Bioremoval of lead, cadmium and nickel by *Chlorella pyrenoidosa* with increase in lipid production for bioenergy application

Monika Prakash Rai*, Anwesha Khanra, Vandit Saxena
Amity Institute of Biotechnology,
Amity University Uttar Pradesh,
Gautam Budh Nagar (Noida), India.
*mprai@amity.edu

Abstract

The discharge of heavy metals into aquatic ecosystems has become a matter of concern over the last few decades. Microalgae play a significant role in metal absorption from contaminated waste water. Algae are ideally suitable to play a dual role of treating wastewater in the process of effective utilization of different constituents essential for growth leading to enhanced biomass and lipid production. Biomass productivity and oil content of microalgae depend heavily on the type of culture media used, mainly composed of metal ions and salts. In the present study, metal ion removal ability of *Chlorella pyrenoidosa* was investigated for its application in waste water treatment. The biosorption of mainly three metal ions Ni^{+2}, Cd^{+2} and Pb^{+2} from artificial wastewaters were investigated in batch experiments. Chlorella sp. showed excellent metal removal efficiency in presence of Cd^{2+}, Ni^{2+} and Pb^{2+} added together in semi-synthetic media preparation. Ni+2were reduced to maximum of 58%, Cd^{+2}were reduced to 66 % and $Pb^{+2}$was reduced to 70% in five days. Three-fold increment in lipid content (18.2 % by dry cell weight) was achieved in culture grown in semi-synthetic media incorporating heavy metals. The results clearly indicate that the alga is significantly utilized for metal removal from waste stream along with the biodiesel production.

Keywords-Microalgae, Waste Water, Metal removal, Lipid, Biodiesel.

Introduction

The release of heavy metals from industries into the environment has resulted in many problems for both human health and aquatic ecosystem (Paul, 2014; Chekroun and Baghour, 2013). A number of methods have been developed for removal of such substances like precipitation, evaporation, ion-exchange etc. However these methods have several disadvantages (Edris, *et al*., 2014). Biosorption using microbioal biomass as the adsorbent has emerged as a potential alternative technique to the existing methods for metal removal (Ozturk, 2007; Terry and Stone, 2002). Among number of microorganisms known for biosorption, microalgae is one of the best source due to its high occurrence, fast growth rate and possibility of large value-added products (Hadiyanto, *et al*., 2014; Rasala, *et al*., 2014).

Microalgae are photosynthetic organisms that grow photo-autotrophically in presence of light and CO2 or they grow heterotrophically in presence of organic carbon. Microalgae can be cultivated under difficult agro-climatic conditions to produce fats, oils, sugars and other bioactive compounds. Microalgae utilise macro and micronutrients for their growth and they accumulate high lipid under stress condition like presence of heavy metals to cope-up with the environment (Osundeko, *et al*., 2013). Algae is proved to be a promising microorganism for biodiesel production as they accumulate cellular lipid and carbohydrate those can be utilized for formation of biodiesel and bioethanol (Kumar, *et al*., 2015; Mata, *et al*., 2010). Third generation biofuel is algal biodiesel, i.e. produced by transesterification (as given below) of algal lipid to fatty acid methyl esters (FAME), which is requisite biodiesel (Hu, *et al*., 2008).

Triglyceride (TG) + R'OH →Diglyceride (DG) + R'COOR1
Diglyceride (DG) + R'OH →Monoglyceride (MG) + R'COOR2
Monoglyceride (MG) + R'OH →Glycerol (GL) + R'COOR3

Chlorella pyrenoidosa is a green microalga and can grow photoautotrophically under nitrogen containing medium. The species has ability to grow and accumulate lipid on cattle waste and the FAME produced has ability to act as biodiesel (Sharma and Rai, 2015). For industrial applications, the use of immobilized algae has more advantages than freely suspended biomass. The benefit of immobilized algae biomass are resistance to chemical environment, easy separation of cells and waste, higher mechanical strength, minimal clogging in continues system and possibility to repeat in many runs (Hameed and Ebrahim, 2007; Horváthová, *et al*., 2009). Cell entrapment is the most widely used technique for immobilization. In this technique, the cells are enclosed in a polymeric matrix which is porous enough to allow diffusion of substrate to the cells. Alginate has been successfully used for cell entrapment and widely used for heavy metals removal from aqueous solution. The cell immobilization is not merely utilized for heavy metals recovery, but it may possible to improve the removal of nutrients available in the waste (Jimenez-Perez, *et al*., 2004). The ability of growing microalgae on waste water stream will help in coupling of the algal biofuel production process with bioremediation.

