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MASTER'S THESIS

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Probing the possiblity of characterization of light induced PSII turn over efficiency via monitoring of the reduction of commonly used exogenous electron acceptors.

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Abstract

Photosystem II is the first protein complex in the Light-dependent reactions. It is located in the thylakoid membrane of plants, algae, and cyanobacteria. The enzyme captures photons of light to energize electrons that are then transferred through a variety of coenzymes and cofactors to reduce plastoquinone to plastoquinol. The light reaction (photolysis of water) is entirely dependent on the amount and intensity of light. It is found possible to use exogenous photosynthetic electron acceptors to study the action and efficiency of PSII. We use two of these exogenous electron acceptors to probe the turnover efficiency of PSII using Fluorolog-3 spectrofluorometer and UV-2401 UV-Vis spectrophotometer. And we found that the PSII turnover was high for the first few measurements. The turnover of PSII shows the efficiency of the PSII in the light reaction.

Acknowledgment

First and for most I thank God for helping me thorough out my life. Second I would like to express my deepest gratitude to my supervisor at the University of Stavanger, Professor Dr. Lutz Andreas Eichacker and Dr. Dmitriy Shevelafor for their great supervision, support and guidance during the work.

I wish also to express my sensior gratitude to My Mother, my wife and all my friends and my colleagues in the lab for been there whenever I need their help

Abbreviations

ATP-Adenosine tri phosphate

BC- Bicarbonate

Chl-chlorophyll

Cyt b_6f -cytochrome b_6f

DCMU- (3-(3,4-dichlorophenyl)-1,1-dimethylurea)

Fd- ferredoxin

FRET- fluorescence resonance energy transfer

$K_3[Fe(CN)_6]$- Potassium ferricyanide

LHCs- light harvesting complexes

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

OEC-Oxygen evolving complex

Pheo- pheophytin

PPBQ-Phenyl-p-benzoquinone

PSI- Photosystem I

PSII- Photosystem II

Q-Quinone

WOC- water oxidizing complex

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1. Introduction

1.1 Overview of Oxygenic Photosynthesis

The word 'photosynthesis' originates from two Greek words 'photo' meaning light and 'synthesis' meaning building up or putting together. Photosynthesis is a complex process performed by plants, green algae and cyanobacteria and responsible for the conversion of light energy into chemical energy (Govindjee et al., 2010, Shevela, 2008, Wollman et al., 1999). Due to photosynthesis and respiration organisms exist in our planet is in balance since inorganic carbon fixed during Photosynthesis is released back to the ecosystem by respiration, and the same is true for oxygen, which is utilized by respiration will be released by photosynthesis. In general a byproduct of one process is the raw material for the other one. Oxygenic photosynthesis depends on two reaction centre complexes, photosystem II (PSII) (Govindjee et al., 2010) and photosystem I (PSI)(Bendall, 2001), that are linked by the cytochrome bf complex and mobile electron carriers. The overall reaction of photosynthesis is the following.

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

Such conversion of carbon dioxide to sugar (carbon fixation) by oxygenic photosynthetic organisms mainly requires a photosynthetic complex enzyme called PSII which is responsible for the utilization of captured light energy (Barber, 2009). All light reactions of photosynthesis take place in the thylakoid membrane of chloroplast.

1.2 Thylakoid membrane as cite of light reactions

Chloroplast is one of many different cellular organelles believed to be originated from cyanobacteria responsible for photosynthesis. The double thylakoid membrane form a sac like structure called thylakoid which are found in the form of pile 'grana'. Chloroplast also has a jelly-like mass of soluble proteins (stroma) where a number of enzymes concerned in the assimilation of carbon and also the photochemical apparatus is embedded(Edwards and Walker, 1983). These chloroplast structures are illustrated in the Fig. 1.

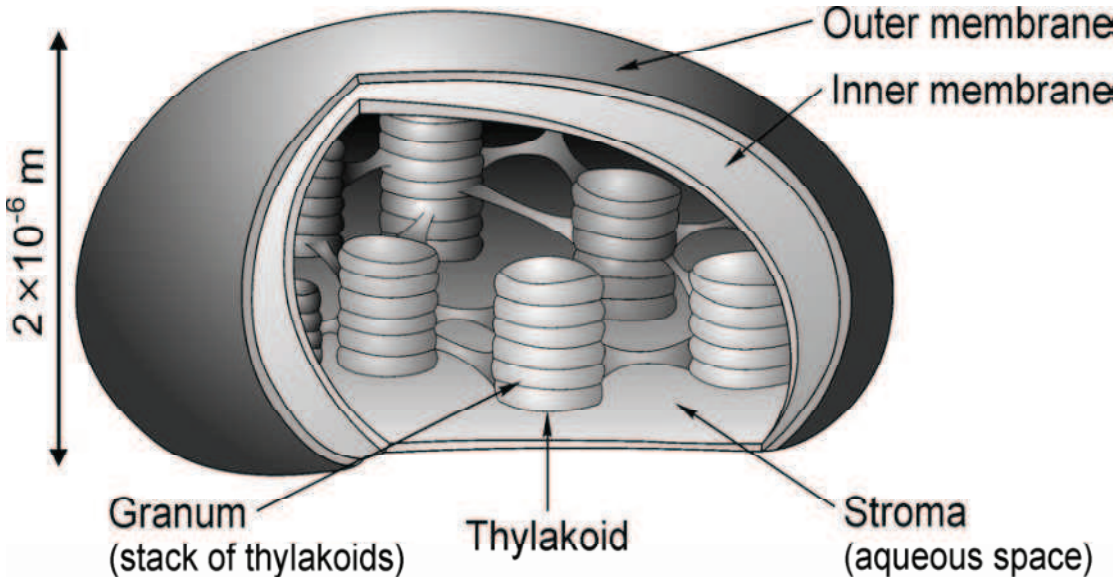


Fig 1. Three dimensional structure of chloroplast showing its structure. The figure is taken from Shevela, 2008.

1.2.1 Harvesting energy of sunlight

Chlorophylls are important in the formation of light harvesting complexes (LHCs,) of higher plants. However, in cyanobacteria the pigment responsible for the formation of antenna complex, called phycobilisomes, are phycocyanobilin and phycoerythrobilin (Bricker et al., 2004, Britt, 2001). LHCs act as antennae; they capture incoming photons and funnel them to respective reaction centers, in which photochemistry takes place (for more detail see Baena-Gonzalez and Aro, 2002, Govindjee et al., 2010, Hillier et al., 2005, Ort et al., 2004, Shevela and Messinger, 2012a, Shevela and Messinger, 2012b, Walker, 2002, Wollman et al., 1999). The major areas of absorption by chlorophylls (a and b) are in the red and blue parts of light spectrum.

Walker, 2002 *stated in his book that "each photon of Red light which is absorbed by chlorophyll molecule raises an electron from a ground state to an excited state and all of available energy is transferred in the process. This excitation brings about an oxidation, as the electron lifted in to higher orbit and a positively charged "hole" is created. A blue light absorption causes even greater excitation but the elevated electron fall back into the red orbit too quickly to permit useful chemical work."*

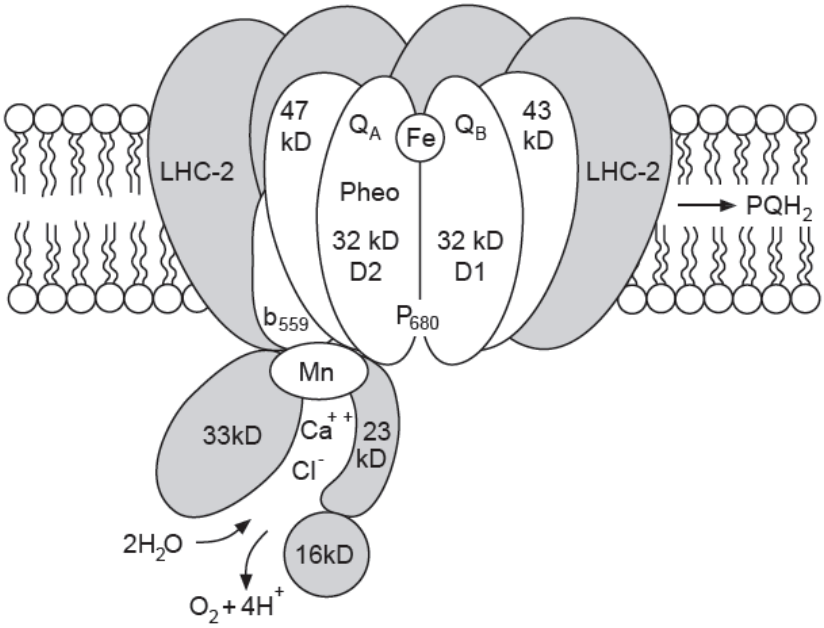


Fig 2. Structure of PSII and its associated components as light harvesting complex in thylakoid membrane. The figure is captured from Walker, 2002.

1.2.2 Overview of light reaction and electron transport system

The thylakoid membrane is the site for light reactions, whereas the stroma is responsible for dark reaction or reduction of carbon dioxide (carbon fixation). There are four thylakoid membranes bound protein complexes associated with photosynthetic energy conversion and ATP production. These protein complexes are PSII, cytochrome b_6f (Cyt b_6f), Photosystem I (PSI) and ATP-synthase. The redox cofactors with PSI, PSII and Cyt b_6f can be arranged as the Z-scheme which allows photo-induced electron transfer from an electron source (water) to final electron acceptor $NADP^+$ through thylakoid membrane (see Fig. 3 and references (Govindjee et al., 2010, Shevela, 2008, Edwards and Walker, 1983).

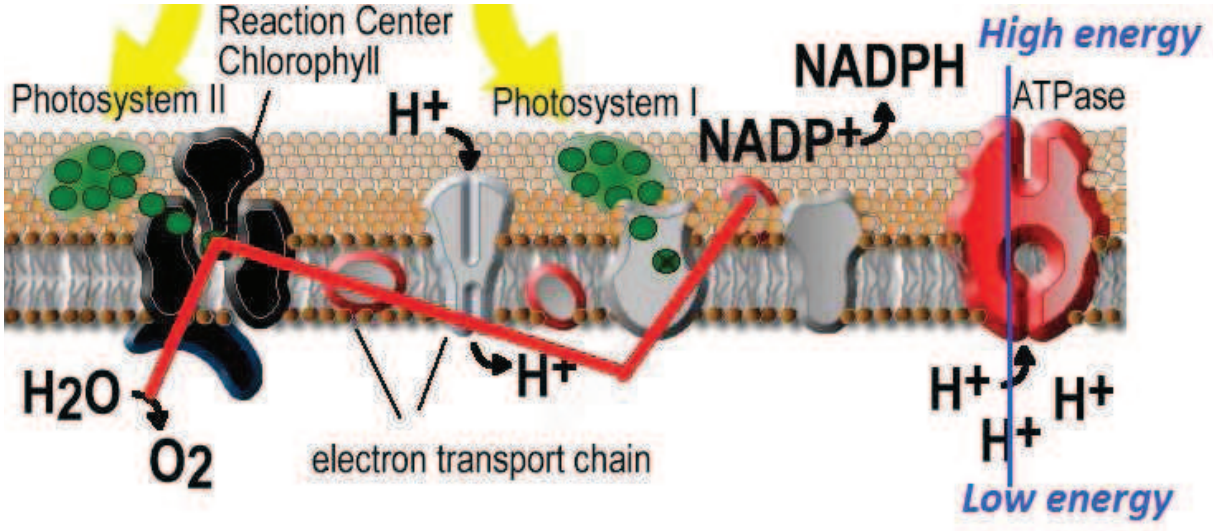


Fig 3. Organization of photosynthetic complexes arranged in the thylakoid membrane taken from Encyclopedia, 2012

The light reaction of photosynthesis features a series of different steps like excitations of antenna system, directed charge separation and subsequent reduction of electron acceptors; and as a result oxidation of water and reduction of $NADP^+$. When PSII uses light energy to oxidize two molecules of water to molecular oxygen 4 electrons extracted from water are transferred to $NADP^+$. The above process (electron transport) creates a proton (H^+) gradient across the thylakoid membrane where this gradient is used for the production of ATP by ATP-synthase from ADP and Pi (Walker, 2002, Rutherford et al., 2012). The light-driven electron transport from H_2O to $NADP^+$ is catalyzed by PSII & PSI and results in the formation of the energy rich compounds NADPH and ATP.

1.2.2.1 The component of the electron transport chain and its functional organization

The photosynthetic electron transport reactions are carried out by five types of membrane spanning protein complexes, associated cofactors and peripheral proteins as illustrated in Fig. 4 Photosystem I (PS I/bound LHC I), Photosystem II (PS II/bound inner antennae) and the peripheral, light-harvesting complex II (LHC II), bind chlorophyll (Chl); the two others, cytochrome b_6f and the ATP synthase, also known as coupling factor(Staehelin et al., 2004). The main electron carriers in PS II include pheophytin, a bound plastoquinone (Q_A) and a dissociable plastoquinone(Staehelin et al., 2004).

Some of the structures associated in the photosynthetic electron transport are a) Manganese (Mn_4O_5Ca) cluster which is responsible for water splitting (Shevela, 2008). b) Cytochrome b_6f contains proteins, metal ions and a special iron-sulfur protein. It also translocates protons across the Thylakoid membrane, and for transfer of electrons from PSII to PSII (Govindjee et al., 2010). c) Q (quencher) it is the main electron acceptor in PSII by quenching fluorescence from Chl *a* on its oxidized state. It is also bound to plastoquinone with single electron reduction to a semiquinone QH (Svensson et al., 1990, Klimov et al., 1995). d) Plastoquinone which is the most abundant electron carrier in chloroplast basically similar with ubiquinone of mitochondrion. e) Cytochrome *f* is associated with PSI by carrying only single electron (Govindjee et al., 2010). f) Iron-Sulphur protein is a structure found between Plastoquinone and cytochrome b_6f. g) Plastocyanin is a two Copper atom per molecule of protein associated with PSI and linking the two systems by carrying a single electron (Barbato et al., 1992). h) Pigment 700 (P700) is a part of PSI that carries single electron and has absorption max at 700 because it contain modified form of chl *a* (Belanger and Rebeiz, 1980). i) Ferredoxin contains two iron per molecule, with flavoprotein it able to catalyze the reduction of $NADP^+$, and it carries single electrons. Ferredoxin serves at a very important regulatory site of the electron transport chain in that it controls the flow of electrons either back to Cyt b_6f. The Cyt b_6f complex consists of four major polypeptides and possibly a small hydrophobic polypeptide (Staehelin et al., 2004). j) Flavoprotein (Ferredoxin-$NADP^+$ oxidoreductase): this is an enzyme that transfer electrons from reduced ferredoxin to NADP and it also act as diaphorase i.e concerned in transferring of electrons from NADPH to artificial electron acceptors (Walker, 2002). k) Nicotinamide adenine dinucleotide phosphate (NADP) is a terminal electron acceptor in photosynthetic electron transport in the Z-scheme. It is entirely confined in the chloroplast (Yamamoto, 2001, Edwards and Walker, 1983, Goldfarb et al., 1997).

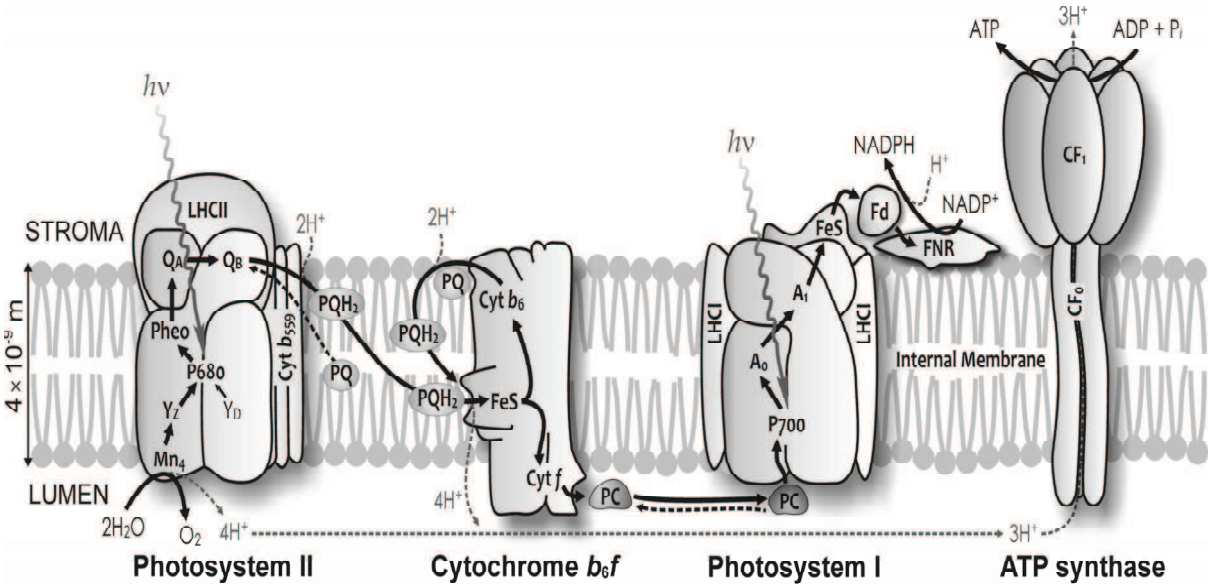


Fig 4. Arrangement of photosynthetic complexes and the light-induced electron transfer from water to $NADP^+$. The figure is captured from Shevela, 2008.

