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Bioflocculation of algae using *Delonix regia* leaf protein

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Abstract

Microalgae are multifaceted photosynthetic microorganisms with incipient business prospective. Harvesting of microalgae from the medium poses a significant obstacle due to their diminutive size and low concentrations. Thus, from carbon capturing and waste management to biofuels, harvesting of microalgae is in an efficient and environmentally-friendly manner, has become the topic of apprehension. Bioflocculation studies of microalgae *Synechococcus*, *Dunaliella*, and *Chlorella* sp. using *Delonix regia* leaf protein was undertaken herein. *D regia* leaf protein had a bioflocculation efficiency of 75%, 76%, and 84% respectively in case of *Chlorella* sp, *Synechococcus* sp, *Dunaliella* sp. As leaf protein is a safe, non-toxic, and environmentally friendly flocculant, it is suitable for effective and ecofriendly microalgal harvesting and has the potential to be upped to an industrial scale.

Keywords: Biofloculation, *Delonix regia*, leaf protein, microalgae

Introduction

Due to the surge in population and improvement in living standards in developing nations, there has been a significant rise in the demand for food, feed, and fuel over the last decade. Industrialization has led to an increased dependence on fossil fuels, which has, in turn, exacerbated global warming and environmental pollution. Microalgae are considered a promising source for production of a variety of valuable products especially in the biorefinery sector, including renewable biofuels and value-added chemicals (Behera et al. 2018a, Khan et al. 2018). This approach addresses the growing demands of the future and offers environmental protection and sustainable benefits (Rangabhashiyan et al. 2017). However, the small size of microalgae cells, combined with their low concentration and the large volume of culture media, which has a density similar to water, leads to increased costs and energy requirements during harvesting (de Morais et al. 2023). Thus, the primary challenge lies in the process of separating the algal biomass from the water or media. Different researchers have discussed a variety of operations and methods (including chemical, mechanical, and biological harvesting), all of which involve substantial costs and energy consumption (Fasaei et al. 2018). The harvesting methods can either be used individually, or in combination, depending on the physiochemical properties of diluted algal suspensions (Matter et al. 2019). Although mechanical harvesting techniques like centrifugation, filter pressing, and electrocoagulation, are easy to apply, they demand significant energy consumption, making them impractical for large-scale use (Wan et al. 2015). Numerous studies have been directed towards sediment and concentrate microalgal biomass using chemical flocculants (Lama et al. 2016). The effectiveness of the chemical coagulation process relies on factors such as algal concentration, pH of the

media, and the amount of flocculants used (Ummalyma et al. 2016). Various studies have investigated polymeric compounds or organic flocculants derived from biological sources. Wan et al. (2015) demonstrated a 60% efficiency in removing algal biomass using polyacrylamide under optimal conditions. Chitosan is also effective as a flocculant, creating larger flocs even at lower dosages compared to other agents (Blockx et al. 2018). Natural plant extracts such as plant leaf protein, fruit waste peels, Moringa seed flour, Soybean seed protein, guar gum, Cicer arietinum seeds, Cactus lactifera, etc. have been utilized for the treatment of highly turbid wastewaters, aiming to eliminate color and turbidity while lowering both biological and chemical oxygen demand (BOD & COD). Plant extracts from cactus (*Ficus indica*), drumstick (Moringa oleifera), leaf protein from *D. regia*, and discarded fruit peels of orange (*Citrus sinensis*), pomegranate (*Punica granatum*), and banana (*Musa acuminata*) were employed in the harvesting process of the mixed microalgal consortium. In bioflocculation, the operational parameters such as eluent concentration, coagulant dosage, pH, and microalgal concentration, need to be adjusted to maximize removal efficiency. This strategy eliminated the use of chemicals and facilitates uniform cultivation conditions, improving the microalgal harvesting process. It is easy, cost-efficient and industrially suitable. Although numerous innovative flocculation technologies for algal biomass harvesting have been developed, ongoing research efforts are focused on cost-reduction and up-scaling. There remain significant challenges in achieving efficient and cost-effective concentration of microalgae biomass through the utilization of flocculation technologies.

D. regia is a flowering plant belonging to the family Fabaceae, subfamily Caesalpinioideae; it is found in tropical and subtropical climates and can be seen in the Indian subcontinent, where it is referred to as Mayflower. An ornamental tree with fern-like leaves and a flamboyant display of phoenix orange-red flowers over summer grant the name flame of the forest, or flame tree. Based on the abundant occurrence, *D. regia* leaf protein was tested for its bioflocculation capacity in this study.

Material and method

Synechococcus sp., *Chlorella* sp., and *Dunaliella* sp. were procured from National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappall, Tamil Nadu, India. The marine microalgae were cultivated under axenic conditions in ASNIII media. The medium consists of 25g/L sea water, 2g/L MgCl2, 0.5 g/LKCl, 0.75g/LNaNO3, 3.5g/L MgSO4, Citric acid, EDTA, trace metal, and ferric ammonium citrate. The cultures were cultivated in a laboratory growth chamber at 24 ± 0.1°C, under a 16:8 light-dark cycle using white fluorescent lights, with a photon flux of approximately 60 µmol photon /m^2 /s^1.