In the present study, *Chlorella pyrenoidosa* was immobilized in calcium alginate beads and bioremoval efficiency for lead, nickel and cadmium were estimated and compared with non-immobilized cells. Biomass and lipid content were measured from the algae established that the Chlorella sp. is potentially used for waste water treatment along with lipid production for biodiesel generation.

MATERIALAND METHODS

Microalgae and growth medium

Chlorella pyrenoidosa*,* obtained from National Chemical Laboratory, Pune, India, was grown photo-autotrophically in Fogg's medium. (Rai, *et al*., 2013). The cells were grown in Erlenmeyer flasks and kept in a temperature controlled incubator room at 25°C providing 24 h fluorescent illumination (40 watt, white tube light) and 3000 lx intensity.

Growth Analysis

Growth curve of the algal species was plotted by measuring biomass production (g/L) on alternate days up to 14 days. The cell dry weight was determined after centrifugation (Shimadzu UV 1650). Growth analysis was also estimated by cell chlorophyll content at the same time interval. Chlorophyll was extracted in a hot water bath in presence of methanol. The absorbance was measured at665 nm (A665) and Chlorophyll a was estimated according to equation (Mackinney, 1941)

Chlorophyll a (mg/L) = 13.42×A665

Bio-sorption of Cd^{2+},Ni^{2+} and Pb^{2+} by Chlorella sp.

The metal processing ability of algal cell was checked by growing it in Fogg's medium containing $NiCl_2$, $CdSO_4$, Pb $(NO_3)_2$ of varying concentrations from 10-600 mg/L. The metal removal efficiency of *Chlorella pyrenoidosa* was also studied in semisynthetic media prepared by taking 50 ppm $NiCl_2$, 100 ppm $CdSO_4$ and 50 ppm $Pb(NO_3)_2$ included in Fogg's media. The algal growth was determined by taking optical density of the sample by UV Visible spectrophotometer at 660 nm. The residual concentration of heavy metals in the medium was estimated on day 0, 3, 6, 9, 12 and 15 by AA1275 Atomic Absorption Spectrophotometer (AAS). Minimum inhibitory concentration (MIC) and bio-removal efficiency (%R) of each metal by algae was estimated by following formula (Edris et al., 2014).

R (%) = (Ci̇ Ce/Ci) X 100

Where, Ci and Ce are initial and final metal concentrations.

Micro-bead Formation (Immobilization of algal cells)

Comparative studies were done to find out a more efficient way of metal processing between free algal cells and Immobilized algal cells. Immobilization of cells was done by formation of micro-beads using 5% sodium alginate and 0.2 M $CaCl_2$ according to the method given by Hadiyanto, *et al*., (2013) with little modification.

Lipid extraction and FAME synthesis

The semi dried weighed algal cells were crushed in presence of 2:1 chloroform-methanol combination with the help of mortar and pestle (Bligh and Dyer, 1959). The extract was collected, dried at 60°C and then stored. The lipid extract obtained was subjected to acid based transesterification in a condenser reflux using sulphuric acid and methanol (Sharma and Rai, 2015). FAME (Fatty Acid Methyl Ester) was produced through this chemical process and separated from glycerol in separating funnel and washed thoroughly.

Result and Discussion

Biomass estimation

Photosynthetic microorganisms, algae can accumulate heavy metals by physical adsorption, ion exchange, covalent bonding, surface precipitation, redox reaction, crystallization on the cell surface (Cechinel, *et al*., 2016; Edris, 2014). Active uptake that often involves the transport of metals into the cell interior is often a defensive tool to avoid poisoning or it serves to accumulate essential trace elements. Advantages of using algae in waste treatment are cost effective, low energy input, reduction in sludge formation, reduction in GHG emission, production of useful algal biomass at low cost media.

