[BIO32] The development of a biosensor for the detection of PS II herbicides using green microalgae

Maizatul Suriza Mohamed, Kamaruzaman Ampon, Ann Anton

School of Science and Technology, Universiti Malaysia Sabah, Locked Beg 2073, 88999 Kota Kinabalu, Sabah, Malaysia.

Introduction

Increasing concern over the presence of herbicides in water body has stimulated research towards the development of sensitive method and technology to detect herbicides residue. Biosensors are particularly of interest for the monitoring of herbicides residue in water body because various classes of herbicides have a common biological activity, which can potentially be used for their detection. The most important herbicides are the photosystem II herbicide group that inhibits PSII electron transfer at the quinone binding site resulting in the increase of chlorophyll fluorescence (*Merz et al*., 1996)

.

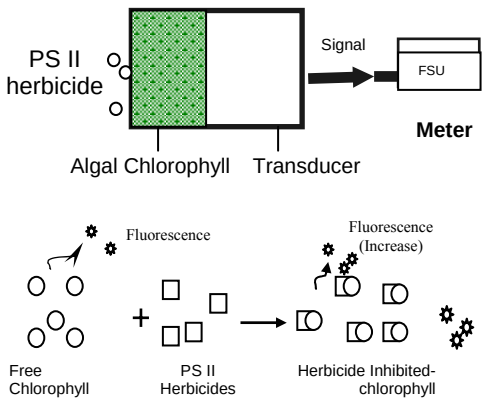


FIGURE 1 Schematic diagram of a basic biosensor for herbicide detection. In the photosynthetic pigments of green alga, absorption of light quantum induces the transition of pigment molecules into the excited state. From the peripheral antenna complexes, excitation is efficiently transferred to the core antenna complexes near photosynthetic reaction centers, where it can be used in the primary photochemical reaction of photosynthesis (dark reaction). A small fraction of the excited energy is reemitted as fluorescence. If the transferred process is blocked (by herbicides), more of the excited energy will be reemitting as fluorescence (light).

Material & Methods

Equipments and Chemicals

Fluorometer used was TD700 by Turner Designs with 13mm borosilicate cuvettes. Excitation and emission wavelength were 340nm-500nm and 665nm. Lamp was daylight white (185-870nm). Equipment for photographing algae was Nikon Photomicrographic Equipment, Model HIII (Eclipse 400 Microscope and 35 mm film photomicrography; prism swing type, automatic expose and built-in shutter). Chlorophyll standards for fluorometer calibration were purchased from Turner Designs, USA. PS II herbicides used were diuron (3-(3,4-dicholorophenyl)-1,1 dimethylurea or DCMU), and propanil (3′,4′-dichloropropionanilide). Non PS II herbicides used as comparison were 2,4-D (2,4-dichlorophenoxy)acetic acid) and Silvex (2,4,5-trichlorophenoxypropionic acid) (Aldrich Sigma). Stock solutions of both herbicides were prepared in the range of 0.001mM to 100mM in 50% (v/v) ethanol and 50% (v/v) DMSO. Media used was modified Bristol solution (K_2HPO_4, KH_2PO_4, $MgSO_4$, $NaNO_3$, NaCl, and CaCl; prepared in separate stock solutions)

Test organisms

Six species of unicellular microalgae from phylum of Chlorophyta (class of chlorophyceae) were used in the study (Table 1).

TABLE 1 Microalgae used in the development of the biosensor.

Algal species	Order	Family
Scenedesmus dimorphus	Chlorococcales	Scenedesmaceae
Chlorella sp.	Chlorococcales	Chlorellaceae
Pediastrum sp.	Chlorococcales	Hydrodictyaceae
Kircheriella sp	Chlorococcales	Oocystaceae
Selenestrum sp.	Chlorococcales	Oocystaceae
Coelastrum sp.	Chlorococcales	Scenedesmaceae or Coelastraceae

The microalgae originated from a lake near Likas Bay, Kota Kinabalu, Sabah and were obtained from The Biotechnology Research Institute, Universiti Malaysia Sabah. They were cultivated in a 500 ml blue capped bottle in the modified Bristol media (Stein, 1973). Starter cells density for all species was approximately 1000 cells/ml. The temperature and light intensity of the test chamber were set at 25°C +/- 2°C and 4000-5000 lux (12h day: 12h night). The growths of all microalgae were monitored by cells counting using haemocytometer. Chlorophyll concentration was determined by 90% (v/v) acetone extraction method suggested by Strickland and Parsons, 1968.