1.3 Photosystem II its function

Photosystem II is a multi-subunit enzyme that carry electrons from an external source (Water) to a small organic molecules (Quinone) that catalyze one of the most important process that depend on the energy input from light which drives oxidation of water to molecular oxygen and reduction of plastoquinone to plastoquinol (Goldfarb et al., 1997, Drath et al., 2009)

The overall reaction process carried out in Photosystem II could be summarized as:

$$2H_2O + 2\text{QUINONE } B_{OX} + 4H^+_{out} + 4h\nu \xrightarrow{\Delta 4\ electrons} O_2 + 4H^+_{in} + 2\text{QUINONE } B_{red}$$

This process was a corner stone for a number of reasons for photosynthetic organisms to colonize the our planet, formation of ozone and also plays a critical role in the development of multicellular organisms.

Excitation of reaction center chlorophyll (called P680) causes rapid transfers of an electron to a nearby pheophytin *a*. The electron is then transferred to a tightly bound QA of the D1 protein subunit. The electron is then transferred to an exchangeable secondary quinone Q_B of the D2 subunit. The arrival of a second electron to the QB site with the uptake of two protons from the stroma produces plastoquinol, PQH_2. When the electron is rapidly transferred from P680* to

pheophytin *a*, a positive charge is formed on the $P680^{+}$. $P680^{+}$ is an incredibly strong oxidant which extracts electrons from water molecules bound at the inorganic water-splitting manganese cluster. The structure of this manganese cluster includes 4 Manganese ions, a calcium ion, and probable a chloride ion. The positively charged P680 extracts an electron from the manganese center (Belanger and Rebeiz, 1980).

4 photochemical steps are required for the oxidation of two water molecules. The Manganese center oxidized one electron at a time, until two molecules of H_2O are linked to form O_2 which is then released. The electron transport from Mn cluster to P680 is linked by tyrosine residue.

1.3.1 Structure of PSII

The oxygen-evolving site of PSII is located on the inner water phase (lumen), while the plastoquinone-binding site near the outer water phase (stroma in eukaryotes and cytoplasm in cyanobacteria) (See Fig. 5 and Govindjee et al., 2010). PSII is often called as water/plastoquinone photo oxidoreductase. It performs 2- electron chemistry on the quinone-acceptor side and its 4-electron chemistry on the water-oxidizing side (Cardona et al., 2011). Photosystem II composed of central reaction core center which is surrounded by light harvesting antenna system. Where the core of PSII is formed by two subunits D1 and D2 polypeptide that bind to cofactors responsible for charge separation and electron transfer for oxidation of water upon reduction of plastoquinone. The antennae system of PSII consists of light absorbing molecule mainly chlorophyll and phycobilins (Govindjee et al., 2010, Groot et al., 2005, Goldfarb et al., 1997).

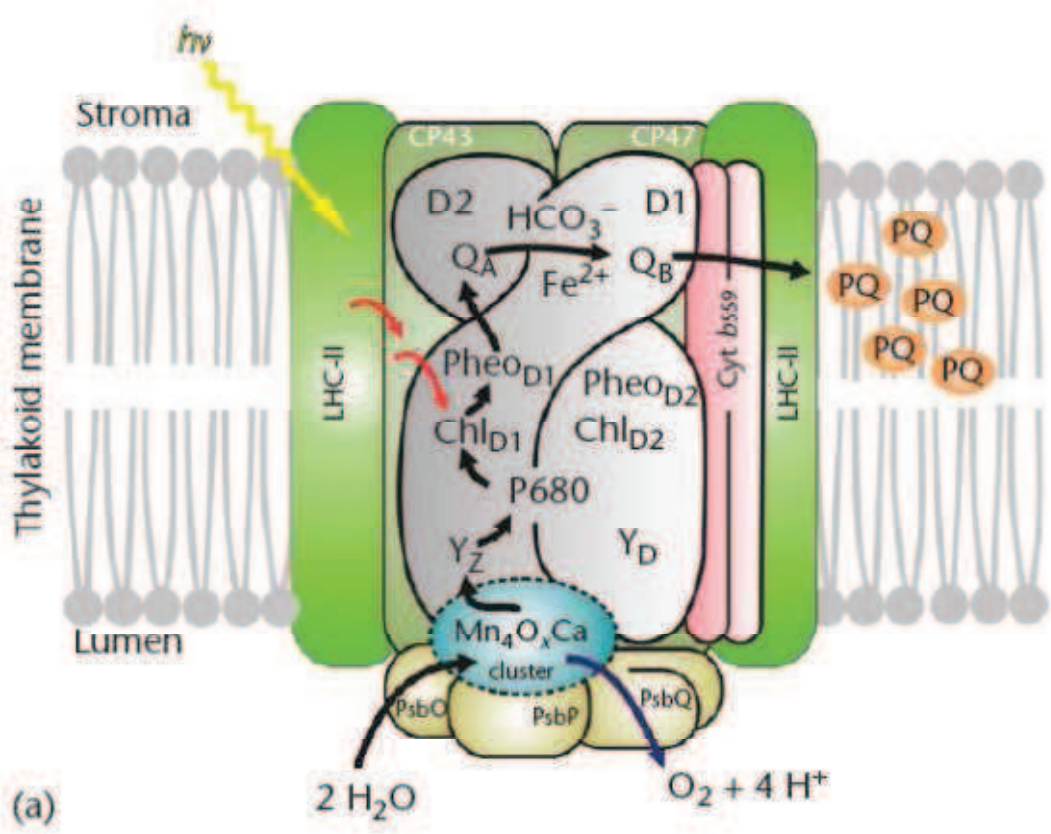


Fig 5. A schematic diagram showing the structure of the photosystem II complex. The figure is dopted from Govindjee et al., 2010.

PSII contains the following redox components: chlorophylls, pheophytin, Quinones QA and QB, redox active tyrosine, Mn cluster, non-heme iron, cytochrome b559 and carotenoids(Govindjee et al., 2010) (See Fig. 5).

1.3.1.1 Electron acceptor side of PSII

The acceptor side of PSII composed of Pheophytine, Q_A, Q_B, and non-hem iron in between these two quinones. Pheophytine is a chemical compound that serves as the first electron carrier intermediate in the electron transfer pathway PSII in plants, and the photosynthetic reaction center (RC P870) found in purple bacteria (Yamamoto, 2001). In the past few decades a number of studies have been done to study the compounds of the electron acceptor side of PSII. Thus, it has been found that hydrogen carbonate (bicarbonate (BC)) bound to the non-heme iron between QA and QB, plays a key role in protonation of PQ (reviewed in Shevela et al., 2012 and McConnell et al., 2012). The Q_B binding site serves

essentially as a 'loading dock' for the sequential transfer of two electrons onto PQ, which, through the binding of two H^+, is converted to PQH_2 (Staehelin et al., 2004).

However, depletion of BC has effect both on the acceptor and donor side of PSII. BC evidently has two sites of action: the first accelerates the electron flow beyond Qa, and the other stimulates it between the secondary electron donor, Yz, and QA (the site between the primary electron acceptor, pheophytin, and QA was suggested for the latter case (Klimov et al., 1995). Acceptor-side photoinhibition involves the interaction between the plastoquinone electron acceptor QB and molecular oxygen (Yamamoto, 2001). The functional analogies between PSII and the bacterial reaction centre seem to be restricted to the primary donor P680, the intermediate acceptor pheophytin and the first and second quinone acceptors (QA and QB) on the reducing side of the reaction centre(Svensson et al., 1990).

1.3.1.1 Electron donor side of PSII and the Oxygen evolving complex (OEC)

The electron donor side of PSII comprises oxygen-evolving complex (OEC), two tyrosine residues namely Y_z(D1-Tyr161), Y_D(D2-Tyr161), and P680 (Bricker et al., 2004). BC like the acceptor side it has also a role in the donor side of PSII, some of these roles of BC on the donor side of PSII are: 1) it serves as an electron donor to the Mn containing oxygen evolving center. 2) it converts the non-oxidizable aqua ions of Mn^{2+} into easily oxidizable form. 3) is essential constitute of the water oxidizing Mn cluster. 4) it increases the binding of Mn^{2+} and the formation of the cluster. Thus increase the capacity of PSII complex to bind Mn^{2+} (Klimov et al., 1995). $P680^+$ and the oxidized tyrosine electron donor Tyr Z^+ are long-lived when the photosystem II complexes loses the catalytic Mn cluster or when the final Oxygen-evolving step is inhibited, for example, under low chloride conditions (Yamamoto, 2001, Bricker et al., 2004).

The order of susceptibility of photosystem II components to photodamage in donor-side photo inhibition was suggested as chlorophylls / carotenoids > Tyr_Z> Tyr_D >> P680, Pheophytin, Q_A (Yamamoto, 2001).

The OEC is part of PSII that contains a tetra nuclear Mn cluster, a redox active tyrosine, and required cofactors Ca^{2+}, and Cl^- ion (Drath et al., 2009, Hillier et al., 2005). The oxygen-evolving complex can exist in one of the five oxidation states, labeled S_0, S_1, S_2, S_3 and S_4. Each photochemical reaction removes a single electron from the water-oxidizing complex, which advances PSII to the next higher S state, until four oxidizing equivalents are accumulated, leading to the oxidation of two molecules of water(Govindjee et al., 2010). The OEC can be made non-functional by the treatments with hydroxylamine, azide, hydrazine, aging, by storing

leaves in the cold, by exposure to high concentrations of Tris buffer and by a chloride deficiency. (Lefebvre-Legendre et al., 2007)

CP47 and CP43 are chlorophyll proteins which play are interior transducers of excitation energy from the light-harvesting pigment proteins (LHCP in plants, the phycobilisomes in cyanobacteria and red algae) to the photochemical reaction center (Bricker et al., 2004). A large (190 amino acid residue) extrinsic loop is one of the most intriguing structural features of CP47. Cross linking experiments demonstrated that domains on this loop are easily cross-linked to the 33 kDa extrinsic protein of PS II. It contains either 10-12 or 20-25 bound chlorophyll *a* molecules. CP47 and CP43 transfer excitation energy from the exterior antennae (LHCP or phycobilisomes) to the chlorophylls of the photochemical reaction center of PS II. Both CP47 and CP43 required for the assembly of oxygen-evolving PSII centers and for the stability of PSII (Bricker et al., 2004).

1.4 Photosystem I and its endogenous electron acceptor and donors

The final stage of the light reactions is catalyzed by PSI which is a plastocyanin /ferredoxin photooxidoreductase (Walker, 2002, Rutherford et al., 2012). It has two main components forming its core, psaA and psaB. These two subunits are quite a bit larger that the core components of PSII. It primarily composed of antenna complex, proteins, ions, a molecule called phylloquinone, a reaction center chlorophylls chl a/chl a' (called P700). Upon excitation- either by direct absorption of a photon or exciton transfer- P700* transfers an electron through a chlorophyll and a bound quinone (QA) to a set of 4Fe-4S clusters. From these clusters the electron is transferred to ferredoxin (Fd). Fd is an iron-containing molecule that receives an excited electron from PSI and provides it for the reduction of $NADP^+$(Edwards and Walker, 1983).There are sufficient similarities between PS I and RCs of green sulfur bacteria and heliobacteria to suggest a common lineage for all type I RCs In PS I, the primary electron donor is P700, a Chl *a* dimer, and binding sites for the electron acceptors A_0 (a Chl *a* monomer), A_1 (phylloquinone), and F_x (a 4Fe-4S cluster) are generated by a heterodimeric structure comprised of *psaA and psa*B gene products (Wolfe et al., 2004).

The cytochrome complex and its counterparts in other membrane-bound electron transfer systems (the complexes of bacterial plasma membrane and mitochondrial inner membranes) are comprised of a single *c*-type heme, 2 *b*-type hemes and an unusual Fe/S center (termed a 'Rieske' center after its discoverer), characterized by a positive, rather than negative, reduction potential (Ort et al., 2004). The ability of the cytochrome b_6f complex to catalyze diverse quinone oxidation reactions is central to thylakoid activity. Cyt b_6f has two PQ binding sites associated with the polypeptide, the q-site that binds quinol and is located close to the lumenal

surface, and the n-site that binds quinone and is closer to the stromal surface (Staehelin et al., 2004). The oxidized PS I is rereduced by an electron provided by reduced plastocyanin. As the excited electron moves through PS I across the membrane to ferredoxin, it passes through a number of prosthetic groups including a Chl known as A_0, bound phylloquinone and iron sulfur centers (Staehelin et al., 2004).

1.5 Artificial Electron Acceptors and Donors

There are a number of compounds that are used as an exogenous electron acceptors and donors, inhibitors and "uncouplers" in the study of photosynthetic electron transport system. Electron acceptors are those compounds that accept electron from a compound and made the compound oxidized while they get reduced in the process. Electron donors are compounds that are associated with donating electrons and get oxidized. Generally inhibitors of photosynthetic electron transfer chain inhibit the transfer of electrons from one compound to the other (Edwards and Walker, 1983). Oxidation always involves electron transport, where electrons are transported from the atoms or molecules which are being oxidized to corresponding electron acceptors whereby it makes it more negatively charged and become reduced (Walker, 2002).

Table 1 Exogenous electron acceptor donor or inhibitor in photosynthetic electron transport system in the thylakoid membrane of a chloroplast collected from van Rensen, 2002, Shevela & Messinger,BBA 2012, Edwards and Walker, 1983, Klimov et al., 1995

Compound name		
Electron donor	**Electron acceptor**	**Inhibitor**
ASC (Ascorbate)	FeCy (potassium ferricyanide)	rotenone,
DPC (diphenylcarbazide)	BQ (benzoquinone)	amytal
	DAD (diaminodurene(tetramethyl-*p*-phenylenediamine))	antimycinA
NQ (naphtaquinone)		cyanide
	DCBQ (2,5-dichlorobenzoquinone)	azide
	PPBQ (phenyl-*p*-quinone)	atrazin
	SM (silicomolybdate)	KCN (Potassium cyanide)
	MV (methyl viologen)	DCMU (diurone(3-(3,4-dichlorophenyl)-1,1-dimethylurea)
		DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone)
		carbon monoxide
		NH_2OH
		Tris buffer

Below we provide more detailed information for the most used exogenous electron acceptors in photosynthesis research and the sites of action for some of the above compounds given in table 1.