The present investigation revealed that the microalga *Chlorella* pyrenoidosa can grow autotrophically and the growth was affected in presence of heavy metals. There was decrease in growth after 9th day in presence of Cd^{2+}. The stationary phase remained up to 12th day of inoculation in presence of Ni^{2+} and Pb^{2+}. Figure 1 and 2 depicts the growth of *Chlorella sp* in autotrophic cultivation mode and in presence of heavy metals. *Chlorella vulgaris* is known for heavy metal removal from textile industry effluent (Hadiyanto, *et al.,* 2014). Cu and Cr were removed around 90% by growing the Chlorella cells in 25% textile waste water.

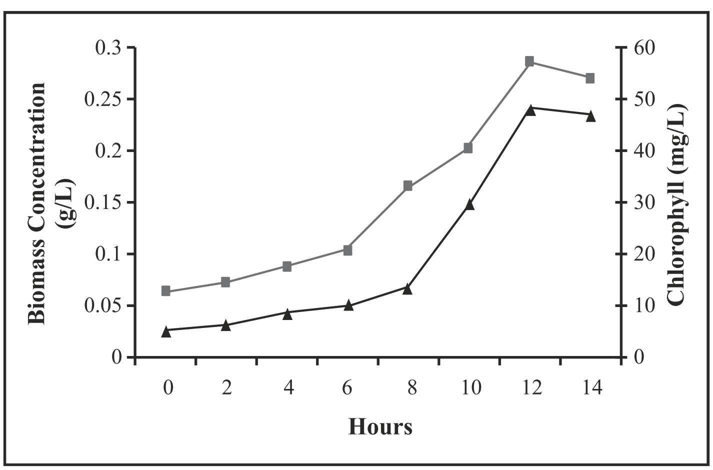

Figure 1: Growth of *Chlorella sp.* under autotrophic condition (▲ by chlorophyll estimation; ■ by biomass estimation).

