of crude POD

The same reaction time (10 minutes), the higher activity of crude POD has been removal efficiency than lower activity of crude POD. Increasing the activity of enzyme proportionally increased the enzyme efficiency.

In case of the same activity of crude POD (2 units/ml) the reaction time is the highest removal of efficiency. The best condition of reaction time was at 80 minutes and 8 units/ml activity of crude POD, the removal efficiency is 100%. The variation of reaction time is the fast initial reaction followed by a very slow removal process had been attributed to the chemical coupling of peroxidase-generated free radical. The drastic slowdown was mainly attributed to the simultaneous decrease of all reacting species or enzyme inactivation. [1]

4.7 Kinetic study of phenol removal

The kinetics of the reaction was studied for the progress of the reaction. The removal phenol by POD using hydrogen peroxide as an oxidizing agent was examined. The reaction was monitored by spectrophotometer.

Conversion of substrate was calculated using their initial concentration at 0 min (C_0) and concentration after t min reaction (C_t). Conversion x is expressed as equation, assuming that the volume and mass of the reaction mixture were constant.

$$x = \frac{C_0 - C_t}{C_0}$$

The wavelength at maximum absorption of the reaction was determined at 510 nm.

Table 4.2 Kinetic study for removal of phenol

Time (minutes)	Absorbance	Phenol (μmol)
0	1.312	1.065
10	0.932	0.756
20	0.617	0.500
40	0.171	0.138
60	0.025	0.020
80	0.000	0.000

Reaction conditions: 5 mL of 20 ppm. Phenol, 5 mL of 0.1% w/w H_2O_2, 5 mL of 6 units/g of plant crude POD, pH 7, 1-h treatment at room temperature

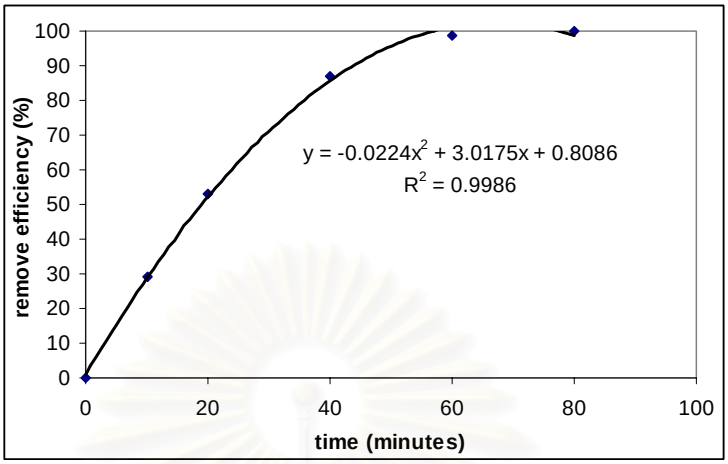


Figure 4.14 Kinetic studies for removal of phenol

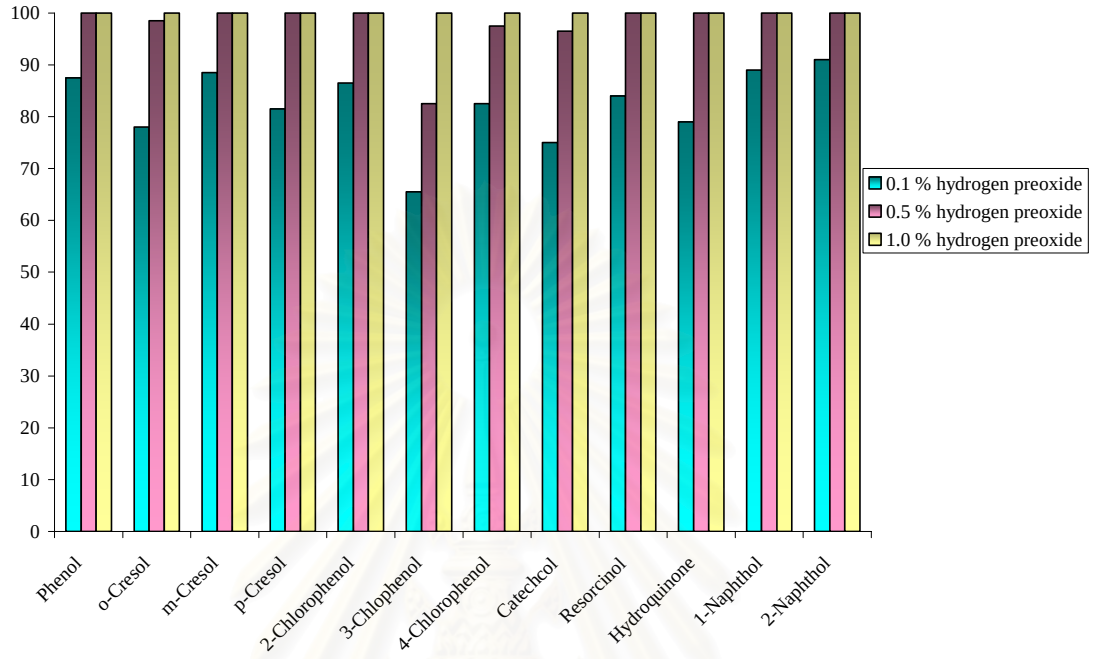
From data in Table 4.2 and Figure 4.14 with variation of time, it was found that remove efficiency of reaction was decrease with the reaction time were more than 40 minutes. The time around 20 minutes was adequate for removal of phenol at room temperature (30^0C). Rate of conversion of phenol under this condition was 1.045 μmol per hr.

4.8 Searching for the optimum conditions for removal of phenols

This section involves with systematically examination on the removal of phenols consist *o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, catechcol, resorcinol, hydroquinone, 1-napthol, 2-napthol using crude POD as an oxidizing catalyst. The effects of pH and H_2O_2 concentration were thoroughly investigated.

4.8.1 Effect of hydrogen peroxide concentration

Effect of hydrogen peroxide concentration can be seen from the experimental results. Hydrogen peroxide concentration was varied: 0.1, 0.5 and 1.0 % w/w H_2O_2. The results are collected in Figure 4.15.



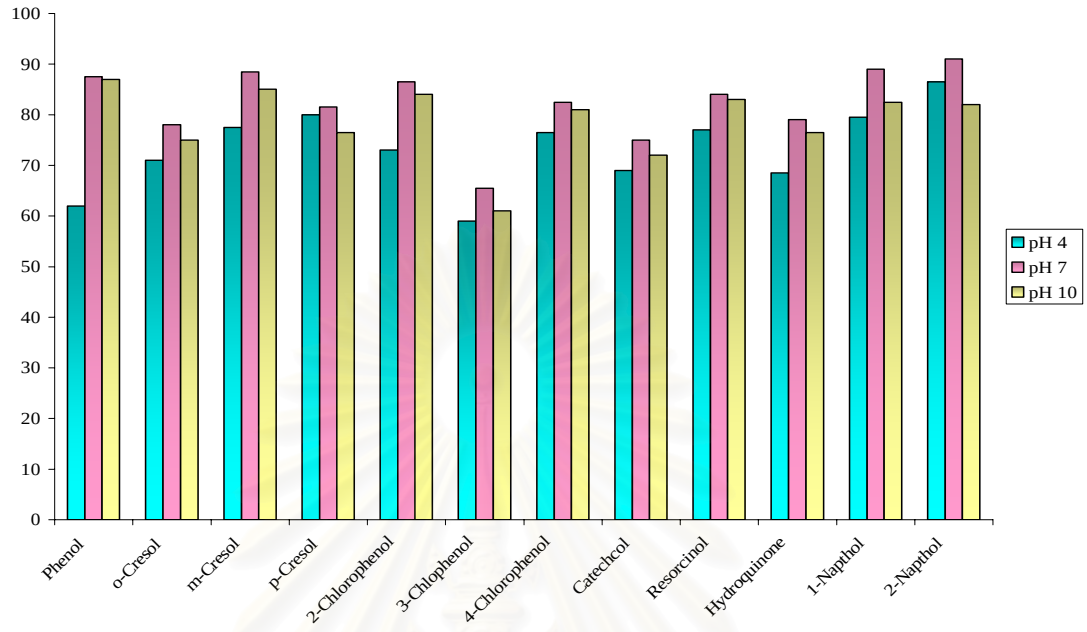
Reaction conditions: 5 mL of H_2O_2, 5 mL of crude POD, pH 7, 1-h treatment at room temperature (30^0C)

Figure 4.15 Effect of hydrogen peroxide concentration for removal of phenols by crude POD

The efficiency of peroxidase was investigated as a function of hydrogen peroxide concentration. The removal efficiency gradually increased the concentration of H_2O_2 from 0.1- 1.0 % w/w. The results show that the removal efficiency gradually increases upon increasing the hydrogen peroxide concentration as oxidizing agent of reaction.