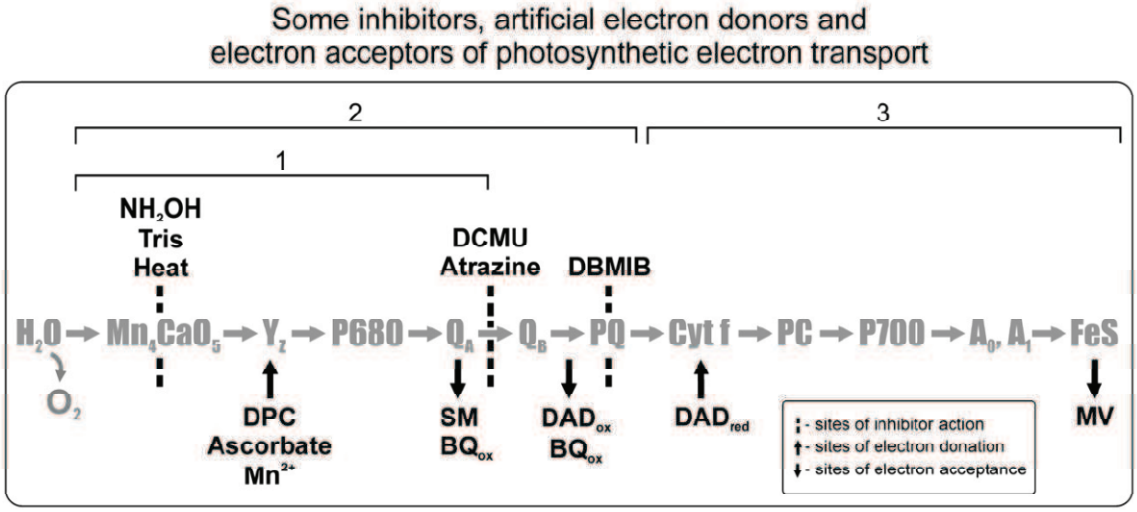


Fig. 6 Sites and action of some exogenous electron acceptors, donors and inhibitors. The figure is taken from Shevela & Messinger,BBA 2012

1.5.1 Potassium ferricyanide ($K_3[Fe(CN)_6]$)

Potassium ferricyanide (FeCy) also known as Potassium hexacyanoferrate(III) having molecular weight 329.24 g/mol is a bright red salt contains the octahedral coordinated $[Fe(CN)_6]^{3-}$ ion which is soluble in different solvents like water, acetone, different forms of acids and slightly in alcohol. its solution shows green-yellow fluorescence(Jacobs, 2011). The chemical structure is given in Fig. 7.

The action of FeCy in the photosynthetic electron transport occurs in two sites: one accepting electron from Q_B which the acceptor side of PSII and the other is from the FeS center (See Fig. 6 and Lavergne et al., 2004, Shevela and Messinger, 2012b). It is a quite good widely used electron acceptor (for instance, see Diner et al., 2004, Whitmarsh et al., 2004). For out study Fecy has been chosen that it gives emission spectra which is different from emission spectra of thylakoid membrane of chloroplast (Horio and Sanpietro, 1964).

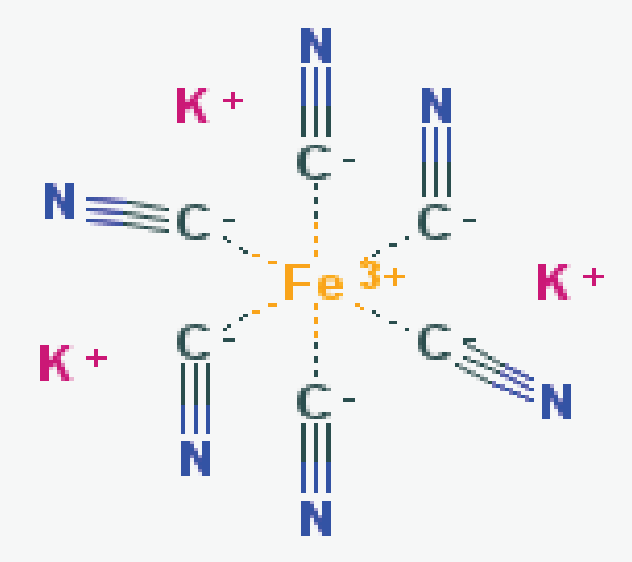


Fig 7. Chemical structure of Potassium ferricyanide adopted from ncbi, 19/07/2005

1.5.2 Phenyl-p-benzoquinone (PPBQ)

Phenyl-p-benzoquinone (PPBQ) is a dark yellow (yellow-brown) powder with molecular formula $C_{12}H_8O_2$ and molecular weight 184.19 g/mol, which is highly sensitive to light and slightly soluble in water but highly soluble in alcohol and has a green yellow florescence. The chemical structure of the compound is given in Fig. 8.

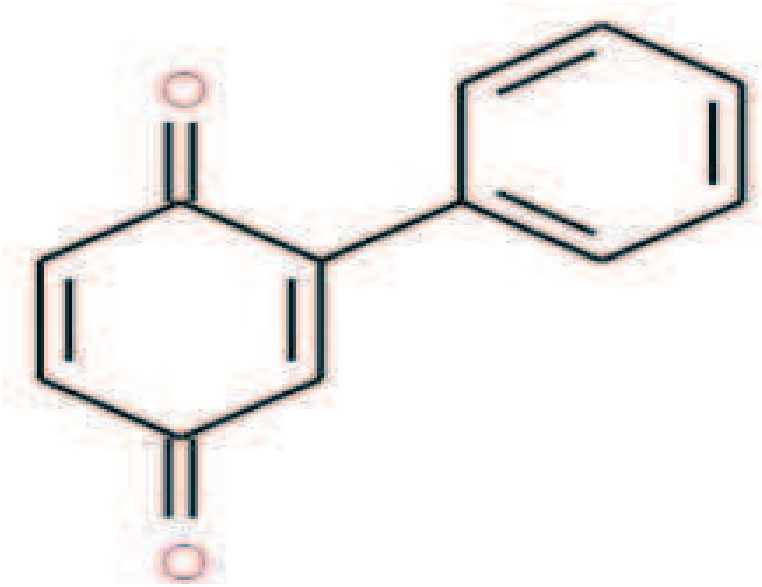


Fig 8. Chemical structure of phenyl-p-benzoquinone (PPBQ) taken from ncbi, 26/03/2005

PPBQ like FeCY is an exogenous electron acceptor in the photosynthetic electron transport in the thylakoid membrane of a chloroplast (Edwards and Walker, 1983, van Rensen, 2002). It accepts electron from Q_B pocket (Shevela and Messinger, 2012b). The reason to use PPBQ in the study of photosynthetic electron transport in the thylakoid membrane it has different absorption spectra from the chloroplast, which mean the emission spectra of the compound is different from the emission spectra of thylakoid membrane of chloroplast solution this is true for FeCy as well.

1.6 Objective of the Work

The purpose of this research project is to probe the possibility of characterization of light induced PSII turn over efficiency via monitoring of the reduction of commonly used exogenous electron acceptors (such as $K_4[Fe(CN)_6]$ and PPBQ) by UV/Vis and fluorescence spectroscopy.

2. Materials and Methods

2.1 Fluorescence and UV/VIS Spectroscopy

Fluorescence spectra were recorded in Fluorolog-3 spectrofluorometer of the company Horiba Jobin Yvonr. The spectrophotometer used was UV-2401 PC UV-Vis recording spectrophotometer of Shimadzu Company. The short introduction about the development and the function of the instrument is given below.

The emission spectrum provides information for both qualitative and quantitative analysis. When light of an appropriate wavelength is absorbed by a molecule (i.e., excitation), the electronic state of the molecule changes from the ground state to one of many vibrational levels in one of the excited electronic states. The excited electronic state is usually the first excited singlet state, S1. Once the molecule is in excited state, relaxation can occur via several processes. Fluorescence is one of these processes and results in the emission of light. Moreover, fluorescence has an average lifetime from $<10^{-10}$ to 10^{-7} sec from singlet states. This is shown in the Fig. 9.

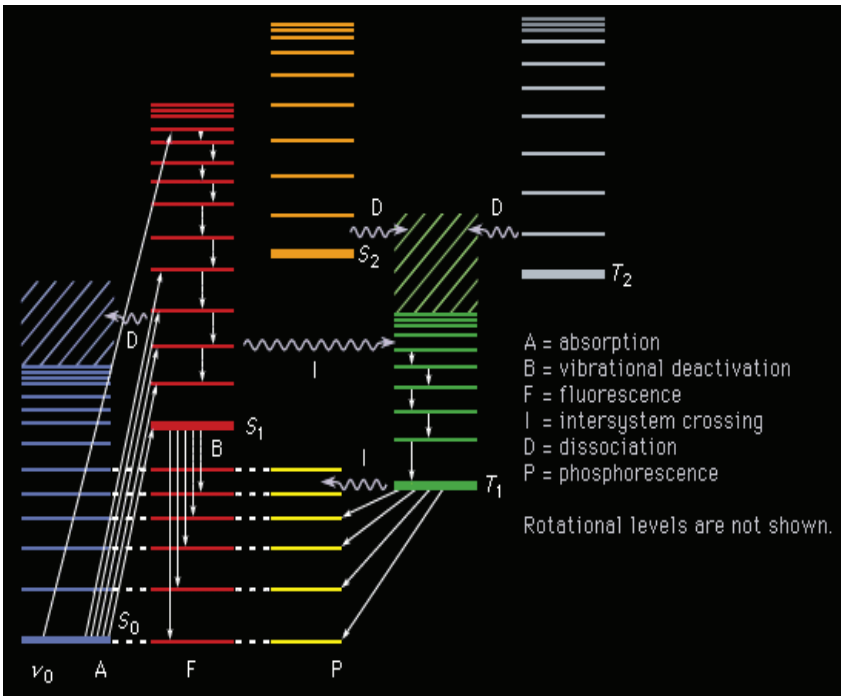


Fig 9. Possible Electronic transition energy level of an excited atom taken from Encyclopedia, 2012

Fluorescence uses for many Biochemical processes including sensitivity to local electrical environment (polarity, hydrophobicity), track biochemical reactions, measure local friction

(micro viscosity), track solvation dynamics and measure distances using molecular rulers fluorescence resonance energy transfer (FRET) (Lakowicz, 2006). Our fluorescence instrument setup can be represented by the following diagram:

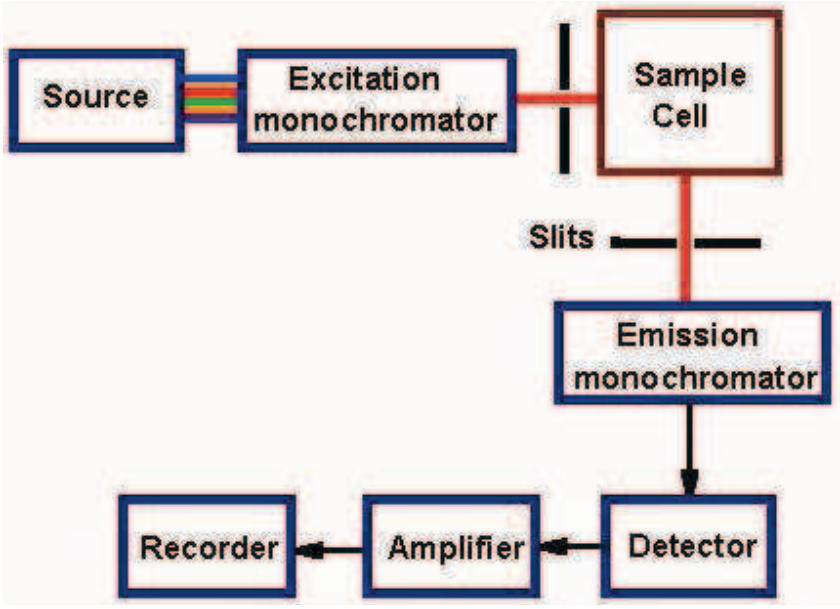


Fig 10. Fluorescence instrumental setup adopted from Chemistry, 2012

The measuring parameters of the spectrofluorometer were the following: Instrumental setup for spectroflorometry (Florolog-3 spectrofluorometer) was at right angle mode, stirrer speed 7, slit widths 4-5 nm, integration time 0.1, temperature 5^{o}C, graph resulted from S_{1c}/R_{1c}, excitation wave length range 350-478 nm, emission wavelength range 250-550 nm, reaction mixture 3 ml.

The instrumental set up for UV-2401 UV/Vis fluorescence spectrophotometer was; reaction mixture 3 ml, slit width 5 nm, wavelength 250-550 nm and the sampling interval was 1.0. All continuous spectrophotometric measurements were performed using standard 1 cm quartz curettes in a total reaction volume of 3 ml.

2.2 Reagents

All chemicals used in this study were of analytical (≥ 98%) or higher grade (99.97% - 99.99%). The reagents were obtained from different reagent manufacturers like Sigma, Merck, Applichem, Fluka etc. if not stated otherwise, For all aqueous solutions deionised and filtrated (Millipore Quality, MQ) water was used.

2.3 Buffers and Stock Solutions

Buffers

1. Grinding buffer (pH 6.5) composition
   1 mM EDTA
   4 mM $MgCl_2$ X $6H_2O$
   50 mM HEPES
   400 mM NaCl
   100 mM sucrose
   5 mM Sodium Ascorbate*
   2 mg/ml BSA*

   * The substances were added to the buffer shortly before pH was adjusted

2. Washing buffer (pH 6.5) composition
   50 mM HEPES
   8 mM $MgCl_2$ X $6H_2O$
   150 mM NaCl

3. Storage buffer (pH 6.5) composition
   40 mM MES
   5 mM $CaCl_2$ X $2H_2O$
   5 mM $MgCl_2$ X $6H_2O$
   15 mM NaCl
   400 mM Sucrose

Stock solution

$K_3[Fe(CN)_6]$: 50 mM K3[Fe(CN)6] was dissolved in MQ water and kept on ice until use.

PPBQ: 20 mM PPBQ was dissolved in DMSO and kept on ice until use.

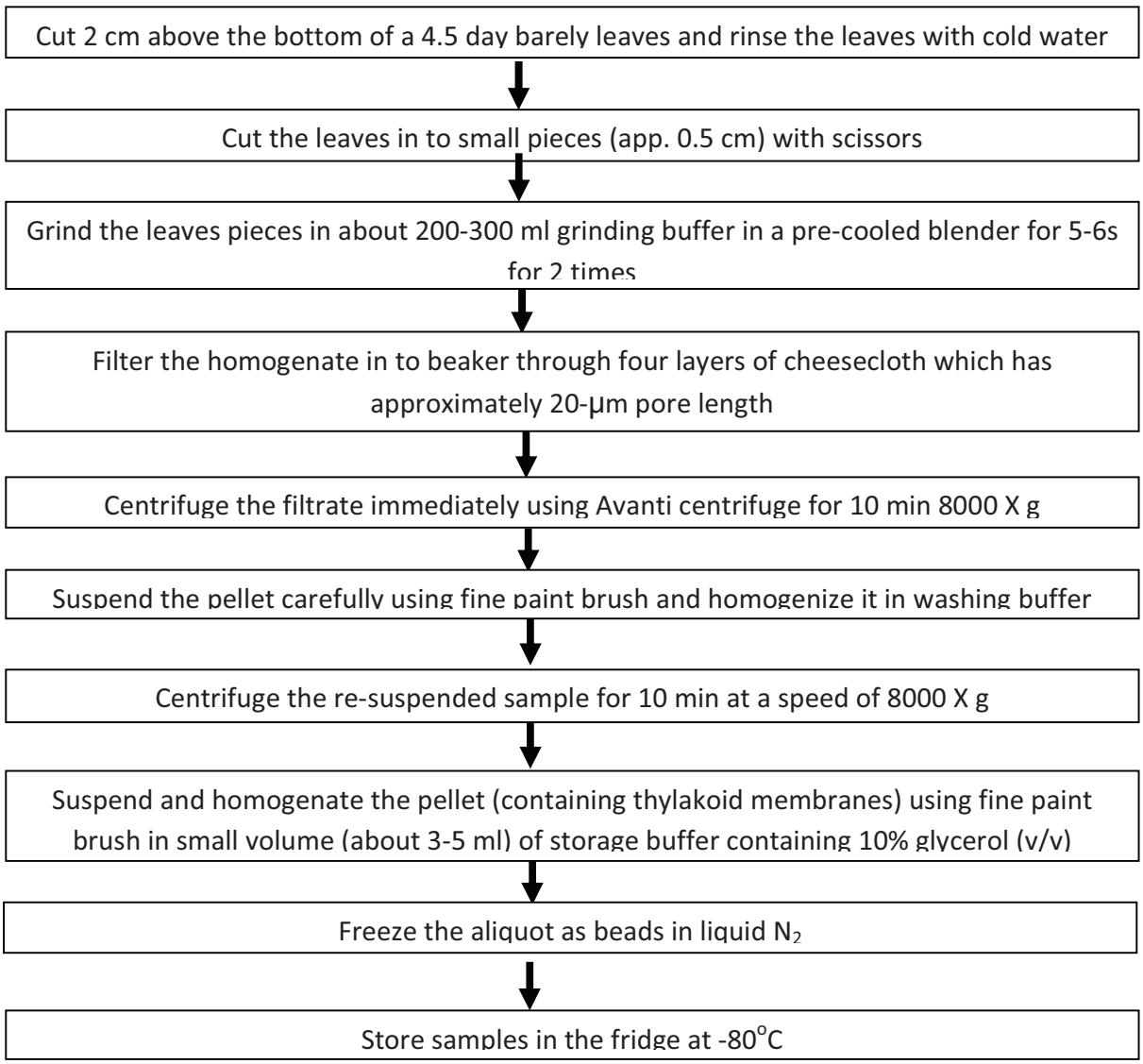
Ascorbic acid: 50 mM ascorbic acid was dissolved in MQ water and kept on ice until use

DCMU: 80 mM DCMU was dissolved in ethanol and kept on ice until use

2.4 Isolation of Membrane Thylakoids from Barley

A 4.5 day barley (*Hordeum vaulagare)* leaves which was grown under light were cut 2 cm above the bottom and rinsed with cold water then it was cut in to pieces to have a size of 0.5 cm with scissor. The leaves were grind with cooled blender in 200-300 ml grinding buffer for 5-6 second for two times. The homogenate was filtrate in a beaker through a 4 layer 20 µm pore length cheese cloth after that the filtrate was centrifuged and using fine paint brush. The pellet was suspended in washing buffer for two times and the final pellet was re-suspended and

homogenized in storage buffer with 10% glycerol (v/v), and stored in -80^{o}C as a bead in liquid nitrogen until use. The general flow chart of the isolation procedure is given below.