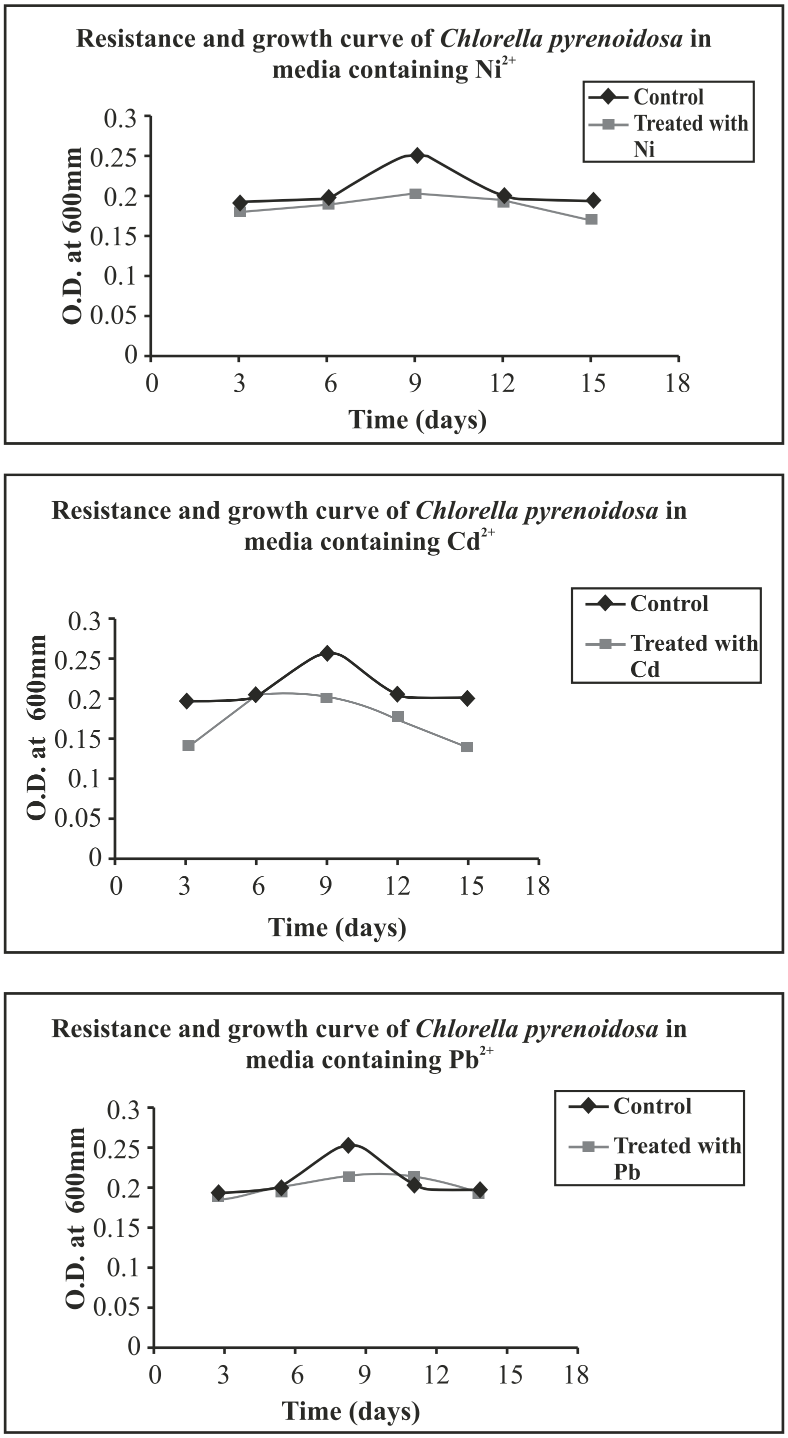

Figure 2: Growth curve of *Chlorella sp.* in presence of heavy metals (100 ppm concentration each)

Metal removal by Algae

Algae are known for their capacity to accumulate heavy metals from wastewater since heavy metals such as Zn, Cu, Fe, Mn, Co and Mo are required as essential nutrients (Gadd, 1990). The algae are known to remove heavy metals from waste stream like Pd, Cd, Hg (Inthorna, *et al.,* 2002). In the present study, potential of *Chlorella pyrenoidosa* was determined for removal of Pd, Cd and Ni. Figure 3 determine the bio-removal efficiency of *Chlorella sp.* for Cd^{2+}, Ni^{2+} and Pb^{2+} during 15 days at fixed time intervals.

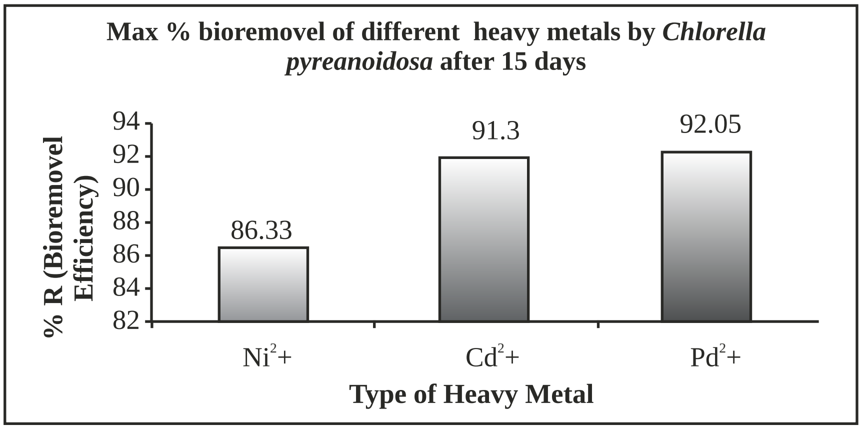

Figure 3: Bio-removal efficiency of *Chlorella sp.* for Cd^{2+}, Ni^{2+} and Pb^{2+} (in 15 days)