4.8.2 Effect of pH

The pH in the removal reactions of phenols: *o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, catechcol, resorcinol, hydroquinone, 1-naphthol and 2-naphthol were varied from 4, 7 and 10. The results are collected in Figure 4.16.



Reaction condition: 5 mL of 0.1% w/w H_2O_2, 5 mL of crude POD, 1-h treatment and at room temperature (30^0C)

Figure 4.16 Effect of pH for removal of phenols by crude POD

The results show that the removal efficiency of crude POD at pH 7 is higher than those at pH 10, 4, respectively. The removal efficiency of enzyme was decreased. The reason was possible due to the pH decreased and became more acidic there was a gradual loss of enzyme efficiency compared to the sudden change at pH above 7.0. Therefore, in an acidic environment it can be speculated that the crude POD is denaturing but not as quickly as in a basic solution.

4.9 General procedure for removal of phenols

4.9.1 Nuclear magnetic resonance spectrometer (NMR) and Fourier transform infrared spectrometer (FT-IR)

Product of phenol: yellow liquid (100 % yield); R_f = 0.75 (ethyl acetate : hexane 1:1), R_f = 0.33 (ethyl acetate : hexane 2:8), R_f = 0.19 (ethyl acetate : hexane 15:1), IR (NaCl cell, cm^{-1}) : 3349 (br, O-H str), 2956 (w, C-H str of aromatic), 1700,1598 (s, c=c str of aromatic) and 1229 (s, c-o str); ^{1}H-NMR (DMSO-d_6) δ ppm: 9.65 (s, O-H), 7.14 (t, 2H, J=15.9 H_z, aromatic), 6.76 (d, 3H, J=7.71 H_z, aromatic)

Product of 4-chlorophenol: yellow liquid (100 % yield); R_f = 0.75 (ethyl acetate : hexane 1:1), R_f = 0.33 (ethyl acetate : hexane 2:8), R_f = 0.19 (ethyl acetate : hexane 15:1), IR (NaCl cell, cm^{-1}) : 3375 (br, O-H str), 2776 (w, C-H str of aromatic), 1695,1598 (s, c=c str of aromatic) and 1229 (s, c-o str); ^{1}H-NMR (DMSO-d_6) δ ppm: 9.69 (s,O-H), 7.15 (d, 2H, J=8.80 H_z, aromatic), 6.73 (d, 2H, J=8.79 H_z, aromatic)

Product of *o*-cresol: yellow liquid (100 % yield); R_f = 0.75 (ethyl acetate : hexane 1:1), R_f = 0.33 (ethyl acetate : hexane 2:8), R_f = 0.19 (ethyl acetate : hexane 15:1), IR (NaCl cell, cm^{-1}) : 3408 (br, O-H str), 2735 (w, C-H str of aromatic), 1594,1462 (s, c=c str of aromatic) and 1236 (s, c-o str); ^{1}H-NMR (DMSO-d_6) δ ppm: 9.22 (s, O-H), 7.01 (d, 1H, J = 7.34 H_z, aromatic), 6.95 (t, 1H, J = 15.17 H_z, aromatic), 6.74 (d, 1H, J = 7.92 H_z, aromatic), 6.65 (t, 1H, J = 14.7 H_z, aromatic)

Product of 2-naphthol: yellow liquid (100 % yield); R_f = 0.75 (ethyl acetate : hexane 1:1), R_f = 0.33 (ethyl acetate : hexane 2:8), R_f = 0.19 (ethyl acetate : hexane 15:1), IR (NaCl cell, cm^{-1}) : 3349 (br, O-H str), 2956 (w, C-H str of aromatic), 1700,1598 (s, c=c str of aromatic) and 1229 (s, c-o str); ^{1}H-NMR (DMSO-d_6) δ ppm: 9.79 (s, O-H), 7.83 (t, 2H, J = 14.95 H_z, aromatic), 7.74 (d, 1H, J = 3.00 H_z, aromatic), 7.72 (d, 1H, J = 3.81 H_z, aromatic), 7.63 (d, 1H, J = 8.22 H_z, aromatic), 7.36 (t, 1H, J = 14.95 H_z, aromatic), 7.28 (d, 1/2H, J = 8.78 H_z, aromatic), 7.23 (t, 1H, J = 14.96 H_z, aromatic), 7.09 (d, C-H, J = 1.80 H_z, aromatic), 7.07 (d, 2H, J = 2.27 H_z, aromatic), 7.05 (s, C-H)