Algae Morphology

The photomicrograph of all the algae were taken using Nikon Microphotographic Equipment, model H-III.

Assay

Two types of PS II herbicides (diuron and propanil) with six different concentrations (0.001mM to 100mM) were used. In order to study the effect of algal age, algae at four different culture ages; 7, 14, 21 and 28 days were tested. The algal suspension was dark adapted for 10 minutes prior to assay. 10 µl aliquot of herbicide solution was added to 8ml of algal suspensions and mix by swirling the tube. Fluorescence was measured before and immediately after herbicide was added. For control, 10 µl of 50% (v/v) solvent was used as replacement to herbicides stock. All experiments were carried out at room temperature with minimum expose of light. Fluorescence yields of inhibition were recorded for 200-400 seconds. Three replicates were made for each assay. The fluorescence yield was than plotted as a function of time (seconds)

Calculations and statistical Analysis

Inhibition curves were fitted using CurveExpert v1.36. All calculations and statistical analysis were computed using SigmaPlot 6 (SPSS Inc., USA) and Microsoft Excel (Microsoft Corporation). All data were mean of three replicates.

Result and Discussion

Growth of Microalgae

The growths of the microalgae were similar to the growth of other microorganism. Each growth curve showed a semi sigmoidal curve with 3 growth phase: a log phase, a lag phase and a stationary phase.

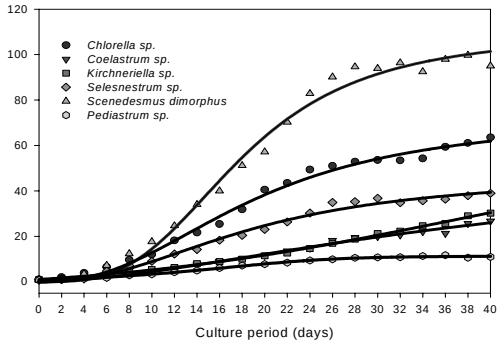


FIGURE 2 Growth curves of all microalgae used. All species have a semi sigmodial curve with different period of growth phases and different growth rate. *Scenedesmus dimorphus* has the highest growth rate ($4.44x10^4$ cells/day) followed by *Chlorella sp*. ($2.57x10^4$ cells/day), *Selesnestrum sp*. ($1.51x10^4$ cells/day), *Kirchneriella sp*. ($7.7x10^3$ cells/day), *Coelastrum sp.* ($7.7x10^3$ cells/day). *Pediastrum sp.* has the lowest growth rate compared to all species ($4.1x10^3$ cells/day). In addition, Pediastrum sp. and Coelastrum sp culture suspensions were easily contaminated with other microalgae.

Alga Morphology

Coelastrum sp. was spherical cells. They formed colonies of a fixed number of cells (4, 8, 16 and more) arranged in 2 or 3 dimensional. The colonies are spherical with inner empty space; cells attached to each other by a protrusion of cell wall, arranged in a single layer (figure 3a). *Selesnestrum sp*. was unicells. The body is crescent in shape; tapered at both ends (figure 3b). *Chlorella sp.* was single cells with round in shape. The chloroplast was cup-shape (figure 3c). *Scenedesmus dimorphus* was usually arranged linearly in a colony of 4 members. The cell bodies were ellipsoidal and crescent in shape (outer members of the colony) (figure 3d). Colonies of *Pediastrum sp* were crown like shape. Each colony consists of 4 or more fixed cells. Cell body was polygonal in shape, with horn-like projections (resemble tooth shape) (figure 3e). *Kirchneriella sp*. was unicells

with bean shape (long cylindroid, strongly curved) (Figure 3f).