2.5 Determination of Chlorophyll Concentration

12.72 µL volume of thylakoids which is equal to 1 X 10^8 plastid per µL was taken and diluted 100 X with 80% buffer acetone solution (2.5 mM Na_2HPO_4/H_2NaPO_4 . $2H_2O$ pH of 7.8) and the mixture was centrifuged for 1 min at 10000 X g and discarded the pellet then the absorption spectra of the supernatant was measured against 80% buffered acetone solution. Absorption spectra measurement was taken at 646.6, 663.3 and 750 nm using UV-2401 absorbance spectroscopy and finally the chlorophyll concentration was calculated using the following equation:

$[17.75 (A_{646.6}-A_{750}) + 7.34 (A_{663.3}-A_{750})]$ K= [1 mg Chl_{total}/ml] = [chl a+chl b]

Where K is dilution factor ($K=\mu L_{solution}/\mu L_{sample}$)

3. Result and Discussion

In this experiment we focused on probing the possibility of characterization of light induced PSII turn over efficiency via monitoring the reduction of commonly used exogenous electron acceptors (such as $K_3[Fe(CN)_6]$ and PPBQ) by UV-2401 UV/Vis fluorescence spectroscopy and Fluorolog-3 spectrofluorometer. There was a need to determine the absorption maximum of these compounds, reduction rate upon illumination and by addition of external electron acceptor to probe the change and compare results obtained with two compounds.

3.1 Determination of Chlorophyll Concentration

The chlorophyll concentration of the isolated thylakoid membrane (12.72 µL of thylakoids which is equal to 1 X 10^8 plastid per µL) was determined using UV/Vis and fluorescence spectroscopy and the following measurements were taken and chlorophyll concentration was calculated using the formula in Methods and Material section. The purpose of determining the chlorophyll concentration was to find out the amount of chlorophyll to be used for other measurements (measurement with Fecy and PPBQ). The chlorophyll concentration of the isolated thylakoid membrane was 100.1 mg chl/ml.

Trial	**A_{750} nm**	**$A_{663.6}$ nm**	**$A_{646.3}$ nm**
1	0.000	0.058	0.031
2	0.001	0.059	0.032
3	0.000	0.060	0.033
Average	**0.000**	**0.059**	**0.032**

$[17.75(A_{646.6}-A_{750}) + 7.34(A_{663.3}-A_{750})]K = [1\ mg\ Chl_{total}/mL] = [chl\ a + chl\ b]$
100 [17.75 (0.032-0.000) + 7.34 (0.059-0.000)]
100 [0.568 + 0.433]
1.001 X 100
100.1 mg chl/mL

3.2 Potassium Ferricyanide ($K_3[Fe(CN)_6]$) Measurements

A number of absorbance, emission and excitation spectra of potassium ferricyanide were taken in order to understand the changes and other properties of FeCy in the presence and absence of thylakoid membrane, the main results and discussion is provided below.

3.2.1 Determination of the Absorbance Spectra of Different Concentration of FeCy

The absorbance spectra of 25, 50, 100, 250, and 500 µM, 1 and 2 mM Potassium ferricyanide ($K_3[Fe(CN)_6]$) were measured using UV-2401 absorbance spectroscopy against 10% buffered glycerol storage buffer. The purpose of this measurement was to find the maximum absorption peak and the response of the compound in relation to different concentration. What was expected that the compound might give absorbance around a wavelength of 420 nm. And all measurements will give absorbance below 1. However, Based on the absorbance spectra result obtained for the above concentrations of Potassium ferricyanide gave absorbance above 1 for concentrations of 1 and 2 mM $K_3[Fe(CN)_6]$ and that of 25 µM is not consistent. Hence they were not taken for further measurement of $K_3[Fe(CN)_6]$ in relation with different concentration. This is shown in Fig. 11. Unlike to a single absorbance peak expected Fecy gave three absorbance peaks. Which were at 260, 302, and 420 nm. But still the absorption maximum was at 420nm.

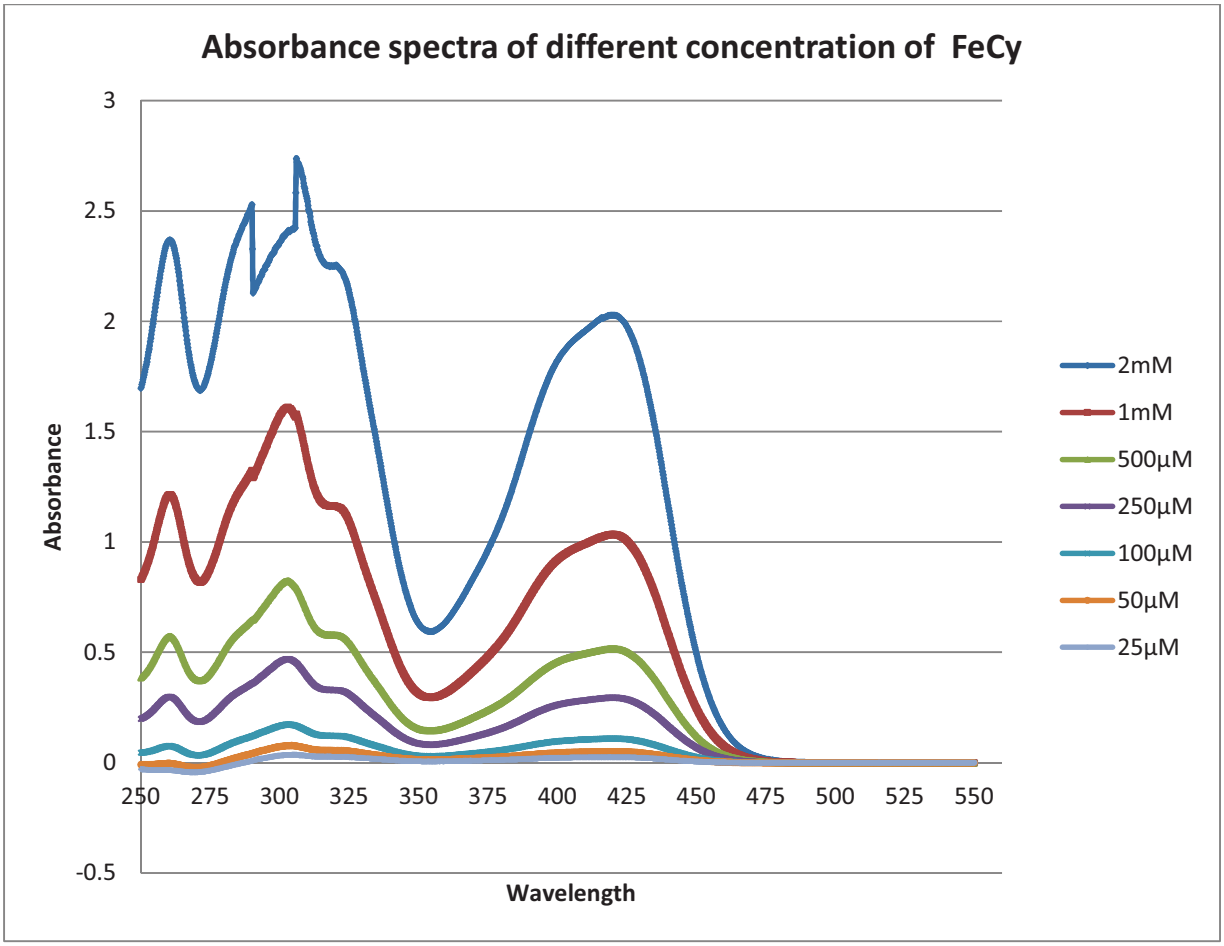


Fig 11. Absorbance spectra of different concentration of potassium ferricyanide

In general the compound gives three absorbance peaks at different wavelengths. These are 260, 302 and 420 nm as it can be seen from the figure 11 given above.

3.2.2 Excitation spectra of FeCy

50, 100, 250, and 500 µM ferricyanide concentration were prepared in 3 ml reaction mixture using storage buffer (pH of 6.5). The emission spectra were monitored upon exciting the sample at wavelength range of 350-480 nm with increment of 10 nm. The peak and the intensity were recorded using the instrumental setup stated in the method section, and the following results were obtained from the measurements. The main purpose of this measurement was to check whether there was a possibility that the emission spectra are dependent on concentrations and on excitation wavelengths.

Table 2. 50 µM potassium ferricyanide

Excitation wave length	Emission peak 1	Intensity	Emission peak 2	Intensity
350	398	1276650	443	1249400
360	411	1287060	446	1176420
370	424	1226890	450	1085640
380	436	1123730		
390	449	944990		
400	462	713630		
410	476	481710		
420	490	335120		
430	502	222970		
440	516	158250		
450	531	113680		
460	545	73760		
470	558	55270		
480	574	30610		

Table 3. 100 µM potassium ferricyanide

Excitation wave length	Emission peak 1	Intensity	Emission peak 2	Intensity
350	397	120060	424	4126410
360	411	1262540		
370	424	1107200		
380	438	1037870		
390	450	864240		
400	463	674210		
410	476	442640		
420	490	307180		
430	504	203200		
440	517	146720		
450	531	107590		
460	546	70550		
470	558	51570		
480	573	28370		

Table 4. 250 µM potassium ferricyanide

Excitation wave length	Emission peak 1	Intensity	Emission peak 2	Intensity
350	398	920430	448	940640
360	410	871530	450	923240
370	424	803360	453	819950
380	438	795550	458	675420
390	450	659710		
400	462	502260		
410	477	335790		
420	490	231210		
430	503	160740		
440	517	122970		
450	531	97270		
460	545	68110		
470	558	48740		
480	573	26570		

Table 5. 500 µM potassium ferricyanide

Excitation wave length	Emission peak 1	Intensity	Emission peak 2	Intensity
350	397	642280	454	715290
360	410	596350	455	770810
370	424	540070	457	688520
380	438	524780	458	532770
390	451	479490		
400	462	364570		
410	476	241850		
420	489	164520		
430	503	119960		
440	516	103280		
450	530	89680		
460	546	64970		
470	558	48650		
480	574	26550		

The experimental expectation was the emission spectra of FeCy are dependent on the excitation wave length used and concentration as well. However, from the mission spectra measurements we can see that the emission was not dependent on the concentration rather the emission changes as the excitation wave length changes. However the intensity is dependent on the concentration and excitation wavelength but still as the concentration goes high the intensity will also drop down or decreased this may be due to inner filler effect. In general from the above four concentration measurements it shows that excitation wave length 410 and 420 nm gives emission spectra at 476 and 490 respectively in almost all measurements so. These wave lengths were taken for further study of potassium ferricyanide with and without addition of thylakoid membranes.

3.2.3 Determination of Peaks Obtained in Absorbance Measurements is a Result of Fluorescence or Not

The emission spectrum of 50, 100, 250, and 500 µM potassium ferricyanide concentration was measured using fluorescence spectrophotometer to check whether the peaks obtained in absorbance measurements (260, 302 and 420 nm) were a result of fluorescence or not by using these three wavelength as excitation wavelength, and the instrumental setup used was what stated in the method part except, emission wave length range was adjusted to 270-500 nm for 260 nm, 312-500 nm for 302 nm and 430-550 nm for 420 nm. To prepare the sample the following amounts were taken to make the respected concentrations.

Final Concentration	50 mM stock potassium ferricyanide	10% glycerol storage buffer	Final reaction volume
50 µM potassium ferricyanide	3 µL	2997 µL	3 mL
100 µM potassium ferricyanide	6 µL	2994 µL	3 mL
250 µM potassium ferricyanide	15 µL	2985 µL	3 mL
500 µM potassium ferricyanide	30 µL	2970 µL	3 mL

The expectation for this experiment was each absorption peak is a result of florescence. From the measurement It was found that its only peak 420 nm was clearly as a result of florescence where as the other two (260 and 302) were not a result of the fluorescence even though they give fluorescence it's not stable like that of 420 nm. When the emission wave length is set at 490 and measured for excitation wavelength it gives excitation at 420 nm. Furthermore the excitation spectra of 50 µM Potassium ferricyanide with and without 5 µgm chl/mL was measured using the instrumental setup stated in the method part except the emission wavelength was set to 490 nm, excitation wavelength range to 350 - 478 nm, and the measurement was taken for 1 hr with 3 min interval. The result obtained from this confirms that the excitation wavelength of Potassium ferricyanide is 420 nm. This coincide with (Schellenberg and Hellerman, 1958) finding that the excitation and emission wave length of potassium ferricyanide is 420 and 490 respectively.

3.2.4 Reduction of Different Concentration of FeCy over Time in Dark

The reduction of spectral change of emission spectra of 50, 100,250 and 500 µM of Potassium ferricyanide was determined over time for 1 hr in 10 and 3 min interval using the same instrumental setup stated in the method part where the changes were the excitation wavelength was set to 420 nm, emission wavelength range to 432 - 750 nm. The purpose of this experiment is to find out whether FeCy solution is stable or not. The 20 cycle measurements with three minute interval showed that the expectation was right even though there was a slight reduction of potassium ferricyanide measured in the dark, In general the FeCy solution gave constant/stable emission spectra over time.

3.2.5 Emission Spectra of Different Concentration of FeCy with Different Concentration of Chl/mL

The emission spectral change of 50, 100, 250, 500 µM potassium ferricyanide with 5, 10, 15 and 20 µ gm chl/mL was determined by using the instrumental setup stated in the method part. The purpose of this measurement is to find out the effect of different concentrations of thylakoid membrane on the reduction of FeCy upon emission spectral measurements. The expectation was the different combination of FeCy with different concentration of thylakoid membrane will give almost similar result since the measurement was taken in the dark.