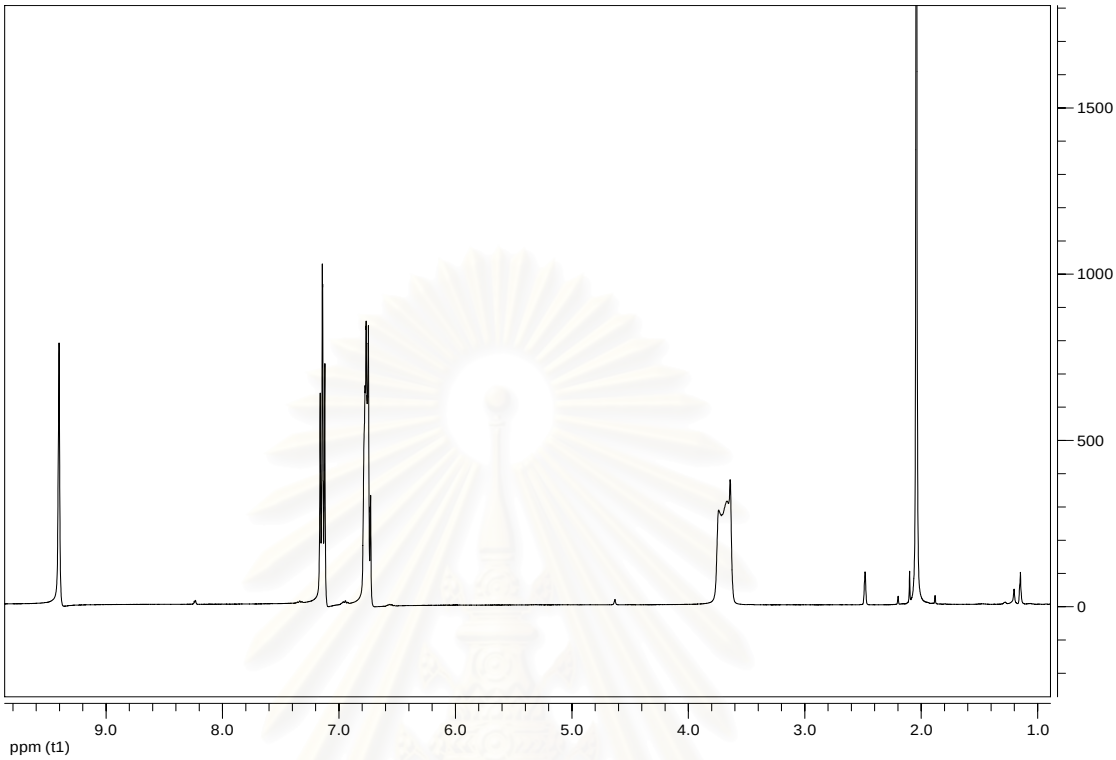


Figure 4.17 ^{1}H NMR spectrum of phenol polymer

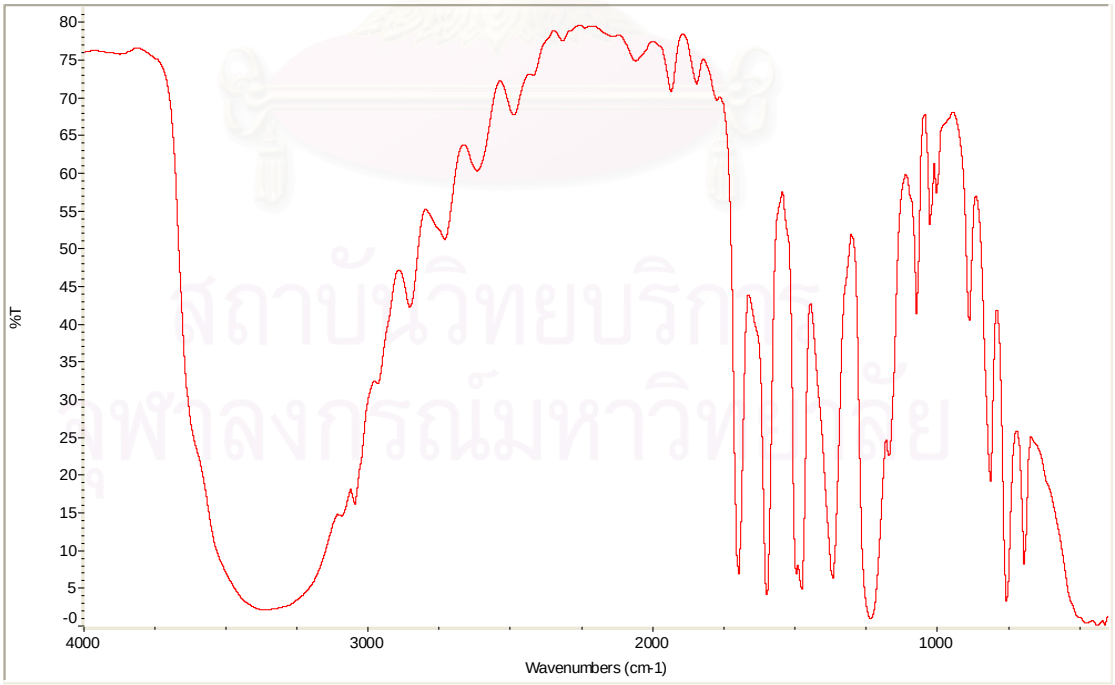


Figure 4.18 FT-IR spectrum of phenol polymer

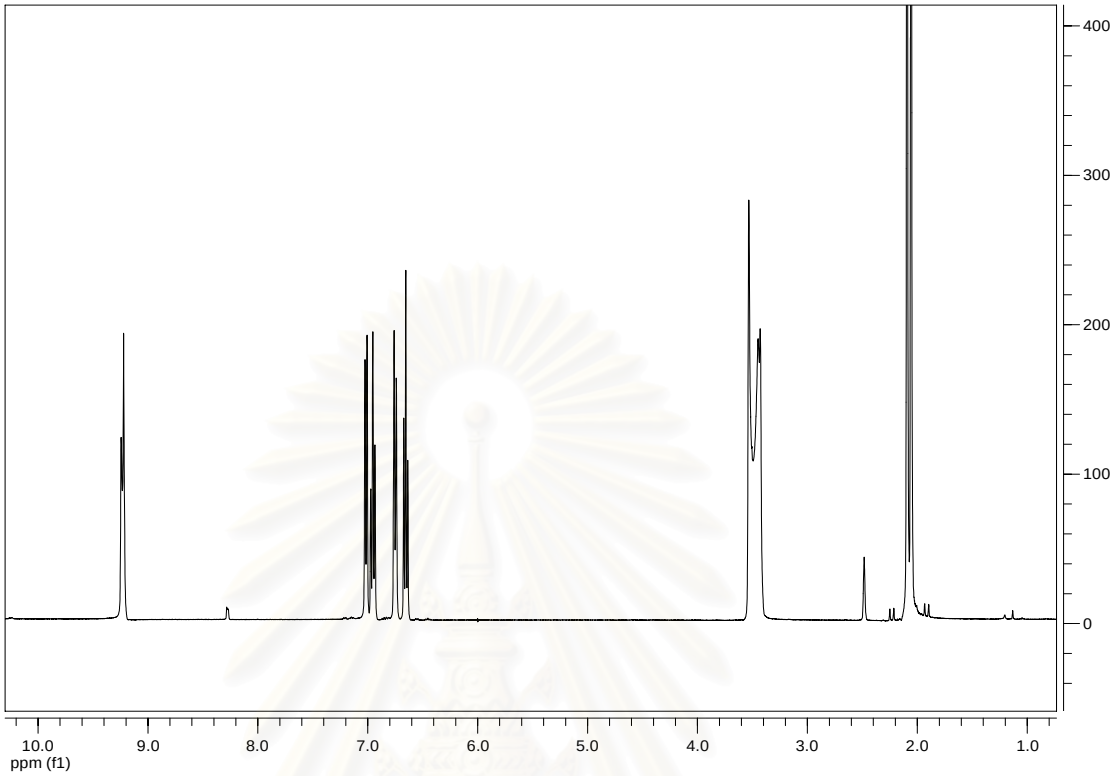


Figure 4.19 ^{1}H NMR spectrum of 4-chlorophenol polymer

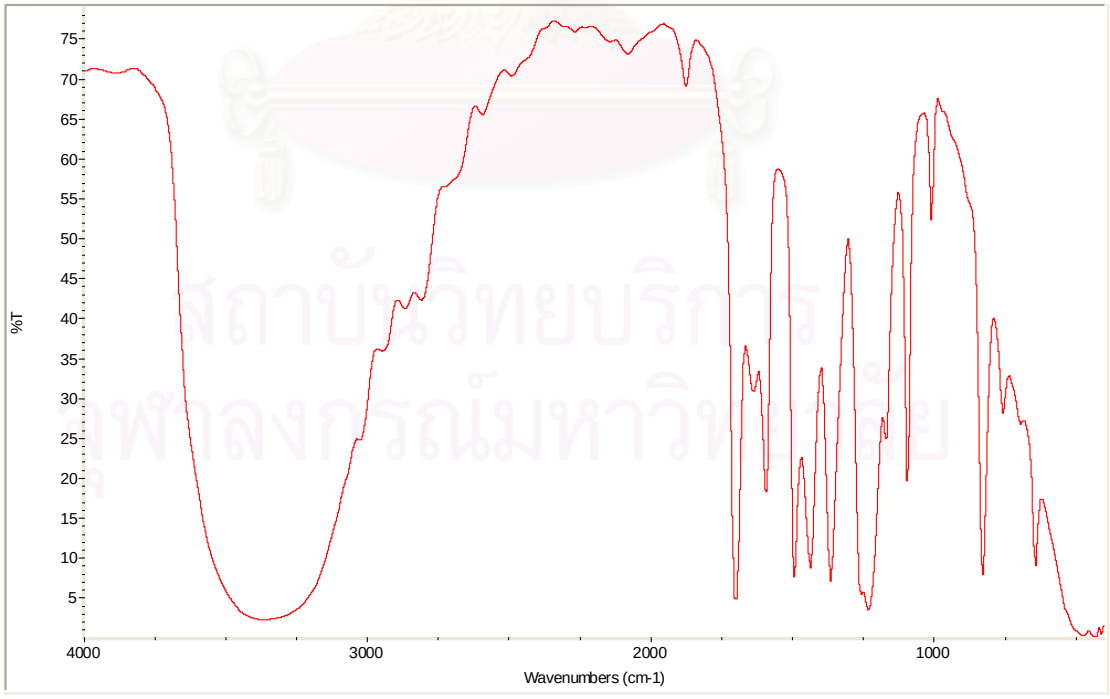


Figure 4.20 FT-IR spectrum of 4-chlorophenol polymer

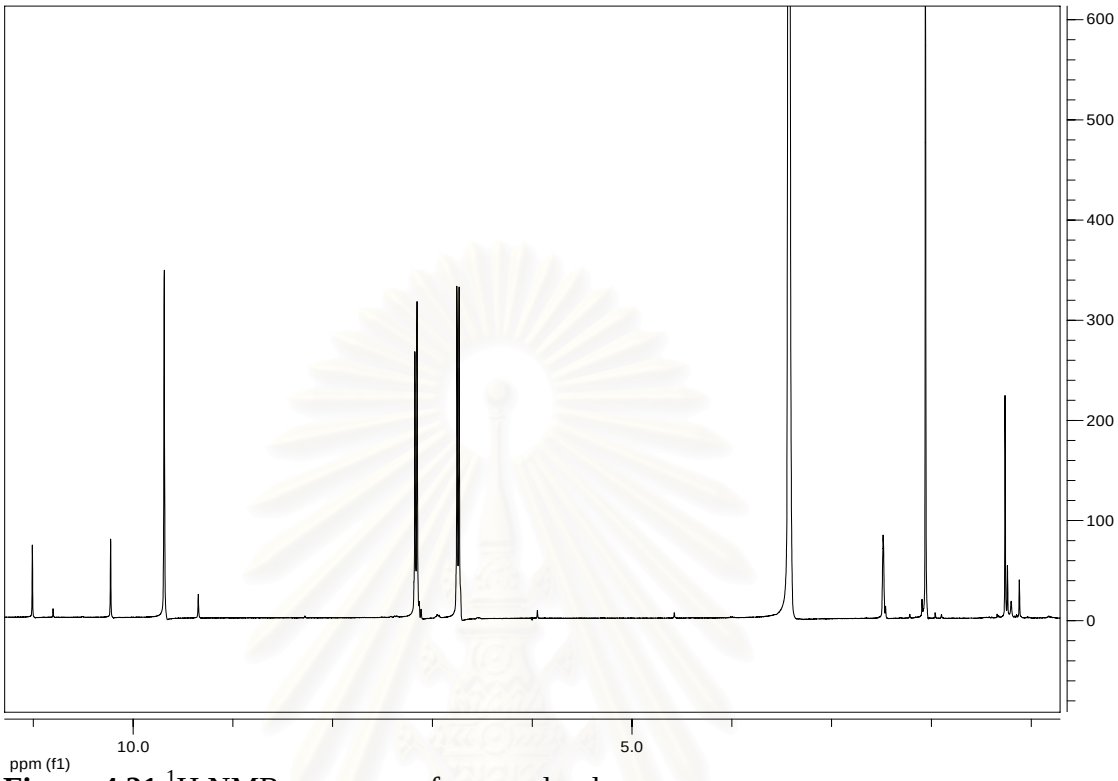


Figure 4.21 ^{1}H NMR spectrum of *o*-cresol polymer

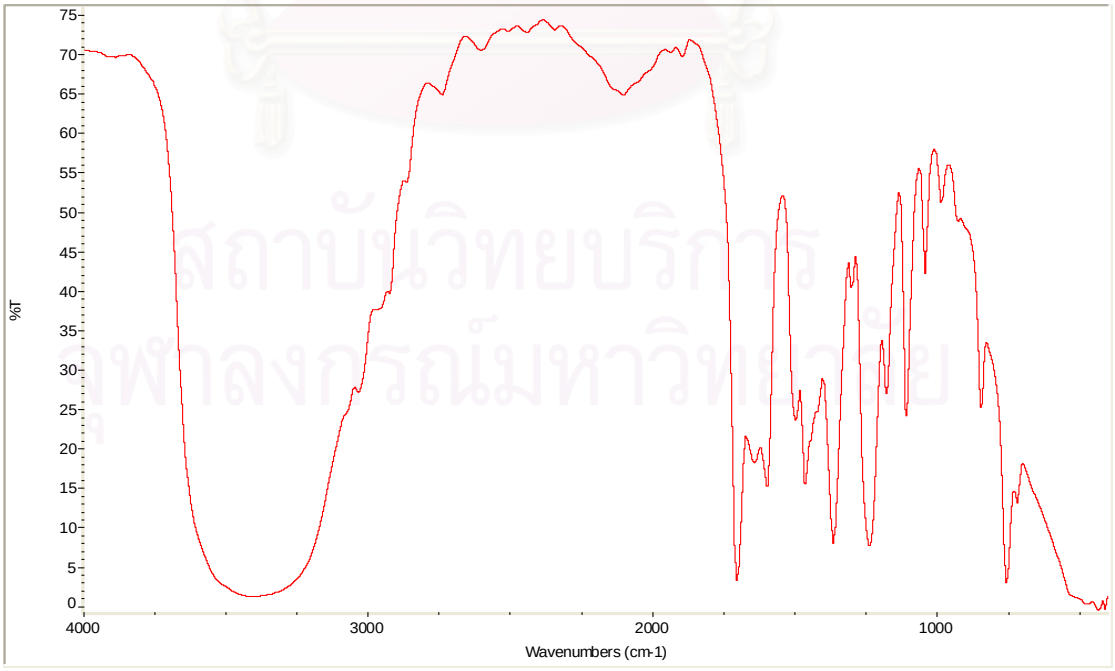