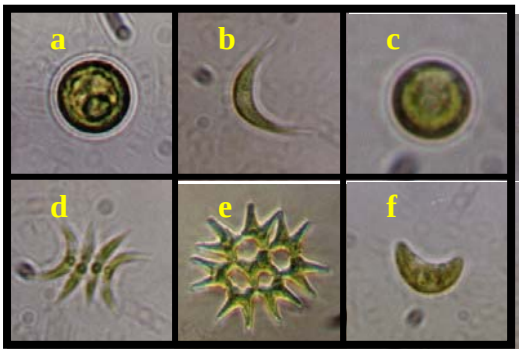


FIGURE ***3*** Photomicrographs of *(a) Coelastrum sp. (100x10*), (b) *Selenestrum sp.(100x10),* (c) *Chlorella sp. (100x10),* (d) *Scenedesmus dimorphus (100x10),* (e) *Pediastrum sp. (40x10),* (f) *Kirchneriella sp. (100x10).*

Chlorophyll a content and fluorescence yield

Chlorophyll **a** content (mg/l) of all algae shows an increase with the increase of culture period from age 7 days to age 21 days. This maybe due to the increase of cell density. *Kirchneriella sp.* has the highest total chlorophyll content 3.4mg/l and 9.5 mg/l for both culture ages (7 and 21 respectively) (figure 4a). *Pediastrum sp.* has the highest chlorophyll content per cell compare to the others (Figure 4b). Because chlorophyll concentration represents the amount of PSII reaction center for the herbicide binding action (Shakinaz, 1997), theoretically we can assume that this species maybe the best algae to be used. However, overall fluorescence yield after herbicide inhibition of this alga was lower than fluorescence yield of others (figure 5a). This suggested that *in vivo* fluorescence of algal maybe not only depend on the amount of chlorophyll but also involve more complex interactions. In addition, the chloroplast condition in the algal cells may result in false result of chlorophyll analysis. For example, *Kirchneriella sp*. has a chloroplast full within the cell. *Scedesmus dimorphus* has one or more chloroplast in plate-like shape. Chlorophyll in *Kirchneriella sp.* can easily be extracted than chloroplast in *Scencedesmus dimorphus.*

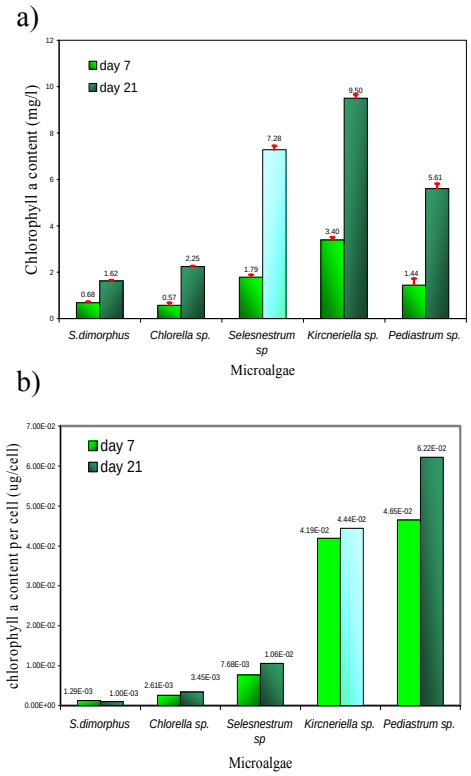


FIGURE 4 a) Chlorophyll concentration of studied microalgae at two culture periods; 7 days and 21 days. *Kichneriella sp.* has the highest chlorophyll content for both days. All species show increased in chlorophyll content when culture age was longer. A chlorophyll concentration for *Coelastrum sp.* was not available due to contamination during cultivation. b) Chlorophyll concentration per algal cell express as ug/cell. *Pediastrum sp* has the highest chlorophyll content per cell maybe due to their large size. *Kirchneriella sp.* also showed high chlorophyll content per cell.

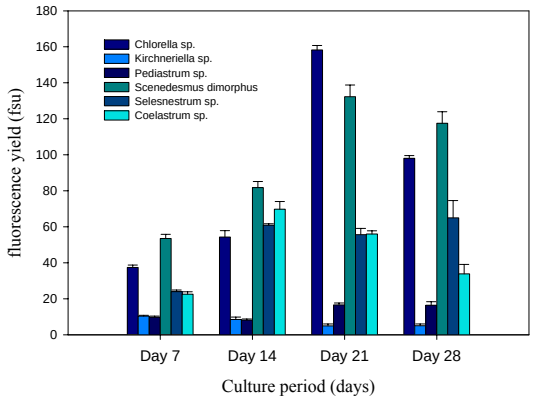


FIGURE 5 a) Chlorophyll fluorescence yield after 50 seconds inhibition of 1mM diuron. The yield was express as fluorescence after 50 sec inihibition – fluorescence before inhibition.