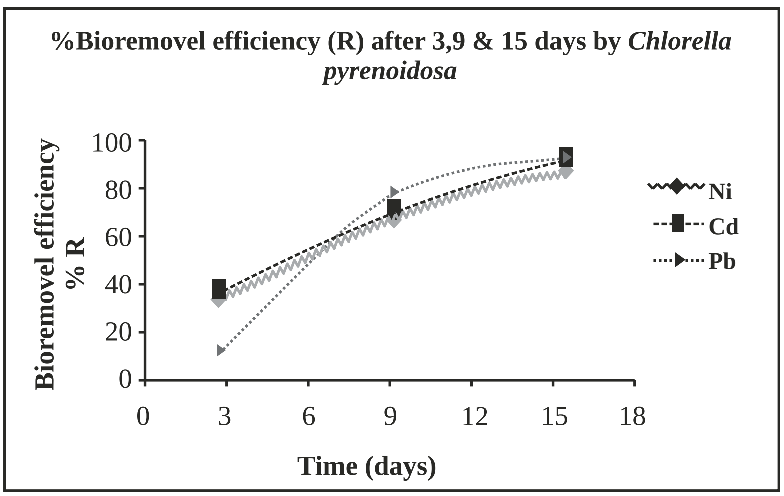

Figure 4: Bio-removal efficiency of immobilized *Chlorella pyrenoidosa* for Cd^{2+}, Ni^{2+} and Pb^{2+} (200 ppm)

Chlorella sp showed excellent metal removal efficiency in presence of those heavy metals added together in semi-synthetic media preparation. Ni^{2+} was reduced to maximum of 58%, Cd^{2+} was reduced to 66% and Pb^{2+} was also reduced to 70% in five days. Kumar and Goyal (2010) have reported removal of lead by 66% from the culture medium of *Chlorella sp.* having the lead concentration of 1 mg l^{-1}. The lead removal efficiency was decreased to about 40% at the concentration of 20 mg l^{-1}.

Figure 4 represents the bio-sorption of Cd^{2+}, Ni^{2+} and Pb^{2+} by the immobilized *Chlorella* cells for different time period. The cells showed more than 90% of removal for each metal species during 15 days. There is not much effect of immobilization on algae removal efficiency rather it worked better under impregnated environment for longer time. *Chlorella pyrenoidosa* immobilized by polyvinyl alcohol-sulphate method was reported to absorb nitrate and phosphorus from waste stream very efficiently (Huang and Wang, 2003). Immobilized Chlorella cells have advantages to be reused in the process for many runs.

semisynthetic media composed of Cd^{2+}, Ni^{2+} and Pb^{2+}. The semisynthetic media was prepared by constituting various concentrations of heavy metals. The overall metal removal for Cd is maximum while final efficiency for Pd is highest as shown in fig.5. In five days metal removal efficiency was estimated about 40-60% for all the metals used in the experiment.

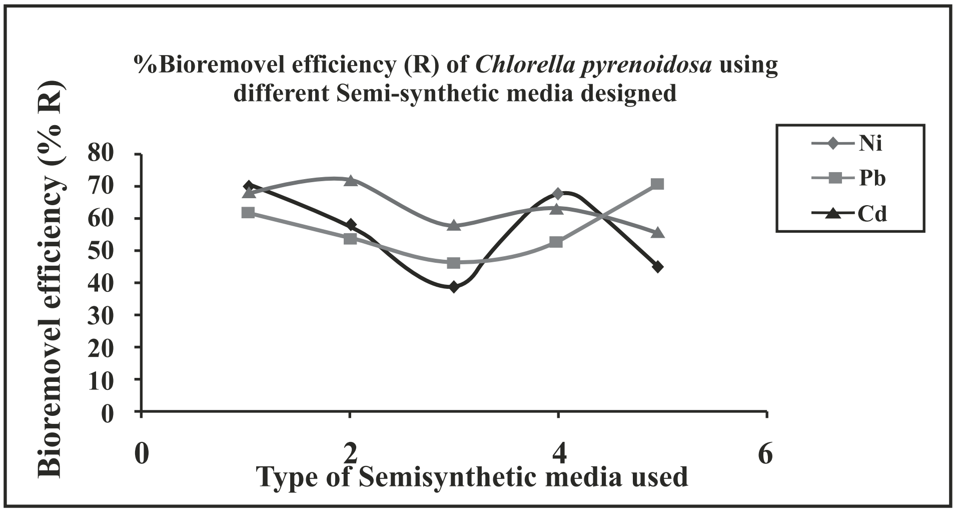

Figure 5: Bio-sorption of Cd^{2+}, Ni^{2+} and Pb^{2+} (200 ppm each) by immobilized *Chlorella sp. under semi-synthetic media.*