Figure 4.22 FT-IR spectrum *o*-cresol of polymer

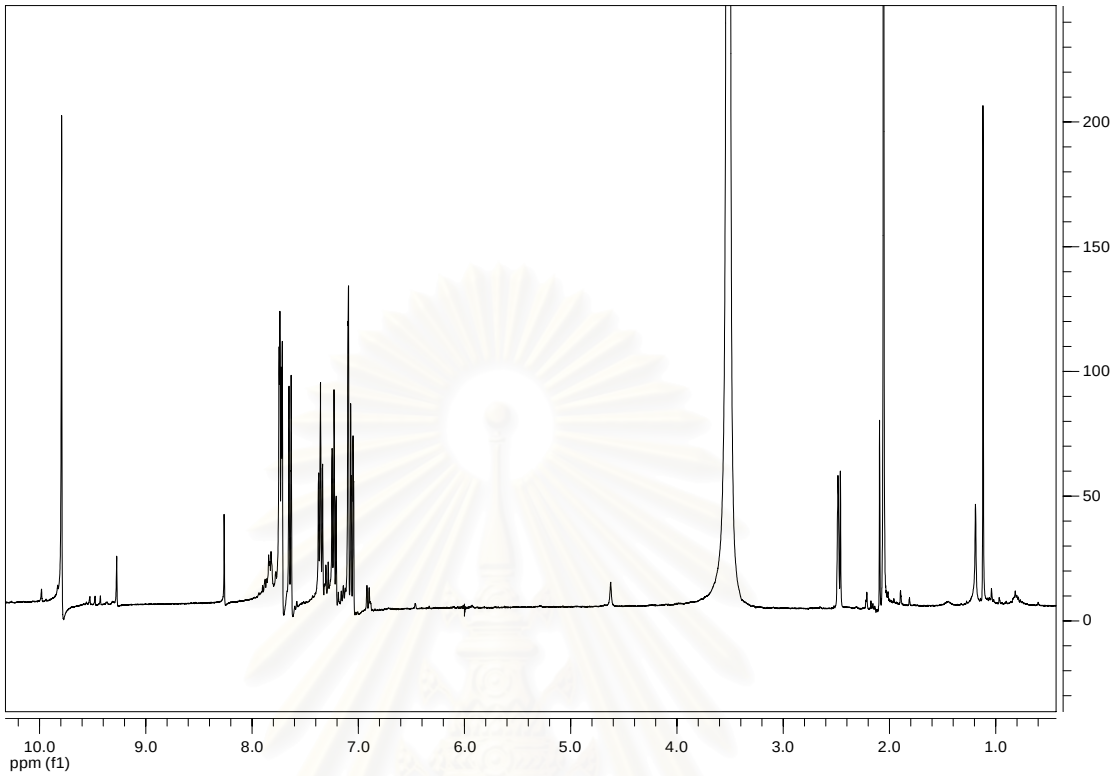


Figure 4.23 ^{1}H NMR spectrum of 2-naphthol polymer

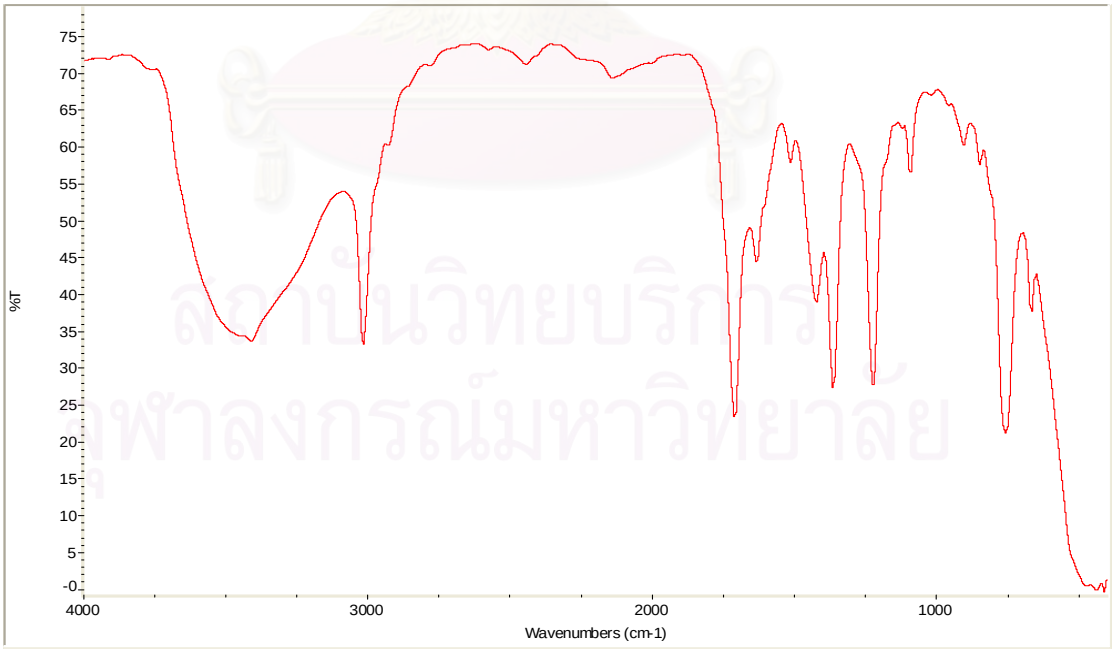


Figure 4.24 FT-IR spectrum of 2-naphthol polymer

4.9.2 Gas chromatography-Mass spectrometry (GC-MS)

The GC-MS is an effective method to identify product of polymer. MS spectra of the phenol polymer are shown in Figures 4.25, 4.26 and 4.27.

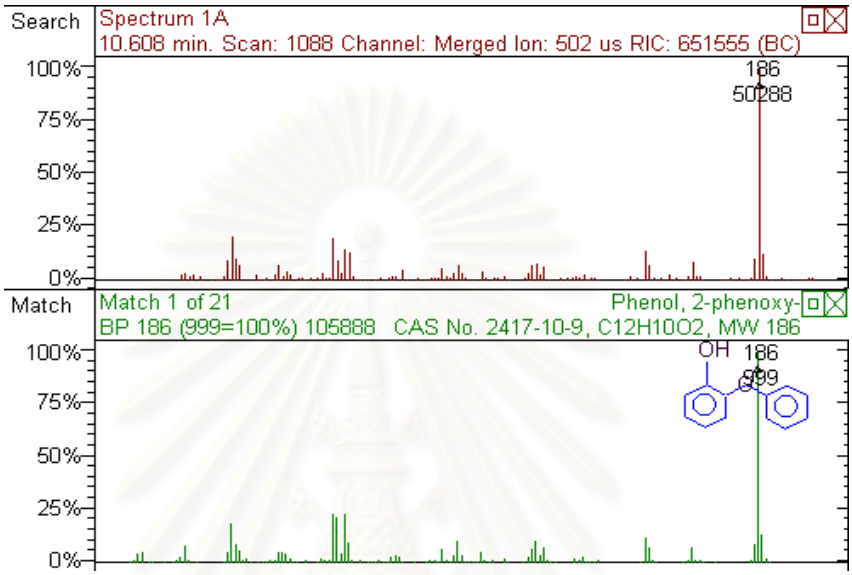


Figure 4.25 GC-MS spectrum of 2-phenoxy-biphenyl

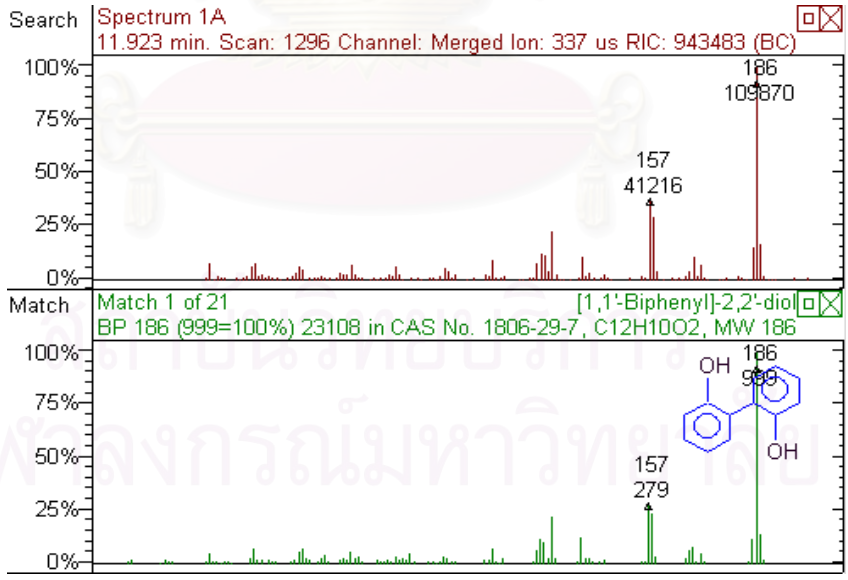