Table 6. Composition of 3 mL reaction mixture

Reaction mixture	plastids	50 mM stock potassium ferricyanide	10% glycerol storage buffer	Final reaction volume
50 µM $K_3[Fe(CN)_6$ with 5 µ gm chl/mL	0.5 µL	3 µL	2997 µL	3 µL µL
50 µM $K_3[Fe(CN)_6$ with 10 µ gm chl/mL	1 µL	3 µL	2996 µL	3 µL µL
50 µM $K_3[Fe(CN)_6$ with 15 µ gm chl/mL	3 µL	3 µLl	2984 µL	3 µL
50 µM $K_3[Fe(CN)_6$ with 20 µ gm chl/mL	6 µL	3 µL	2991 µL	3 µL
100 µM $K_3[Fe(CN)_6$ with 5 µ gm chl/mL	0.5 µL	6 µL	2994 µL	3 µL µL
100 µM $K_3[Fe(CN)_6$ with 10 µ gm chl/mL	1 µL	6 µL	2993 µL	3 µL
100 µM $K_3[Fe(CN)_6$ with 15 µ gm chl/mL	3 µL	6 µL	2991 µL	3 µL µL
100 µM $K_3[Fe(CN)_6$ with 20 µ gm chl/mL	6 µL	6 µL	2988 µL	3 µL
250 µM $K_3[Fe(CN)_6$ with 5 µ gm chl/mL	0.5 µL	15 µL	2985 µL	3 µL
250 µM $K_3[Fe(CN)_6$ with 10 µ gm chl/mL	1 µL	15 µL	2984 µL	3 µL
250 µM $K_3[Fe(CN)_6$ with 15 µ gm chl/mL	3 µL	15 µL	2981 µL	3 µL
250 µM $K_3[Fe(CN)_6$ with 20 µ gm chl/mL	6 µL	15 µL	2979 µL	3 µL
500 µM $K_3[Fe(CN)_6$ with 5 µ gm chl/mL	0.5 µL	30 µL	2970 µL	3 µL
500 µM $K_3[Fe(CN)_6$ with 10 µ gm chl/mL	1 µL	30 µL	2969 µL	3 µL
500 µM $K_3[Fe(CN)_6$ with 15 µ gm chl/mL	3 µL	30 µL	2967 µL	3 µL
500 µM $K_3[Fe(CN)_6$ with 20 µ gm chl/mL	6 µL	30 µL	2964 µL	3 µL

Introducing of different concentration of thylakoid membrane to the reaction mixture resulted in the reduction of potassium ferricyanide over time in the dark. However, since the measurements were taken in the dark the PSII action is low. This is because the photolysis of water ($2H_2O \rightarrow O_2 + 4e^- + 4H^+$) by PSII is a completely light dependent reaction. As we know light is the energy that derives this process. Hence the number of electron available to reduce

potassium ferricyanide becomes few and this made the reduction spectral change become small.

3.2.6 Reduction of FeCy by Adding Hydrogen Per-Oxide upon Illumination

The same procedure which was used in the above measurement (measuring the reduction of potassium ferricyanide without chlorophyll sample) was used with only few changes where the pH of the buffer was adjusted in alkaline pH by adding KOH then the measurement was taken during titrating the sample with 5% Hydrogen peroxide.

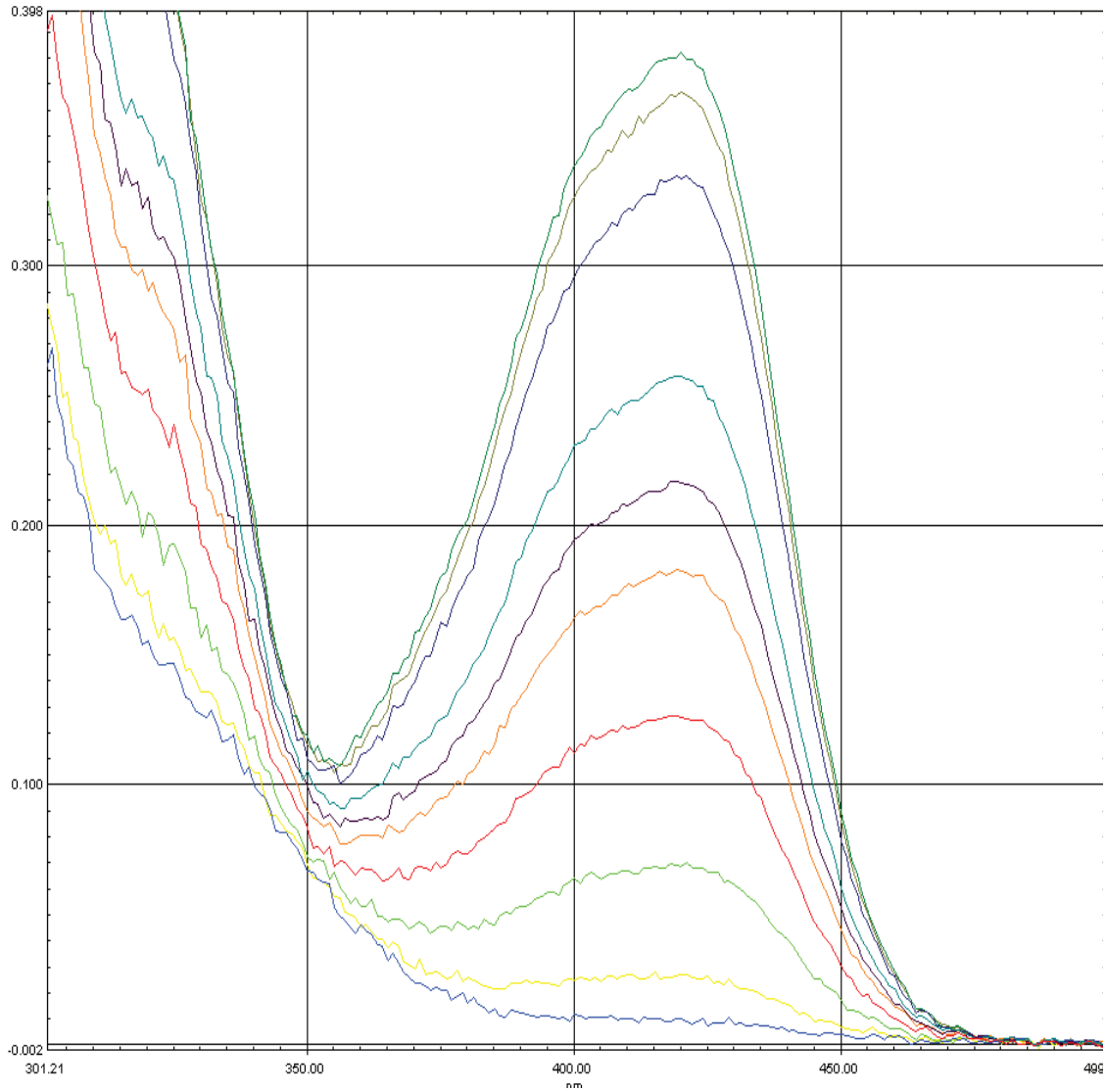


Fig 12. Absorption spectral change of 500 µM potassium ferricyanide upon adding 20 µl 5% H_2O_2 upon illumination

As it can be clearly shown in the figure 12 potassium ferricyanide can be reduced by titrating with hydrogen peroxide while illumination. 500 µM potassium ferricyanide was completely reduced to potassium ferrocyanide. In order to this process to take place the reaction medium should be in alkaline pH range (Park and Johnson, 1949)and this was done by potassium hydroxide (KOH) then once the pH was adjusted in alkaline pH range 20 µM Hydrogen peroxide was added and absorbance spectra was measured till the absorbance is close to zero. The following reaction was taking place when potassium ferricyanide is reduced to potassium ferrocyanide by hydrogen peroxide in alkaline medium.

$$2K_3[Fe(CN)_6] + 2KOH \rightarrow 2K_4[Fe(CN)_6] + H_2O + O \quad \ldots\ldots\ldots\ldots 1$$

$$H_2O_2 + O \rightarrow H_2O + O_2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

Combine 1 and 2 gives $2K_3[Fe(CN)_6] + 2KOH + H_2O_2 \rightarrow 2K_4[Fe(CN)_6] + 2H_2O + O_2$

3.2.7 Effect of Adding Different Concentration of Chl/ml in the Reduction of FeCy

The absorbance spectral change of potassium ferricyanide was measured by using 500 µM $K_3[Fe(CN)_6$ in a 3 mL quartz cuvette with and without thylakoid membrane where the measurement was made against 10% buffered storage buffer and the instrumental setup was set to: wavelength to 250-550 nm, slit to 5 nm, using UV-2401 absorbance spectroscopy for 10 times with 5 min illumination time. The purpose of this experiment was to determine the effect of different concentration of thylakoid membrane on the reduction of 500 µM FeCy using UV-2401 spectrophotometer since the Fluorolog-3 spectrofluorometer measurement doesn't show good result.

The spectrum of the light-induced changes of absorbance difference in barley thylakoids was determined by introducing different concentration of chlorophyll to the reaction mixture. The change in the reduction of the potassium was monitored and it was found that the change in spectra was not dependent on the concentration of potassium ferricyanide in the system rather the change was dependent on the concentration of thylakoid membrane added to the system. For the small concentrations of thylakoid membrane in respect of potassium concentration i.e 50, 100, 250, 500 µM it give small change in spectra. This is because the number of electrons accepted by potassium ferricyanide is dependent on the number of water molecule that under goes photolysis process which in turn dependent on the amount/number of PSII present in the system. Hence a small concentration of thylakoid membranes has small number of PSII so the number of water molecule broken down into Hydrogen and oxygen became few. Whereas, large concentrations of thylakoid membrane in the system has many number of PSII and the

number of electron generated in the photolysis of water became many. So the number of potassium ferricyanide reduced become high this result in giving a big spectral change during measurements.

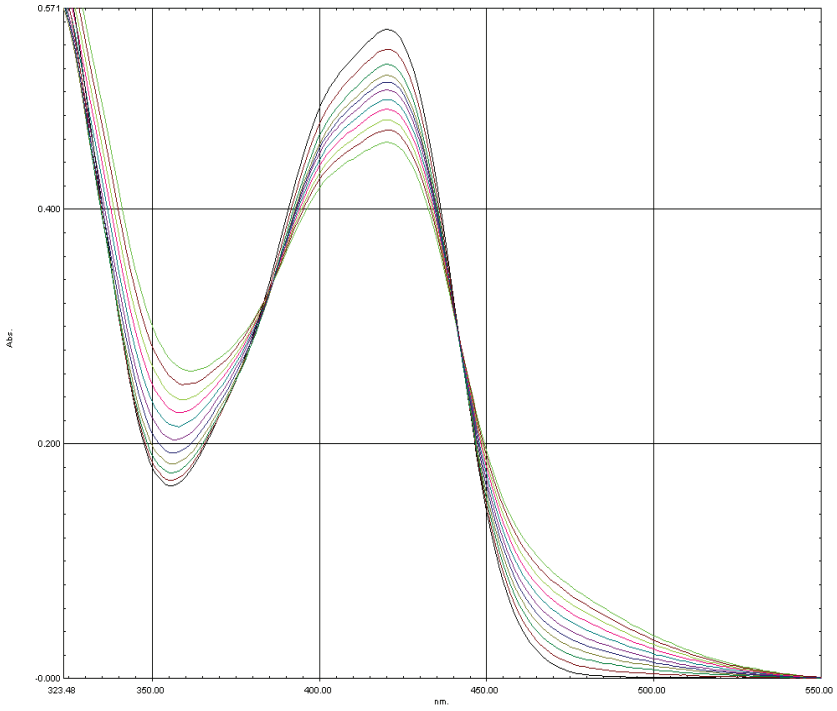


Fig 13. Absorption spectral change of 500 µM potassium ferricyanide without thylakoid membrane upon illumination for 1 hr in 5 min interval

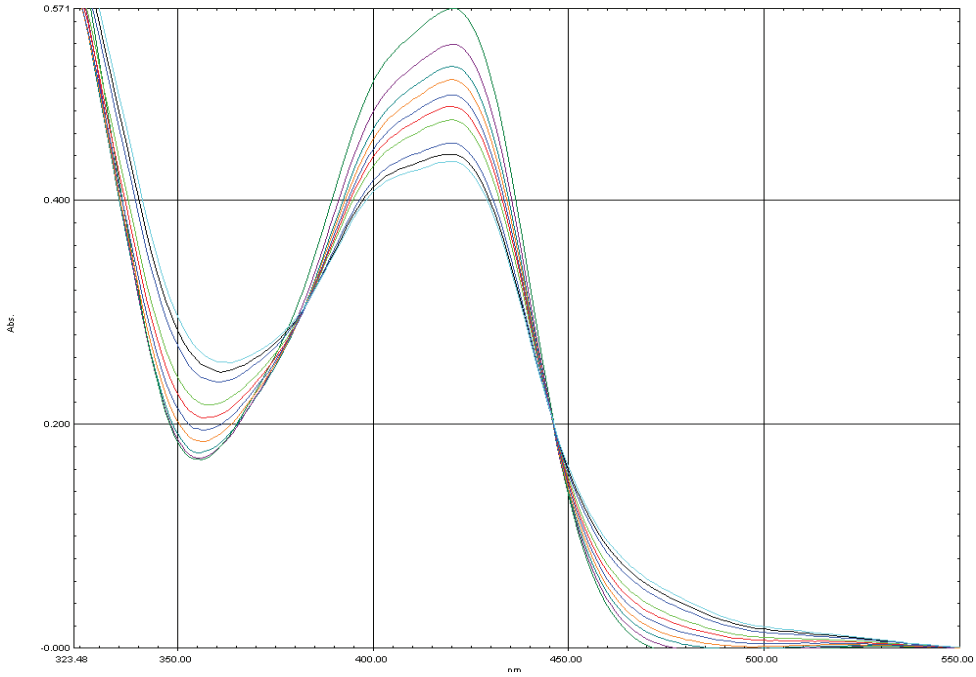


Fig 14. Absorption spectral change of 500 µM potassium ferricyanide with 20 µgm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

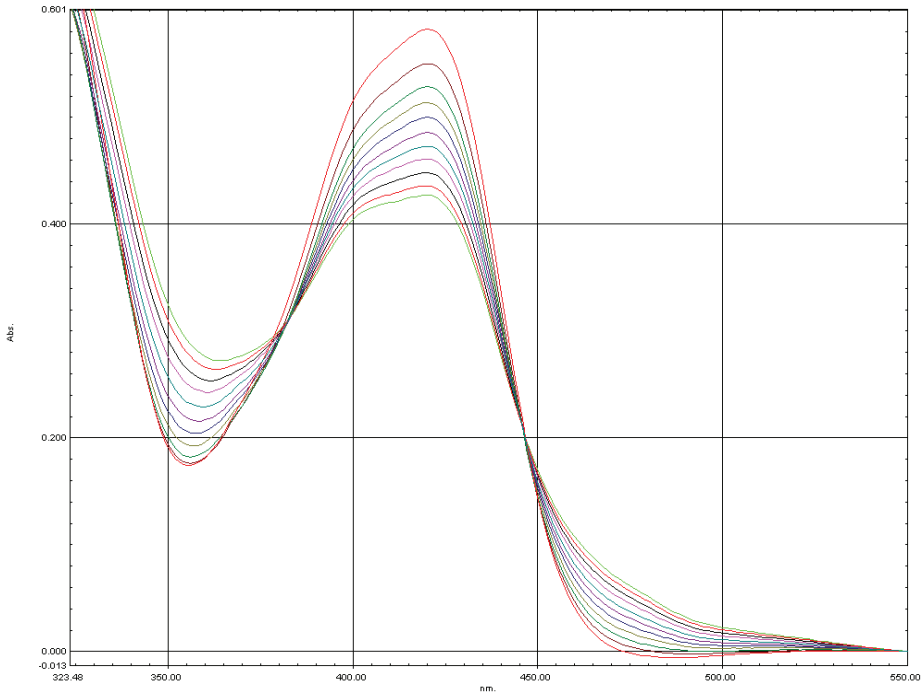


Fig 15. Absorption spectral change of 500 µM potassium ferricyanide with 15 µgm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

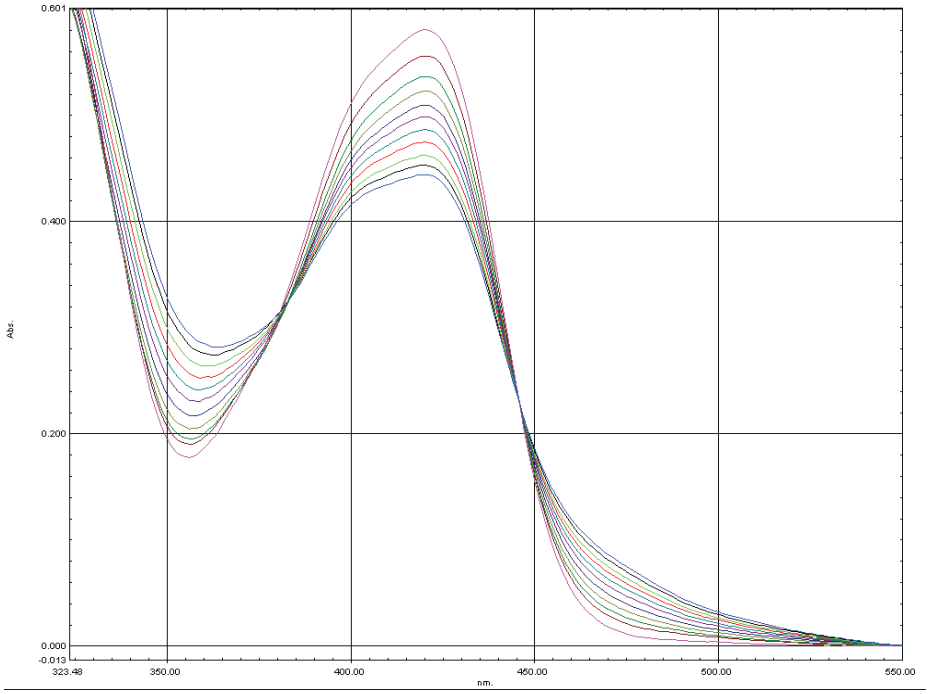


Fig 16. Absorption spectral change of 500 µM potassium ferricyanide with 10 µgm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