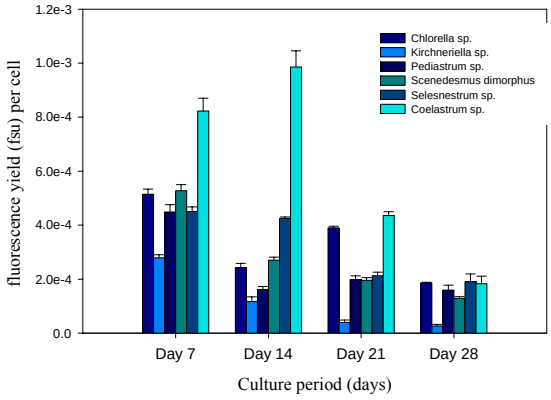


FIGURE 5b) Estimated Chlorophyll fluorescence yield of each algal cell after 50 sec inhibition of diuron.

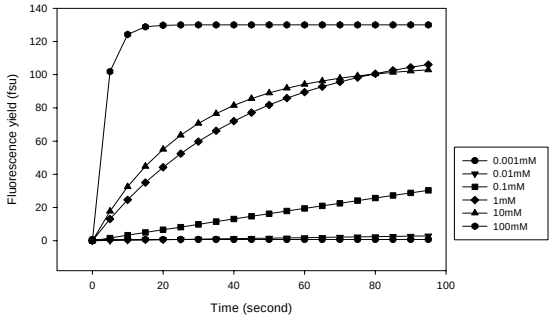
Selection of Best Microalgae

The best microalgae for use as biosensor was selected based on several criteria; a) growth rate, b) easy to cultivate and maintain c) high sensitivity to PSII herbicide. The test algal used in biosensor must have high growth rate, ubiquitous and easily cultivated to make sure the continuous supply of the bio-receptor. *Scenedesmus dimorphus* suited these criteria well. Although the chlorophyll concentration in their cell is low, but the fluorescence yield per cell of this algal was high. *Kirchneriela sp.* has high chlorophyll content but produced low of chlorophyll fluorescence yield. In addition their growth rate was slow. *Coelastrum sp* was difficult to cultivate. Their cells suspension can easily be contaminated with other algal. From the result obtained (figure 5b), the best age of algae to be used as biosensor was 7 days. Fluorescence yield per cell of all algae was highest at this age. For further discussion, *Scenedesmus dimorphus* at age 7 will be used as reference.

Standard Calibration Curve and PSII herbicide detection

After herbicide was added to the microalgae, the fluorescence increased with time until it reached a saturating point. It was assumed that when the fluorescence reached a maximum, all of the chlorophyll pigments in the cells have been inhibited by herbicides. These curves were the same as the kinetic reaction curve. All the algae tested with both herbicides had a similar curve pattern as *Scenedesmus dimorhus*, (figure 6a and 6b). However the rate of the increase varies with the herbicide concentrations, algal species and type of herbicides used. In some species, the increase in the fluorescence was not detected at herbicide concentrations lower than 0.01mM. The detection range was between 100mM– 0.01mM for all algal species. An assay using non-PSII herbicide (Silvex and 2,4-D) showed no inhibition effect.

a)



b)

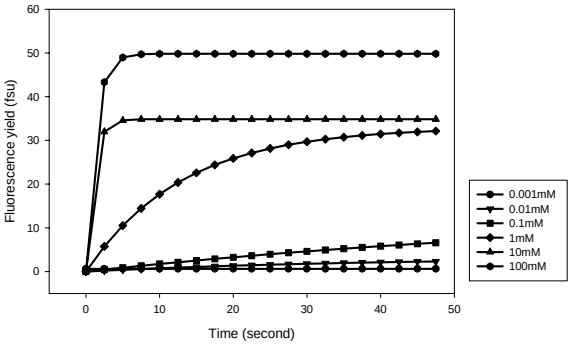


FIGURE 6 Time-dependent Effect of PS II herbicides, (a) diuron (b) propanil to the Fluorescence yield of *Scenedesmus dimorphus*, culture age 7 days. Solvent used was 50% (v/v) DMSO. The plotted data were mean of 3 replicates. Each curve was obtained from non-linear regression of raw data.