Figure 4.26 GC-MS spectrum of 1,1′-biphenyl-2,2′-diol

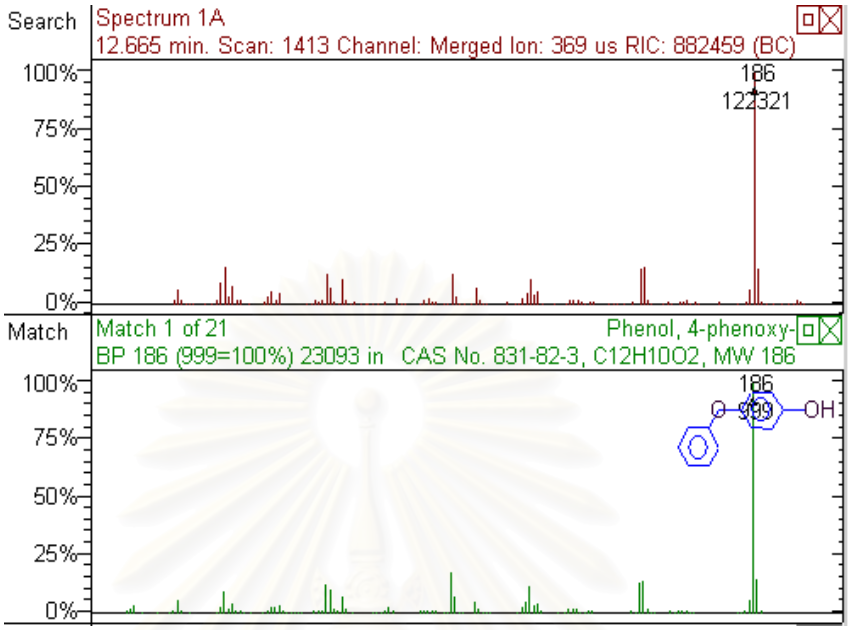


Figure 4.27 GC-MS spectrum of 4-phenoxy-biphenyl

Phenol polymer is the products from the removal of phenols by crude POD. GC-MS spectrum (Figure 4.26) showed m/z=186, which is the molecular mass of 2-phenoxy-biphenyl. GC-MS spectrum (Figure 4.27) showed m/z=186, which is the molecular mass of 1,1′-biphenyl-2,2′-diol and GC-MS spectrum (Figure 4.28) showed m/z=186, which is the molecular mass of 4-phenoxy-biphenyl.