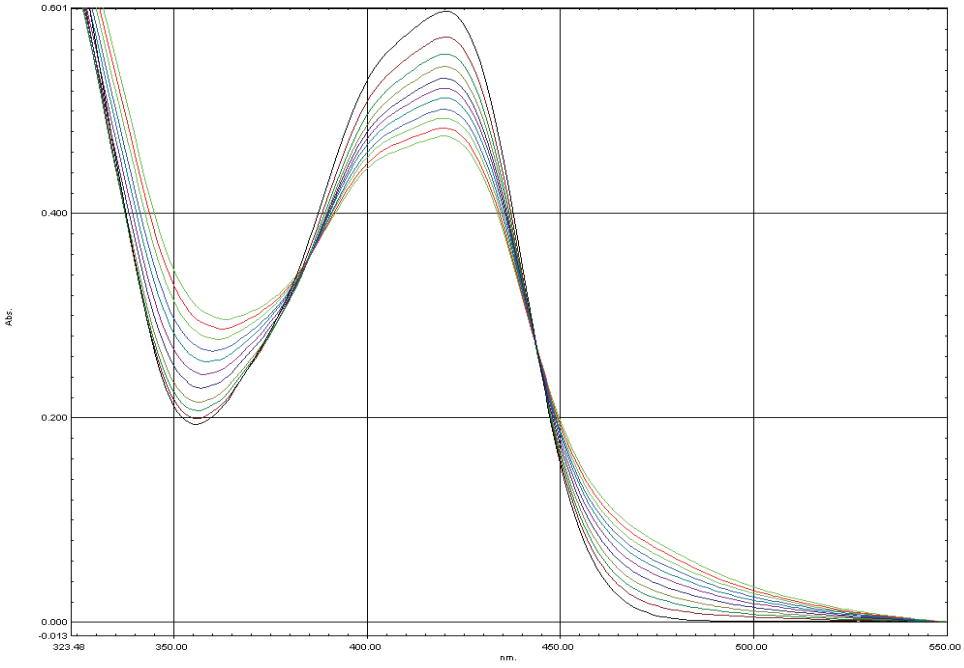


Fig 17. Absorption spectral change of 500 µM potassium ferricyanide with 5 µgm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

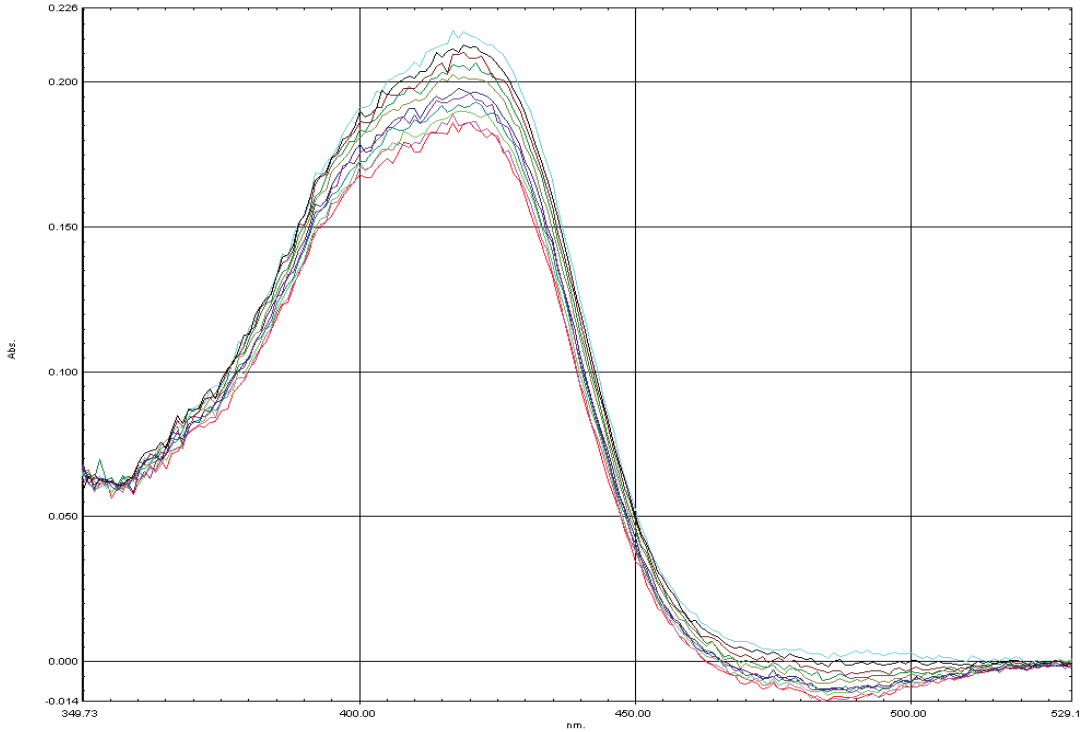


Fig 18. Absorption spectral change of 250 µM potassium ferricyanide with 20 µgm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

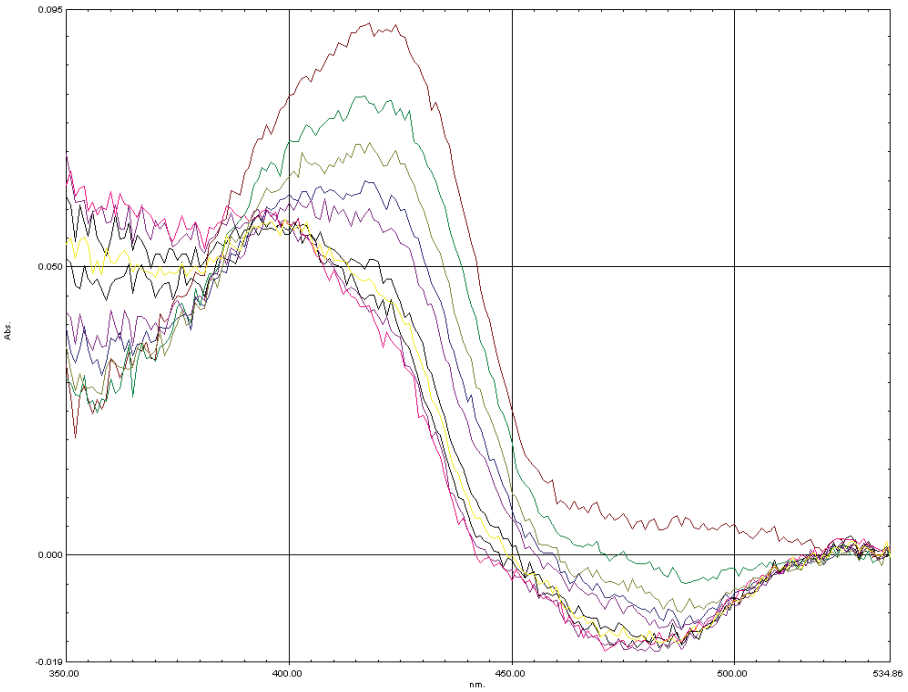


Fig 19. Absorption spectral change of 100 µM potassium ferricyanide with 20 µgm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

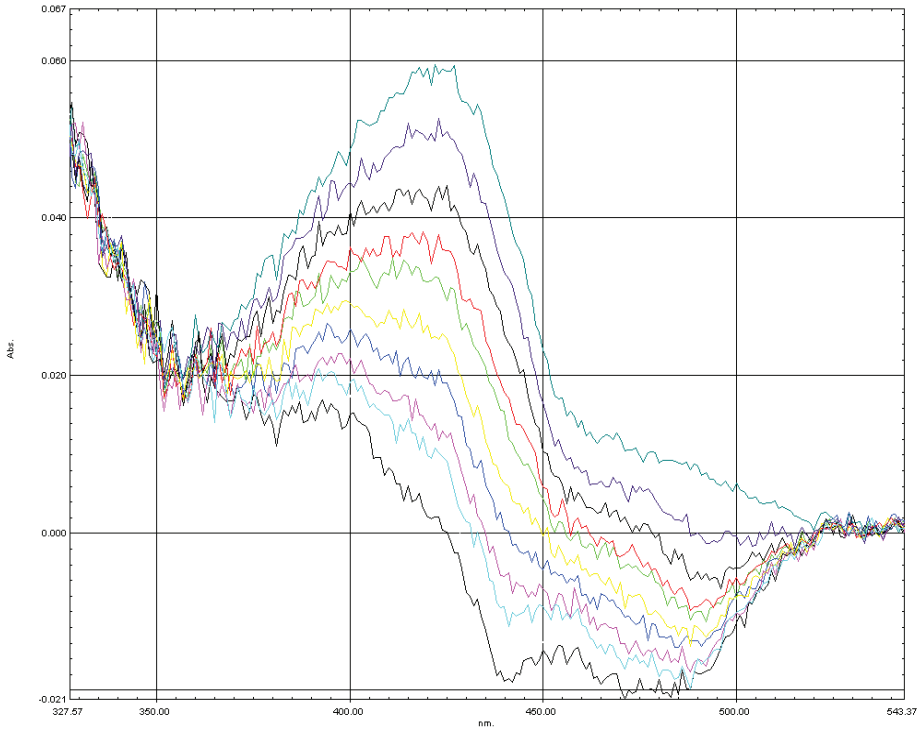


Fig 20. Absorption spectral change of 50 µM potassium ferricyanide with 20 µgm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

Many eukaryotic cells contain a redox system in their plasma membrane capable of reducing extracellular substrates using electrons from intracellular NADH. The system efficiently reduces the impermeable substrate ferricyanide. This is not the natural substrate for the redox system(Van Duijn et al., 1998) The maximum effect with ferricyanide was between pH 6.3 and 7.1(West and Hill, 1967). Photo reduction of ferricyanide and the coupled phosphorylation by chloroplasts requires only the "accessory pigment or short wavelength system." ferricyanide would essentially substitute for the "long wavelength chlorophyll a system"(Horio and Sanpietro, 1964). For ferricyanide reduction by chloroplasts plastoquinone is required (Leo). Action spectra for the photo reduction of ferricyanide and the coupled phosphorylation and found that the resultant action spectra differ from the absorption spectrum of the chloroplasts.(Horio and Sanpietro, 1964). The precise stoichiometry of four ferric ions reduced per oxygen molecule evolved in the ferricyanide hill reaction. This transfer of electrons from water to ferricyanide is accompanied by the formation of one ATP molecule per two electrons if ADP and inorganic phosphate are also present(Leo).

3.2.8 Absorbance Spectral Change of FeCy with Heated Plastid

The reduction of 500 µM $K_3[Fe(CN)_6$ with 20 µ gm chl/ml was determined using the same procedure and instrumental setup which was used for other measurements except here the plastids were heated for 10 min at 60°C in order to destroy or kill PSII. The experimental expectation was since the heating destroyed PSII the Absorbance spectral change of FeCy is self decay (reduction) by light.

The reduction of 500 µM potassium ferricyanide with heated 20 µ gm chl/ml thylakoid membranes upon illumination for 1 hr in 5 min interval gives spectra which are similar to 500 µM absorption spectra measured without thylakoid membrane. Since in both case there is no PSII. Heating the thylakoid membranes solution at 60 °C destroy/kill the PSII(Spikes et al., 1950). Hence there is no production of electron from the photolysis of water by PSII. This result both measurement to have the same spectral change. This can be seen by comparing the fig 13 and 21. And it also showed in the figure 24 the total reduction change between the first measurement (directly after mixing) and the last measurement (after 1hr illumination).

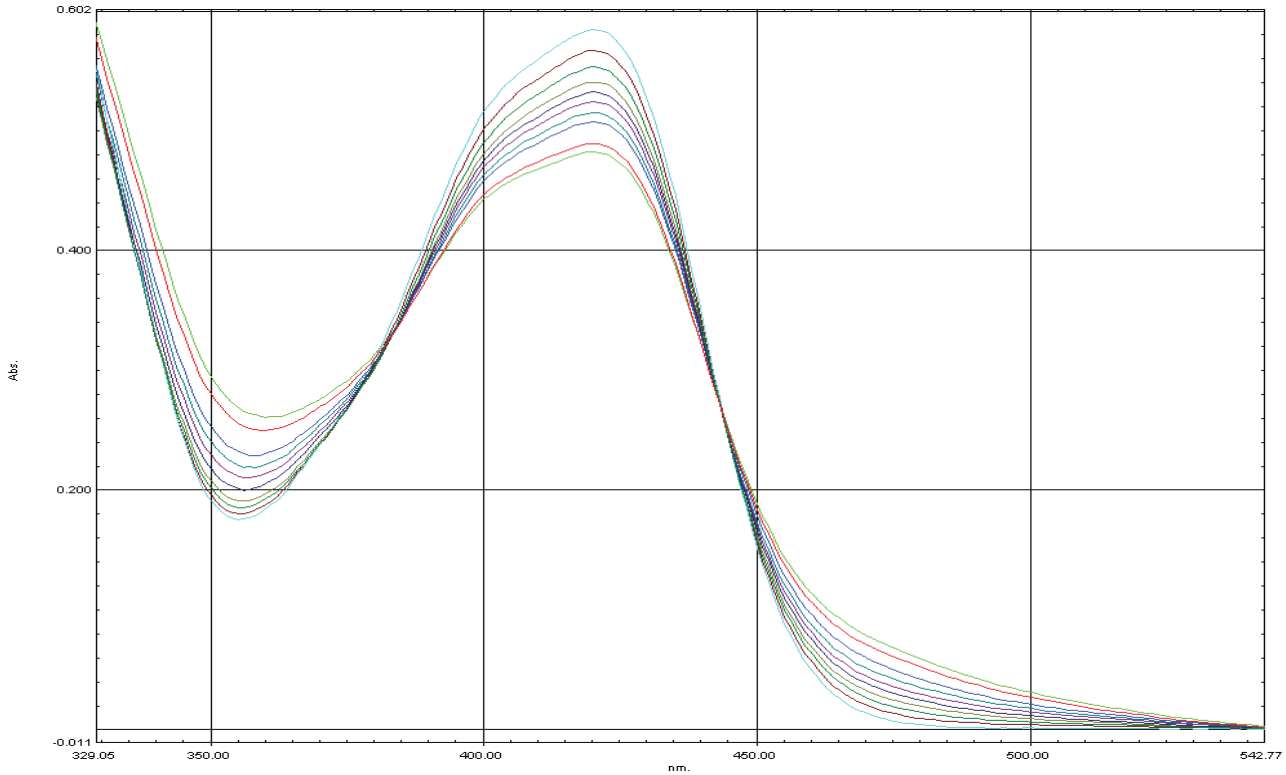


Fig 21. Absorption spectral change of 500 µM potassium ferricyanide with heated 20 µ gm chl/ml thylakoid membrane upon illumination for 1 hr in 5 min interval

3.2.9 Effect of Adding External Electron Donor in the System

The effect of introducing external electron donor was determine by adding 50 µM and 100 µM ascorbic acid to the 500 µM $K_3[Fe(CN)_6$, 20 µgm chl /mL reaction mixture and the measurement was taken for 10 cycles with 5 min interval illumination time, where the instrumental setup were the same with other absorbance measurements. The purpose of this measurement was to check whether FeCy absorbance reduction change will improve when extra electrons are provided by exogenous electron donor.