The presence of PS II herbicides can be detected by the increased *in vivo* chlorophyll fluorescence of the algae. The rate of increased of the chlorophyll fluorescence was proportional to the PSII herbicide concentrations used. By plotting the rate of chlorophyll increases to PS II herbicide concentrations, a standard calibration curve can be obtained (figure 8a and 8b). Concentration of PSII herbicide in the sample can be estimate from the standard calibration curve. The detection was specific to PSII herbicides since no inhibition effect on Hill

reaction centre was observed for herbicides with different mode of action. Thus, no chlorophyll fluorescence increase can be detected.

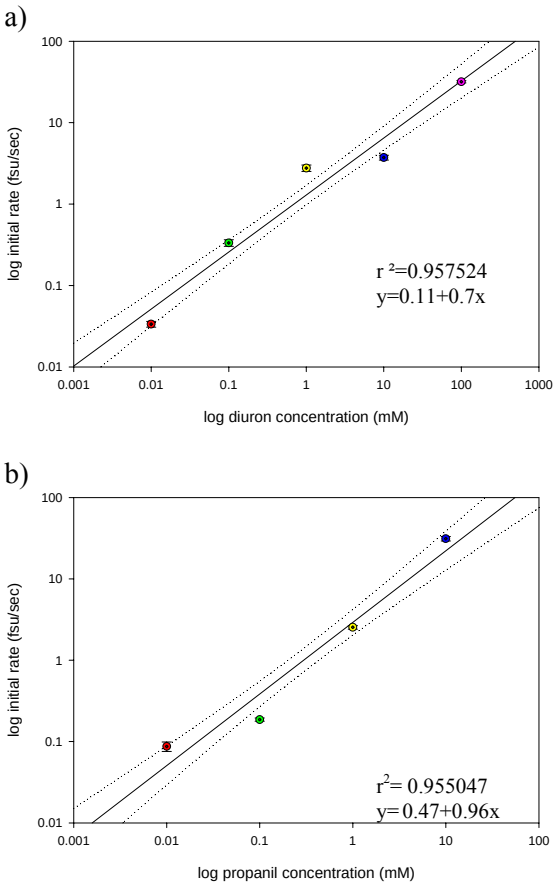


FIGURE 7 Standard calibration curves for a) diuron and b) propanil using *Scencedesmus dimorphus* at the age of 7 days.

The sensitivity of this biosensor was around 10 µl (0.01mM). However, the sensitivity can be further improved with some modification in the assay method. The biosensor can be used to continuously monitoring the presence of PS II herbicides in the water body. Although this biosensor is specific to PSII herbicides, the biosensor can be used as primary detection of herbicides. PS II herbicides such as propanil and diuron are important active ingredient in herbicides used in agricultural area.

Acknowledgements

The authors wished to thank the Ministry of Science, Technology and Innovation (MOSTI), Malaysia for the National Science Fellowship awarded to Maizatul Suriza Mohamed and for IRPA grant awarded to Kamaruzaman Ampon. The authors were also grateful to Assoc. Prof Dr. Amran Ahmed, School of Science and Technology, Universiti Malaysia Sabah, for his expertise and guidance in statistical analysis.

References

Merz, D., Geyer, M., and Moss, D.A. (1996). Chlorophyll Fluorescence Biosensor for the Detection of Herbicides. *Fresenius Journal of Analytical Chemistry* 354: 299-305

Nichols, H.W. (1973). Growth Media–Freshwater. In Stein, J.R. (editor) *Handbook Of Phycological Methods – Culture Methods And Growth Measurements*: 7-24. London: Cambridge University Press.

Shakinaz Desa, (1997). *The Development of Photosystem II-Herbicide Detection Method Using Microalgae as a Biosensor*, Faculty of Science and Environmental Studies, University Putra Malaysia.

Strickland, J.D.H and Parsons, T.R. (1968). Pigment Analysis. In Strickland, J.D.H. and Parsons, T.R. (editors) *A Practical Handbook Of Seawater Analysis*: 186-192. Ottawa: Fisheries Research Board of Canada.