The GC-MS is an effective method to identify product of *o*-cresol MS spectrum of the *o*-cresol polymer was shown in Figure 4.28.

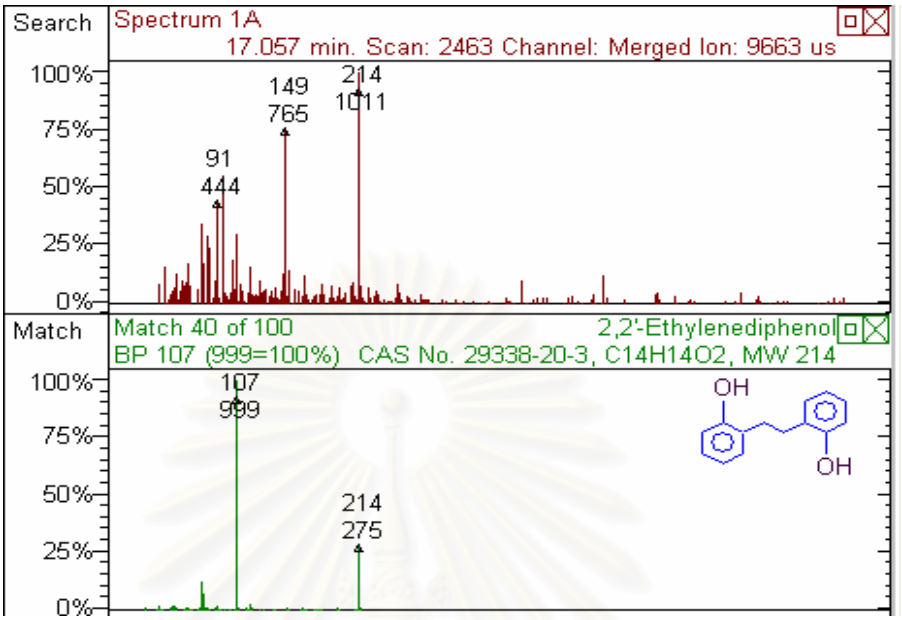


Figure 4.28 GC-MS spectrum of 2.2′-ethylenediphenol

o- Cresol polymer is the product from the removal of phenols by crude POD. GC-MS spectrum (Figure 4.28) showed m/z= 214, which is the molecular mass of 2.2′-ethylenediphenol.

CHAPTER V

CONCLUSION AND SUGGESTION

From the proceeding results and discussion, the main focus of this research is to search for the optimum conditions for the removal of phenols by POD derived from agricultural waste.

In this study, the plant species, solvent types, temperature, pH affected the activity of crude POD. The optimum condition for extraction of crude POD to achieve highest activity was at 30^0C, pH 7-9, peel of sweet potato as plant, and phosphate buffer as solvent. The activity of crude POD showed high activity (activity= 31.505). The stability of crude POD was kept at -5^0C for 1 h was higher than the other condition examined.

The removal of phenol under the optimum condition was found that the plant species, solvent types, temperature, pH, time, oxidizing agent (H_2O_2), phenol, crude POD, PEG affected the removal. The highest yield of the phenol removal was at 30^0C, 100 minutes, pH 7, 20 ppm of phenol, 0.5 % w/w H_2O_2, 8 units/mL of crude POD, 10 ppm PEG-6000, peel of sweet potato as plant, and phosphate buffer as solvent. For other substrates (*o*-cresol, *m*-cresol, *p*-cresol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, catechcol, resorcinol, hydroquinone, 1-naphthol, 2-naphthol), the optimum condition was at 30^0C, 100 min, 1 % w/w of H_2O_2, peel of sweet potato as plant, and phosphate buffer as solvent.

The product from the removal of phenol by POD is polymer consisted of 2-phenoxy-biphenyl, 1,1′-biphenyl-2,2′-diol and 4-phenoxy-biphenyl while that derived from the removal of *o*-cresol by POD is 2,2′-ethylenediphenol.

The kinetic of phenol study was performed with initial rate method using UV-visible spectrometer, It can be found that conversion of phenol increases with time. Rate of conversion of phenol under this condition was 1.045 μ mol per h.

Propose for the future work

For the future work, the removal of other organic compounds by POD as biocatalyst should be attempted such as alcohol. And study rate of the removal between high pure POD and low pure POD.

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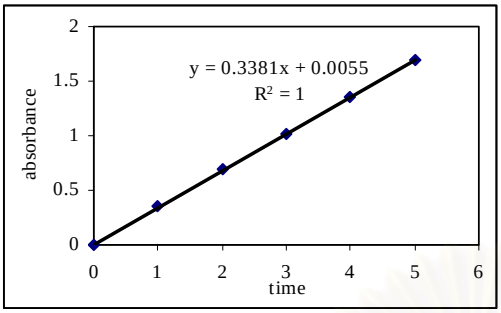
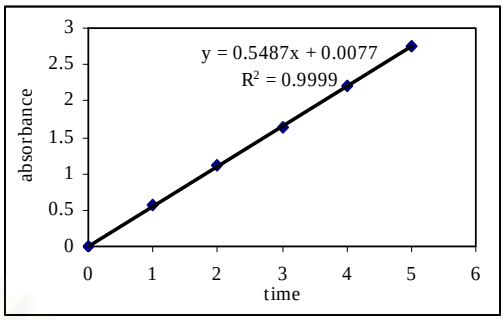
APPENDICES

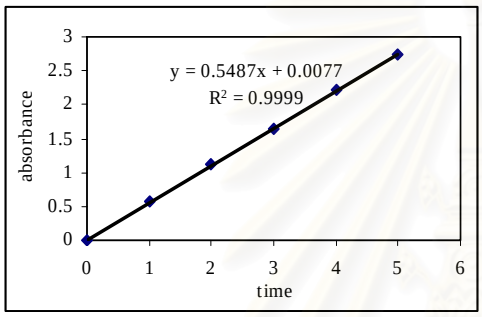
APPENDIX A. Activity assay (Increasing in absorbance at 510 nm)



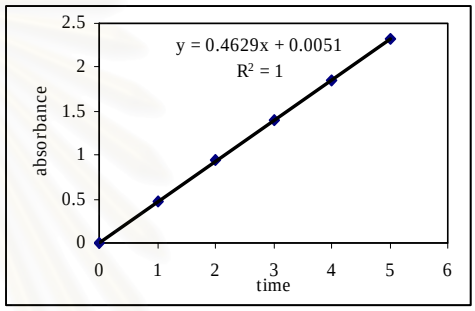
seedcoat of soybean extracted by water



seedcoat of soybean extracted by NaCl



seedcoat of soybean extracted by buffer



peel of sweet potota extracted by water

APPENDIX B. Activity assay

Activity of seedcoat of soybean extracted by water

$$= \frac{\mathbf{0.339}/min}{\mathbf{6.58}ml \cdot \mu mol^{-1} \times \frac{\mathbf{0.01}}{\mathbf{3}} g \cdot of\ plant \cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{15.465}\,\mu mol\, min^{-1} g\ of\ plant^{-1}$$

Activity of seedcoat of soybean extracted by NaCl

$$= \frac{\mathbf{0.400}/min}{\mathbf{6.58}ml \cdot \mu mol^{-1} \times \frac{\mathbf{0.01}}{\mathbf{3}} g \cdot of\ plant \cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{18.219}\,\mu mol\, min^{-1} g\ of\ plant^{-1}$$