Chloroplasts were capable of consuming oxygen in the presence of ascorbate, particularly following a preliminary reduction of quinine in a regular Hill reaction(Leo). Reduction of ferricyanide was carried out using 500 µM FeCy. This reduction of ferricyanide by the thylakoid membrane can be greatly stimulated by the addition of ascorbate and the oxidized form of ascorbate(Van Duijn et al., 1998). Where ascorbate serves as an alternate electron (or hydrogen) donor and ascorbate oxidation substituted for the water oxidation (and oxygen production) (Leo). Ferricyanide reduction by ascorbate was due to a plasma membrane-localized ascorbate free radical (AFR) reductase. It was proposed that ferricyanide reacts with ascorbate to ferrocyanide and the ascorbate free radical. The latter would then be regenerated by the AFR reductase to ascorbate, which can subsequently again react with ferricyanide.(Van Duijn et al., 1998). The reduction of potassium ferricyanide absorbance was determined at 420 nm (Yim et al., 2004). The immediate reaction of ascorbate with ferricyanide caused excessive depletion of ferricyanide from the system at higher concentrations.(Van Duijn et al., 1998).

Introducing external electron donor like ascorbic acid to the reaction mixture resulted in increasing the reduction rate of potassium ferricyanide to potassium ferrocyanide. This reduction rate was doubled when the concentration of ascorbic acid was increased from 50 to 100. The effect can be clearly seen in the following figures 22 and 23.

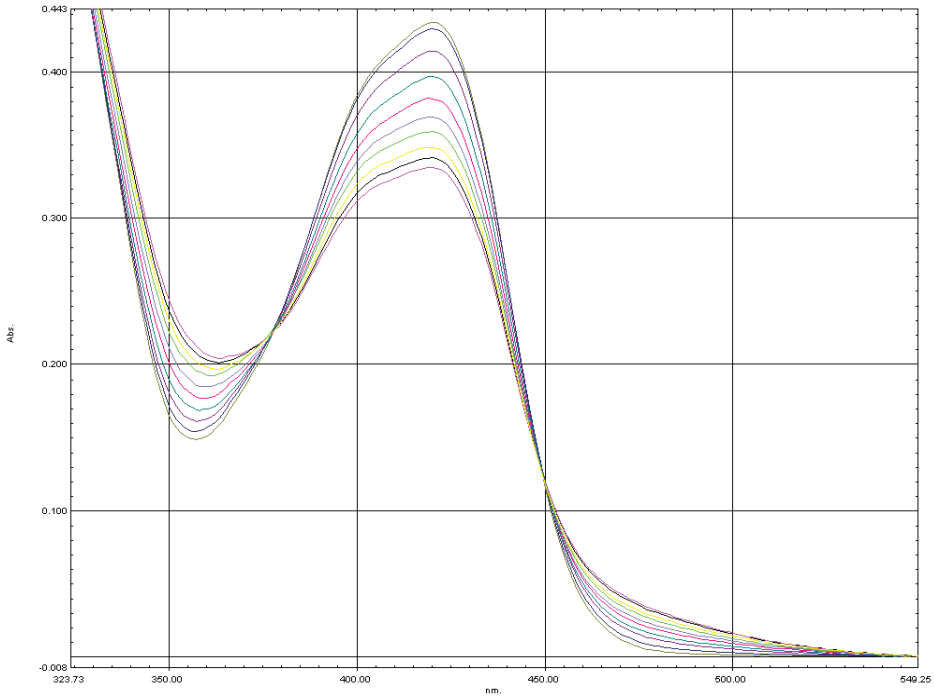


Fig 22. Absorption spectral change of 500 µM potassium ferricyanide with 20 µgm chl/ml thylakoid membrane and 50 µM ascorbic ascid upon illumination for 1 hr in 5 min interval

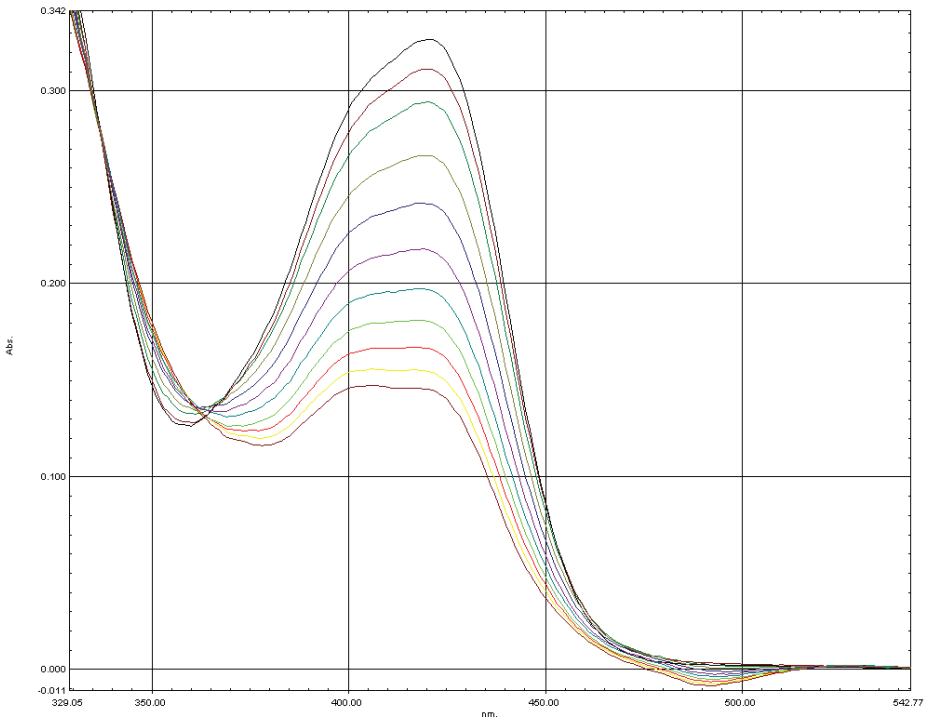


Fig 23. Absorption spectral change of 500 µM potassium ferricyanide with 20 µgm chl/ml thylakoid membrane and 100 µM ascorbic ascid upon illumination for 1 hr in 5 min interval

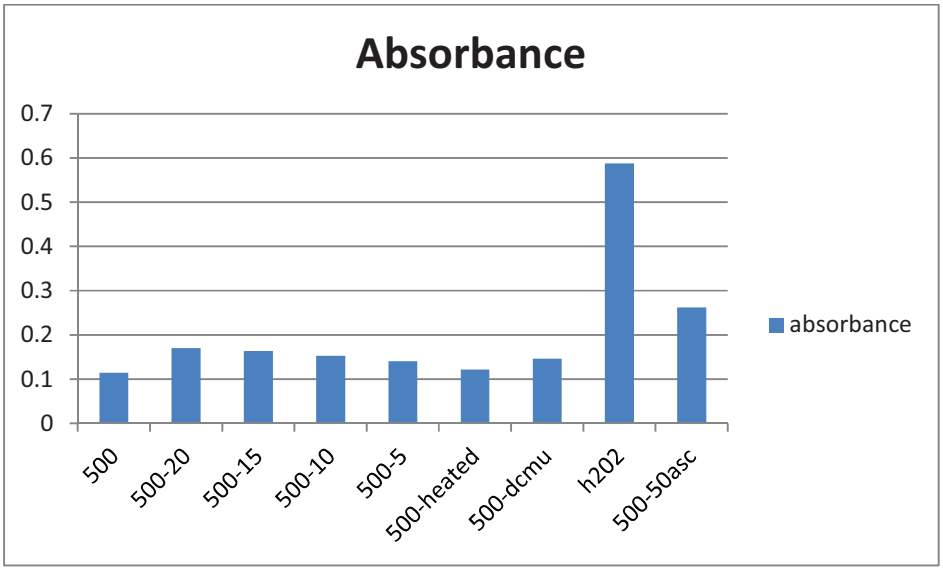


Fig 24. Absorbance difference of 500 µM potassium ferricyanide with 20, 15, 10, 5, µgm chl/ml at 490 nm after 1hr.

From the above graph one can understood that the reduction potassium ferricyanide to potassium ferrocyanide was high when there was external electron donor like 50 µM ascorbic acid shown in the graph as 500-50asc. The reduction difference measurement taken with addition of inhibitor DCMU showed that there is only self reduction of potassium ferricyanide to potassium ferrocyanide which shown in the graph as 500-dcmu. In general it is 500 µM potassium ferricyanide with 20 µgm chl/ml shown in the graph as 500-20 showed a better reduction difference as compared to the other potassium ferricyanide-thylakoid membrane concentrations.

3.2.10 Effect of DCMU (diurone (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea))

The same procedure (effect of adding external electron donor) was used to see the effect of adding inhibitor to the system where the only difference was in this measurement there was addition of 100 µM DCMU to the reaction mixture.

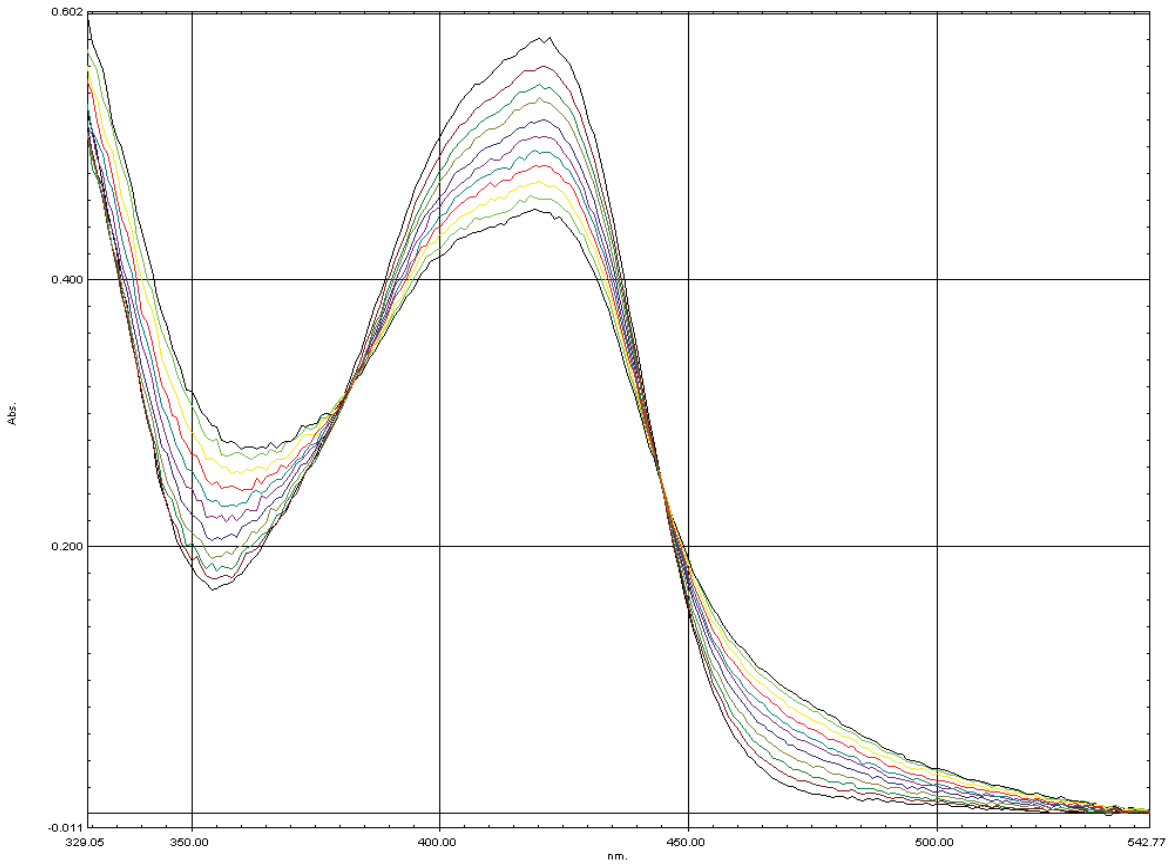


Fig 25. Absorption spectral change of 500 µM potassium ferricyanide with 20 µgm chl/ml thylakoid membrane and 50 µM DCMU upon illumination for 1 hr in 5 min interval

DCMU is known to block the reoxidation of QA – by the secondary quinone acceptor QB (van Rensen, 2002, Groot et al., 2005, Drath et al., 2009) and ferricyanide accepts electrons directly at the QA site (van Rensen, 2002). Hence adding 50 µM DCMU resulted in blocking electron flow from the water splitting complex to the artificial (external) electron acceptor whereby it block the reduction of potassium ferricyanide(Hall et al., 1971). Therefore the reduction measured in UV/Vis absorbance spectrophotometer was entirely due to self reduction of potassium ferricyanide to potassium ferrocyanide by illuminated light.

3.3 Phenyl-p-benzoquinone Absorbance Measurement

The absorbance spectra of different concentration of PPBQ was measured using for UV-2401 UV/Vis fluorescence spectrophotometer with instrumental setups; reaction mixture 3 ml, slit size 5 nm, wavelength 250-550 nm and the sampling interval was 1.0 as it is stated in the method section.

3.3.1 Determination of the Absorbance Spectra of Different Concentration of PPBQ

The procedure and instrumental setup used in measuring potassium ferricyanide was also used in measuring 50, 100, 250 and 500 µM Phenyl-p-benzoquinone absorbance measurement, where the only difference was the illumination time was for 1 min with 10 sec interval during PPBQ measurements. The purpose of this measurement was to determine the wavelength for maximum absorption and which of the concentrations will give absorbance less than one so that it can be used for further measurement. Since 50, 100, 250 and 500 µM FeCy concentrations gave an absorbance less than one the same thing was expected for PPBQ concentrations measured.

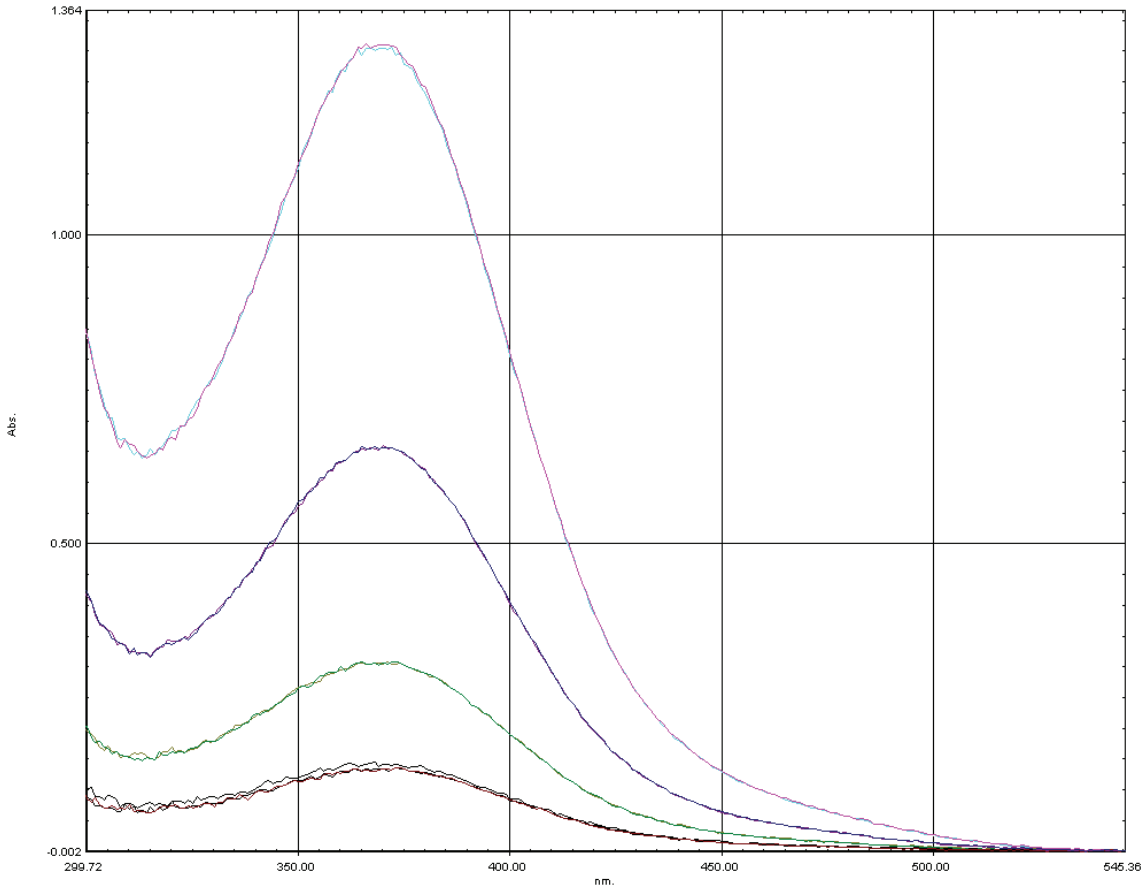


Fig 26. Absorption spectra of 500 µM (pink line), 250 µM (blue line), 100 µM (green line) and 50 µM (brown line) Phenyl-p-benzoquinone

After the absorbance spectra measurement of 500, 250, 100 and 50 µM PPBQ since the absorbance spectra of 500 µM PPBQ is above 1 we took 250 for the rest of absorbance measurement of PPBQ. The absorbance spectrum is given on fig 26. Generally it was found that PPBQ give an absorbance around 371 nm.