Effect of heavy metals on lipid accumulation in Chlorella pyrenoidosa

The algae has ability to grow under unfavorable condition and it try to cope up with the stress environment by accumulating more storage lipid. Similar trend was also noticed in our study for *Chlorella pyrenoidosa,* it grows well in presence of semisynthetic media containing Cd^{2+}, Ni^{2+} and Pb^{2+}. Three fold increment in lipid content (18.2% by dry cell weight) was achieved in culture grown in semi-synthetic media incorporating heavy metals (Cd^{2+}, Ni^{2+} and Pb^{2+}; 200 ppm each). Under photoautotrophic growth the lipid content of the cell is approximately 6%. Table 1 shows the comparison of growth and lipid content of algal species in normal Fogg's and semi-synthetic media. Growth of the Chlorella is slightly affected due to unfavorable condition but accumulate higher lipid. There is significant difference in lipid content of the cell with heavy metal and without using metal ions.

Table 1: Comparison of growth and Lipid Content of *Chlorella sp.* in normal Fogg's medium and semi-synthetic medium

	Autotrophic Culture in normal Fogg's medium	Cells grown in semi synthetic media
Biomass Production (mg/L)	1186	953
Lipid accumulation (%)	6.0	18.2

The algal lipid extract was esterified under acid treatment through a process known as transesterification. Figure 6 represents the preparation and purification of biodiesel. After downstream processing the methyl esters are separated out in organic layer leaving the glycerol content in bottom layer. The methyl esters (biodiesel precursors) were dried, weighed and dissolved in hexane solution. The FAME sample will be characterized in future for its application.

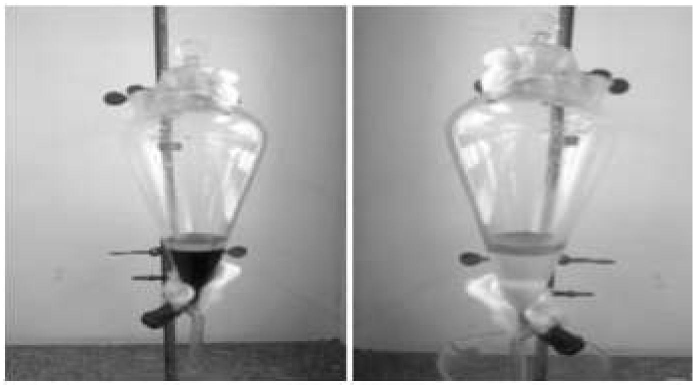

Figure 6: Preparation and purification of Bio-diesel via transesterification

Similar results also obtained with macroalgae sp. *Oedogonium* and *Spyrogyra* for FAME production (Hossain and Salleh 2011). Though, the lipid content of macroalgae is lower than microalgae, but the FAME synthesis from both the sources are very similar. The *Chlorella pyrenoidosa sp.* grown mixotrophically on acetate and glycerol has proved to be a potential source for biodiesel production. In future, GC-MS study will reveal the quality of the biodiesel produced from *Chlorella pyrenoidosa* when grown on semi-synthetic waste water.

Conclusion

As a result of present study, *C. pyrenoidosa* was selected for metal removal of Pd, Cd and Ni those are toxic to aquatic ecosystem. Alga was proved to be a good raw material for bio removal of heavy metals and showed significant increase in lipid accumulation. The cell growth is slightly effected by the use of heavy metals in culture medium but the increase in lipid is more than three folds that would be desirable for biodiesel production. An integrated process would be developed at large scale by incorporating *Chlorella pyrenoidosa* for waste water treatment and biodiesel production simultaneously.

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