Activity of seedcoat of soybean extracted by NaCl

$$= \frac{\mathbf{0.550}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{25.076}\,\mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of peel of sweet potota extracted by water

$$= \frac{\mathbf{0.484}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{21.155}\,\mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of peel of sweet potota extracted by NaCl

$$= \frac{\mathbf{0.540}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{24.620}\ \mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of peel of sweet potota extracted by buffer

$$= \frac{\mathbf{0.691}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{31.505}\,\mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of pulp of sweet potota extracted by water

$$= \frac{\mathbf{0.092}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{4.195}\ \mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of pulp of sweet potota extracted by NaCl

$$= \frac{\mathbf{0.141}/\,min}{\mathbf{6.58}ml \cdot \mu mol^{-1} \times \frac{\mathbf{0.01}}{\mathbf{3}} g \cdot of\ plant \cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{6.429}\ \mu mol\, min^{-1} g\ of\ \ plant^{-1}$$

Activity of pulp of sweet potota extracted by buffer

$$= \frac{\mathbf{0.181}/\,min}{\mathbf{6.58}ml \cdot \mu mol^{-1} \times \frac{\mathbf{0.01}}{\mathbf{3}} g \cdot of\ plant \cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{8.252}\ \mu mol\, min^{-1} g\ of\ \ plant^{-1}$$

Activity of peel of pineapple by water

$$= \frac{\mathbf{0.134}/\,min}{\mathbf{6.58}ml \cdot \mu mol^{-1} \times \frac{\mathbf{0.01}}{\mathbf{3}} g \cdot of\ plant \cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{6.109}\ \mu mol\, min^{-1} g\ of\ \ plant^{-1}$$

Activity of peel of pineapple by extracted by NaCl

$$= \frac{\mathbf{0.181}/\,min}{\mathbf{6.58}ml \cdot \mu mol^{-1} \times \frac{\mathbf{0.01}}{\mathbf{3}} g \cdot of\ plant \cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{8.252}\ \mu mol\, min^{-1} g\ of\ \ plant^{-1}$$

Activity of peel of pineapple by extracted by buffer

$$= \frac{\mathbf{0.221}/\,min}{\mathbf{6.58}ml \cdot \mu mol^{-1} \times \frac{\mathbf{0.01}}{\mathbf{3}} g \cdot of\ plant \cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{10.076}\ \mu mol\, min^{-1} g\ of\ \ plant^{-1}$$

Activity of pulp of pineapple by extracted by water

$$= \frac{\mathbf{0.013}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{0.593}\ \mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of pulp of pineapple extracted by NaCl

$$= \frac{\mathbf{0.017}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{0.775}\ \mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of pulp of pineapple extracted by buffer

$$= \frac{\mathbf{0.033}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{1.505}\,\mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of axis of pineapple by extracted by water

$$= \frac{\mathbf{0.009}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{0.410}\ \mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of axis of pineapple extracted by NaCl

$$= \frac{\mathbf{0.014}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{0.638}\,\mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of axis of pineapple extracted by buffer

$$= \frac{\mathbf{0.021}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{0.957}\,\mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of peanut by extracted by water

$$= \frac{\mathbf{0.024}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{1.094}\ \mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of peanut extracted by NaCl

$$= \frac{\mathbf{0.042}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{8.252}\ \mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

Activity of peanut extracted by buffer

$$= \frac{\mathbf{0.055}/\,min}{\mathbf{6.58}ml\cdot\mu mol^{-1}\times\frac{\mathbf{0.01}}{\mathbf{3}}g\cdot of\ plant\cdot ml^{-1}\ reaction\ mixture}$$