3.3.2 Reduction of PPBQ with thylakoid membrane

The reduction of 250 µM PPBQ in the presence of 20 µgm chl/mL thylakoid membrane was determined using the same procedure and instrumental setup used for the measurements of Potassium ferricyanide. The purpose of this experiment was to see the action of different concentration of thylakoid membrane on the reduction of PPBQ while illumination for 1 min in 10 sec interval.

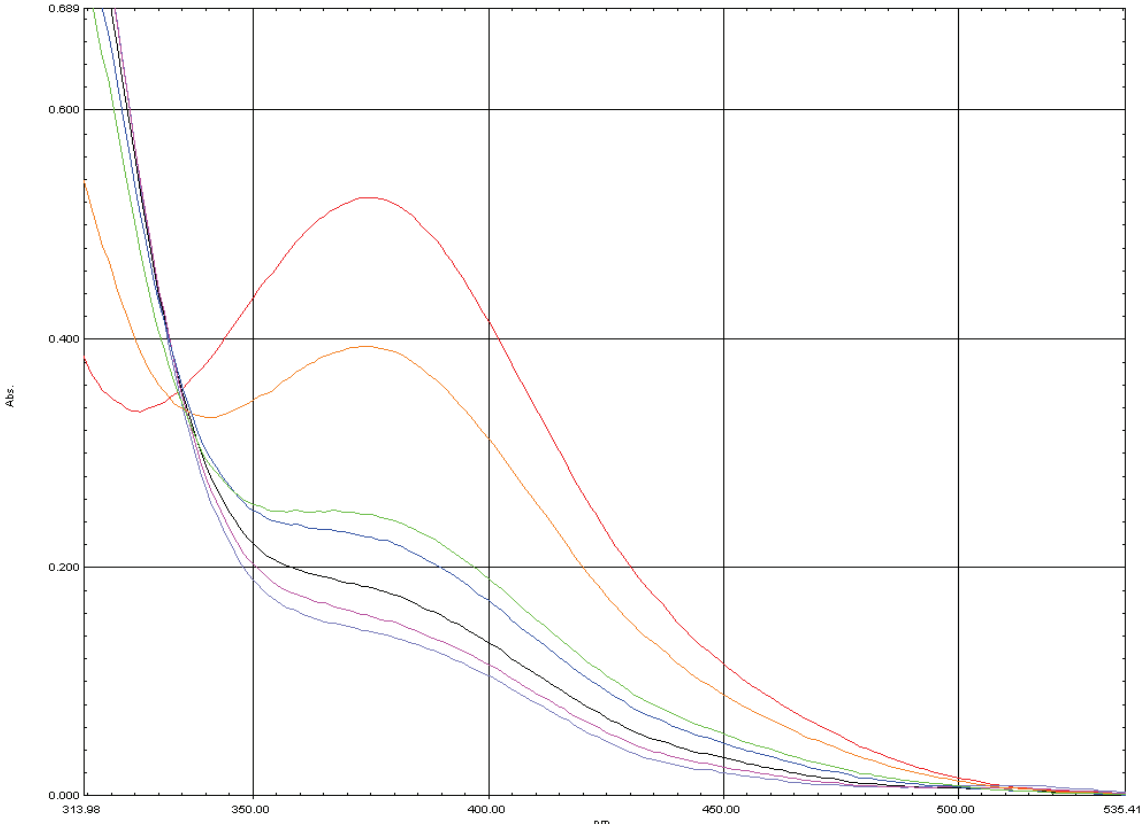


Fig 27. Absorption spectra of 250 µM Phenyl-p-benzoquinone with 20 µgm chl/ml thylakoid membrane upon illumination for 1 min with 10 sec interval

Unlike to Potassium ferricyanide the reduction of PPBQ upon illumination was fast. As it is shown on fig 27 the compound (PPBQ) is completely reduced within a minute unlike to 1hr of

potassium ferricyanide. In the graph given below (fig 28) it clearly showed that the immediate PSII response is high as compared to Potassium ferricyanide.

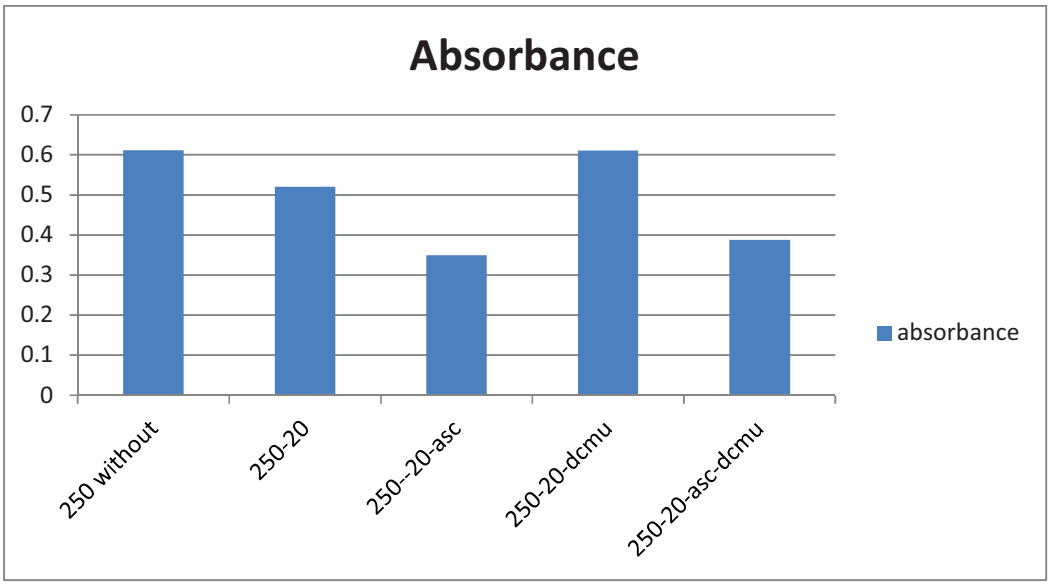


Fig 28. Absorbance of PPBQ measured just after mixing at 371 nm

3.3.3 Effect of Adding External Electron Donor in the System

Here also the same method used which stated in 3.2.9 had been used to see the effect of adding ascorbic acid to the system

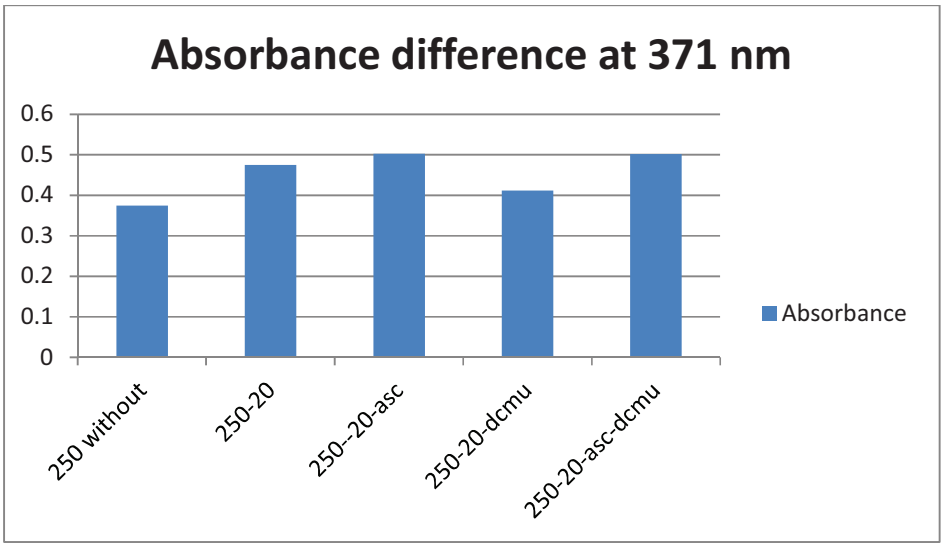


Fig 29. Absorption difference at 371 nm between the first measurement and after 1 min

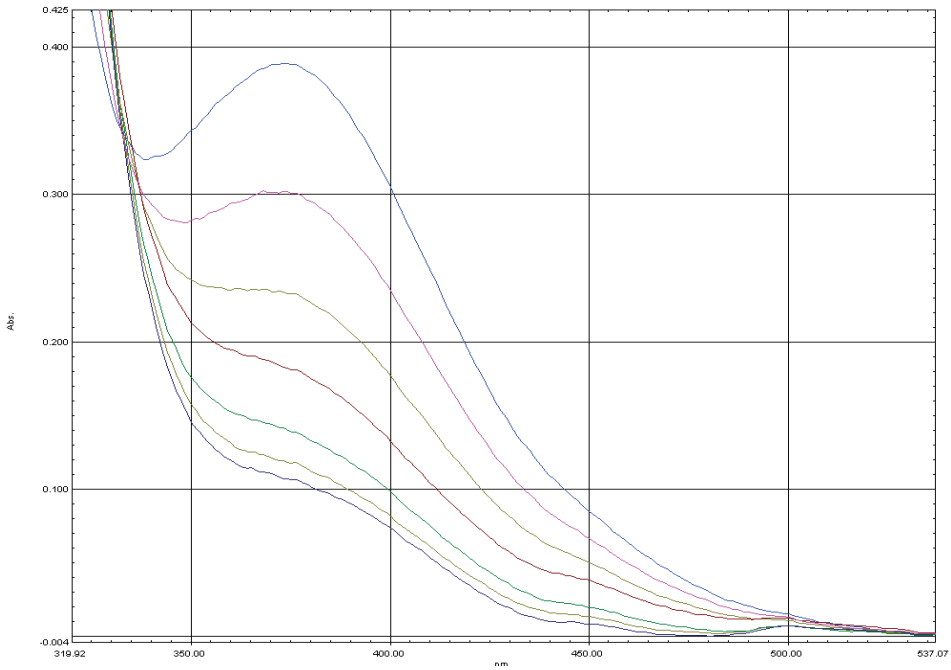


Fig 30. Absorption spectra of 250 µM Phenyl-p-benzoquinone with 20 µgm chl/ml thylakoid membrane and 50 µM ascorbic acid upon illumination for 1 min with 10 sec interval

The effect of adding external electron donor (ascorbic acid) to the reaction mixture showed similar effect found in potassium ferricyanide measurement. As it is known ascorbate provide electron to the tyrosine (Y_z) and cyt *f* where as PPBQ accept electron from Q_A and potassium ferricyanide from QB and FeS cluster. The absorbance change is shown in fig 29 and Fig 30.

3.3.4 Effect of DCMU (diurone (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea))

The same method used for Potassium ferricyanide measurement stated (2.7.13) was used for PPBQ sample where the change is that the concentration of PPBQ was 250 µM and the illumination time was 10 sec for 1 minute.

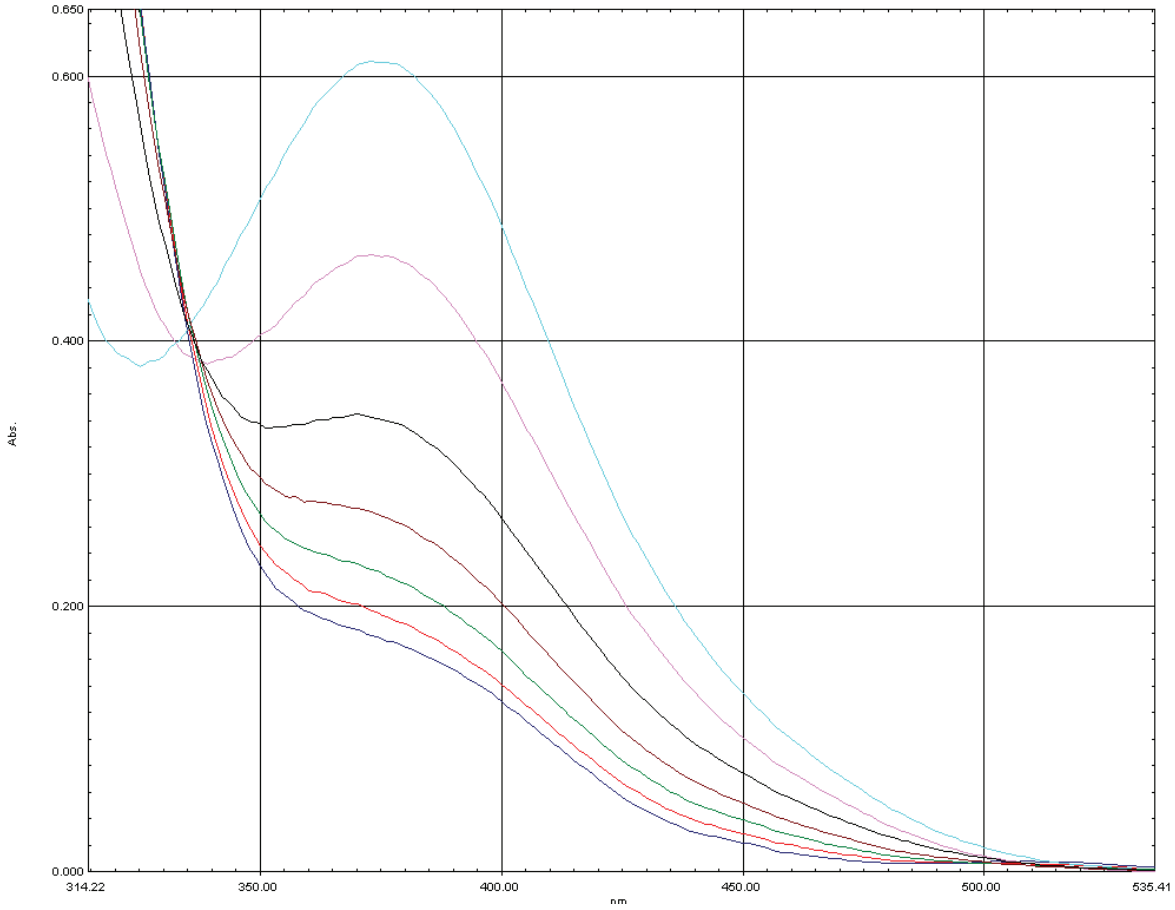


Fig 30. Absorption spectra of 250 µM Phenyl-p-benzoquinone with 20 µgm chl/ml thylakoid membrane and 50 µM DCMU upon illumination for 1 min with 10 sec interval

The action of ferricyanide versus PPBQ on oxygen evolution was quite different in PSII core preparation from plants, and this difference might be due to the properties of the QB site which modified the access of the negatively charged ferricyanide (Nagao et al., 2010). PPBQ accepts electrons from Q_A^- in PSII membrane fragments with similar efficiency as plastoquinone in intact cells(Shevela and Messinger, 2012b). Since DCMU block electron transport from Q_A to Q_B it will not have any role in the reduction of PPBQ. Because PPBQ accept the electron prior reaching the DCMU blocking site. Therefore, the reduction of PPBQ will not be affected by any concentration of DCMU added to the system. This is shown on the graph given in fig 30 where the reduction of 250 µM PPBQ upon addition of 50 µM ascorbic acid (showed in the graph as 250-20-asc) showed same effect with that of the same concentration with additional 50 µM DCMU which is shown on the fig 30 as 250-20-asc-dcmu.

4. Conclusion

The turnover of the PSII found to be high in the first few minutes of FeCy measurement. When the illumination continues the reduction (the absorbance) of FeCy become less and less as shown in the figures. This was true for PPBQ measurement as well. The reduction of the compounds (PPBQ and FeCy) found to be entirely dependent on the concentration of thylakoid membrane added to the system. However, the PPBQ response was fast. In general it was possible to monitor the light induced PSII turnover efficiency. It was found that it's better to use 250 or 500 µM FeCy and 250 µM PPBQ with 20 µ gm Chl/ml in order to study the light induced PSII turnover efficiency. But to be sure one should go for further measurement of oxygen evolution using Clark-electrode.

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