$$= \mathbf{8.255}\,\mu mol\,min^{-1}g\ of\ \ plant^{-1}$$

APPENDIX C. Standard curve of phenols

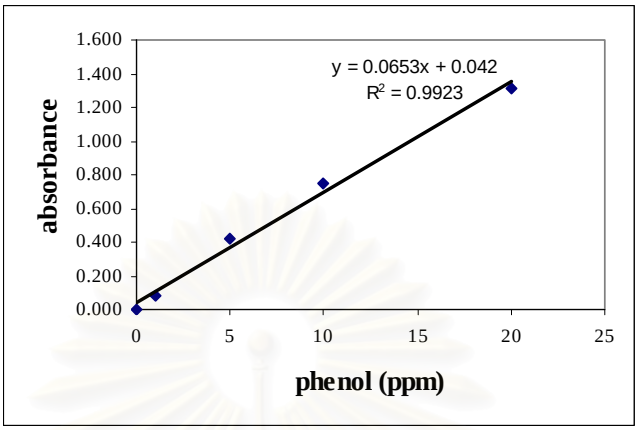


Figure C1. Standard curve of phenol

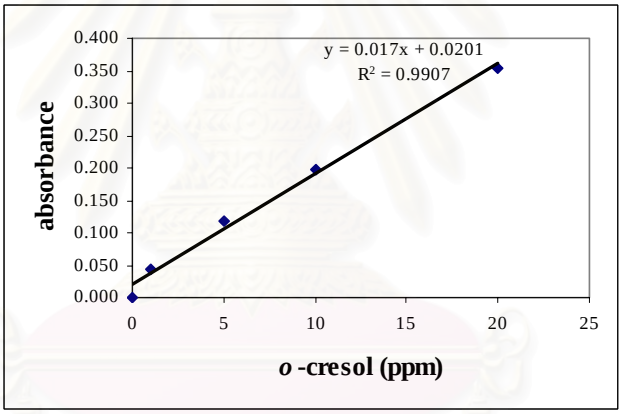


Figure C2. Standard curve of *o*-cresol

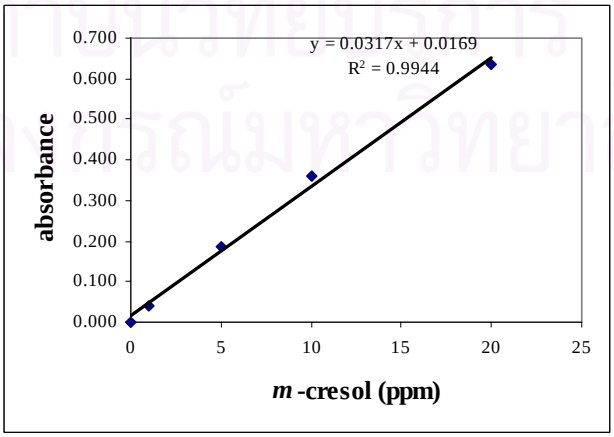


Figure C3. Standard curve of *m*-cresol

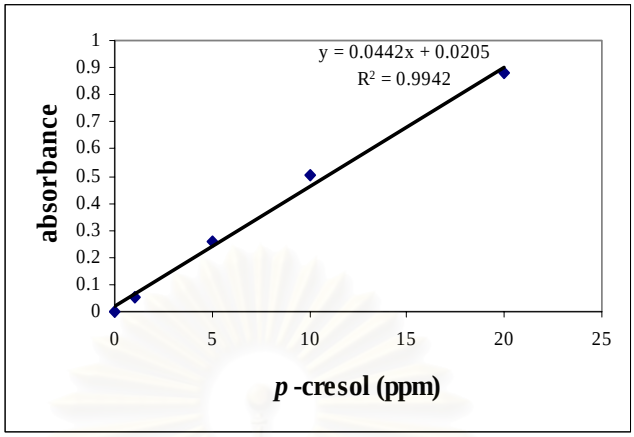


Figure C4. Standard curve of *p*-cresol

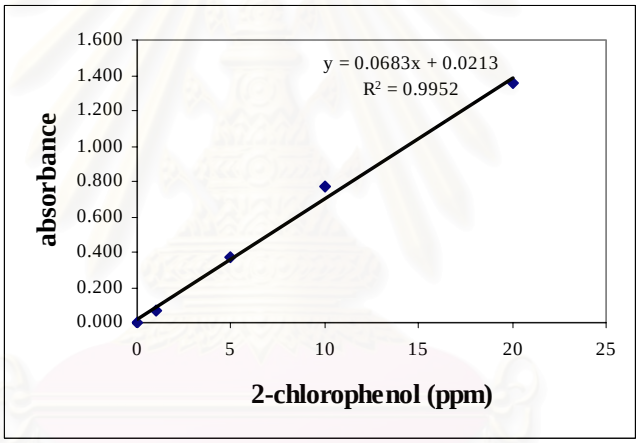


Figure C5. Standard curve of 2-chlorophenol

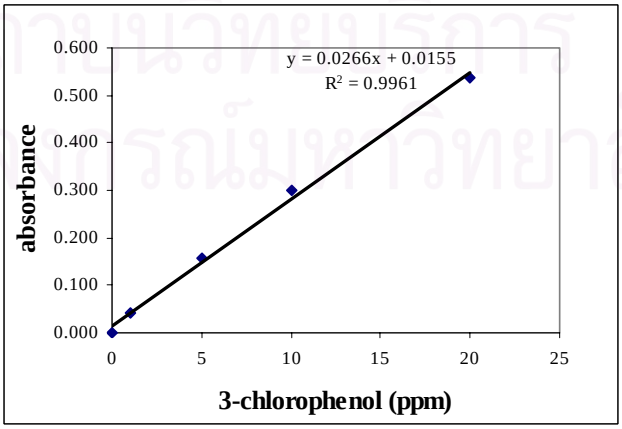


Figure C6. Standard curve of 3-chlorophenol

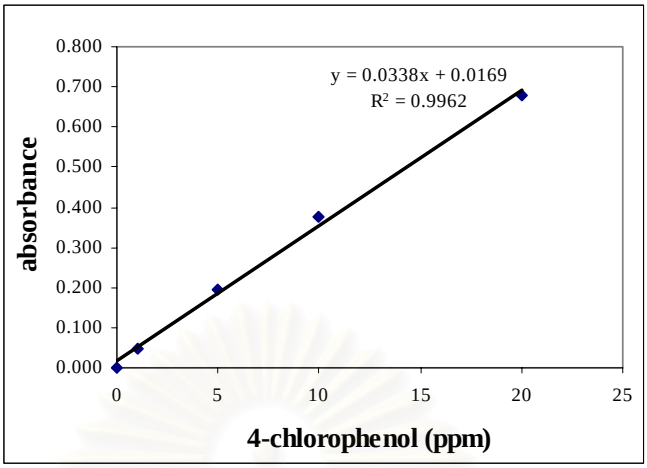


Figure C7. Standard curve of 4-chlorophenol

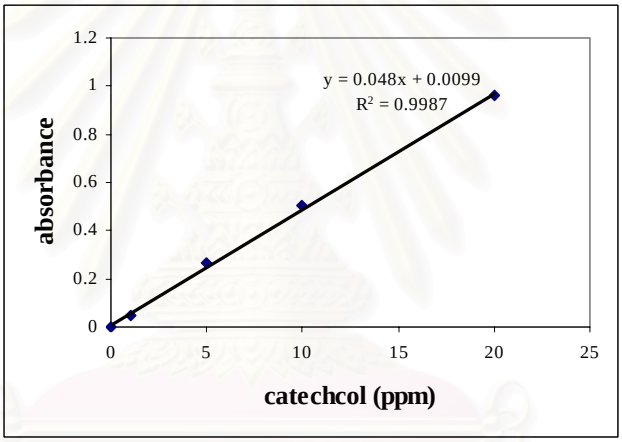


Figure C8. Standard curve of catechcol

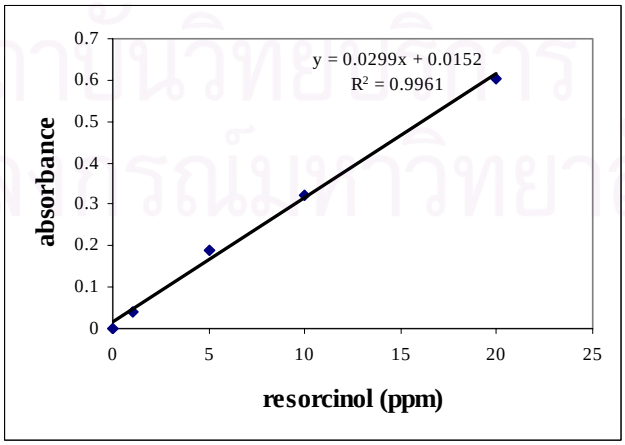


Figure C9. Standard curve of resorcinol

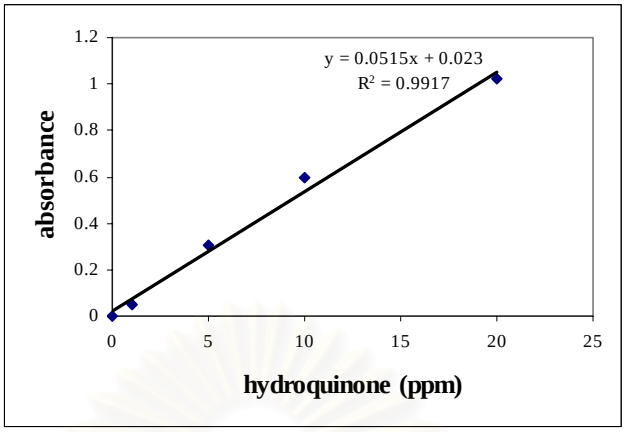


Figure C10. Standard curve of hydroquinone

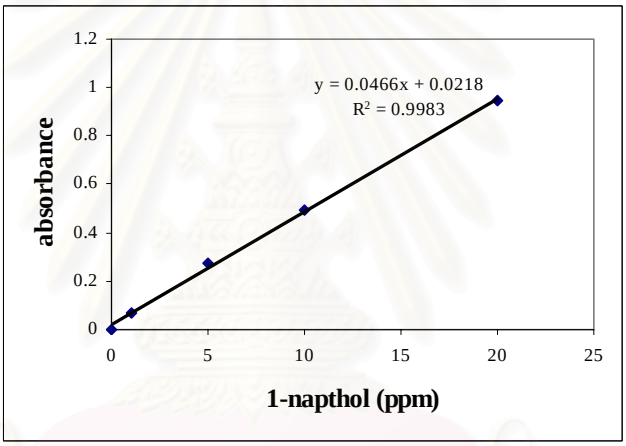


Figure C11. Standard curve of 1-napthol

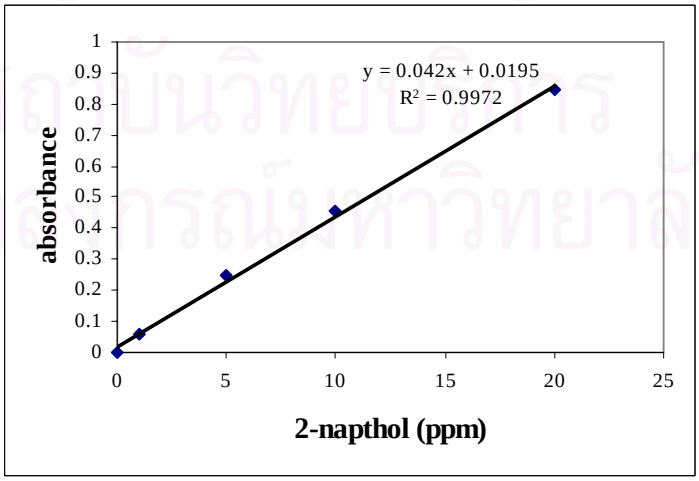


Figure C12. Standard curve of 2-napthol

VITA

Mrs. Chuensumon Yimthin was born on November 23, 1974 in Chainat, Thailand, She received a Bachelor's Degree of Science major in Chemistry from Nakonsawan Rajabhut University in 1996. From 1997, she has worked as a chemist in the Office of Atomic Energy for Peace, Bangkok. In 1999 she worked as a Teacher in Rajamangala University of Technology Suvarnabhum. Since 2002, she has been a graduate student studying in the Program of Petrochemistry and Polymer Science, Faculty of Science at Chulalongkorn University, and completed her M.Sc. degree in 2006.

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