Extraction of Protein from *D. regia* leaf

The extraction of protein from the *D. regia* leaf was carried out according to Niu et al. (2019). Here 500 mg of *D. regia* leaf, was placed into a chilled mortar and pestle after which 0.5 ml of lysis buffer containing trichloroacetic acid was added; this was ground into a uniform paste. Thereafter, the paste was centrifuged at 10,000 rpm for 5 min and maintained the temperature at 4℃. The supernatant was discarded; the remaining pellet which contained the plant protein. The pellet was placed at -20°C for 1h and chilled acetone was added to it. The mixture containing the sample was thereafter vortexed for 5 min, followed by which it was centrifuged at 10,000 rpm (4°C for 5 min). The supernatant was discarded and the pellet was washed it with acetone thrice at room temperature to remove all the plant pigments. After this, the pellet was reconstituted with rehydration buffer. The sample was vortexed until a uniform solution was obtained. The sample was centrifuged at 10,000 rpm for 5 minutes and the supernatant containing total crude plant protein was collected. The percentage of protein was estimated according to Sarkar et al. (2020).

Flocculation Experiment

The flocculation efficiency of the proteins was evaluated using a variant of the jar test method (Gerde et al. 2014), considering flocculant concentration, settling time, and, pH of the algal medium. Here, 1000 mL microalgal suspension was agitated at 300 rpm in a 1.2 L jar, and there was a gradual addition of the flocculant into the algal medium. The stirring was discontinued after 5 min and the mixture was set-aside for 10 minutes. The absorbance of the initial algal medium was noted prior to adding the flocculant. In order to assess the impact of pH on flocculation, pH adjustments were made using either 0.5 N HCl or 0.5 N NaOH. Cell numbers

were inferred from absorbance values using a standard curve generated from algal cell suspensions of varying cell densities.

Flocculation efficiency of microalgae

In general, flocculation is the process of contact and adhesion whereby the particles of a dispersion form larger-size clusters; substances with high flocculation efficiency are sought for. Determination of the flocculation efficiency was carried out using the following equation:

$$\text{Flocculation efficiency (\%)} = \frac{\text{Initial cell concentration- Cell concentration in supernatant} \times 100}{\text{Initial cell concentration}}$$

Result and Discussion

D. regia leaf constituted 5.733±0.989% of total crude protein which was very close to that reported by Ayomide et al. (2022) for *Chrysophyllum albidum* leaf (7.37%).

Effect of pH and flocculant dose on harvesting of microalgae

Several experiments have been conducted to examine the influence of different concentration of leaf protein on the efficiency of flocculation of algae, and it has been recognized that the concentration of dosage is a crucial in flocculation. In our study, preliminary experiments were conducted to determine the optimal dosage of flocculant for effective flocculation of microalgae, considering both the degree and speed of the flocculation reaction. A fixed concentration of algae was used alongside varying doses of protein (ranging from 30ml/L to 150 ml/L), maintaining a pH of 2. As the concentration of flocculant doses increased, the flocculation efficiency of microalgae also increased (Fig. 2 a-c). The maximum flocculation efficiency (76.579%, 84.265%, and 75.925%) for *Synechococcus*, *Dunaliella*, and *Chlorella* respectively was recorded with 150 ml/L doses at pH 2. Figure 1 (a-c) shows the effect of pH on the flocculation efficiency of *D. regia* leaf protein. The flocculation efficiency of leaf protein sharply increased from 40 to 84.265% when the dosage increased from 80 to 150 ml/L. Table 1 compares the effectiveness of D. regia leaf protein as a bioflocculant as compared to other reports. Our study indicates that a concentration of 150 ml/ Lof *D. regia* leaf protein could represent an optimal dosage for achieving enhanced flocculation of algal species at pH 2. The increased efficiency of leaf protein flocculation at pH 2 might result from the protonation of amino groups within the protein, enhancing its interaction with the anionic charges present on the algal cell wall. As the pH rises, the carboxyl group of the protein acquires a negative charge (COO^-), potentially leading to electrostatic repulsion. Wongasgonsup et al. (2005) observed a decreased coagulation efficiency of rice protein at higher pH levels. Several studies have identified active coagulant proteins derived from various seed extracts, containing charged groups comprising charged amino acids as well as some charged functional groups.

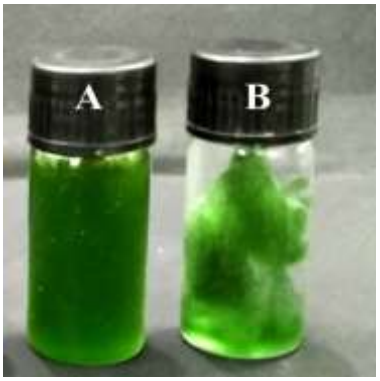

Fig1(a): Bioflocculation of *Synechococcus* sp (A) Control (B) *Delonux regia* leaf protein at pH 2

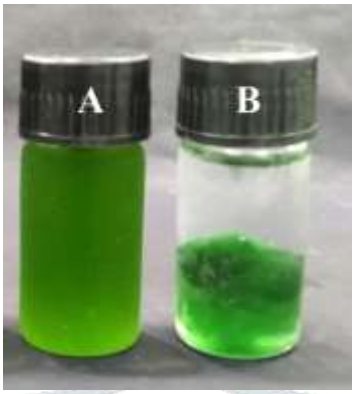

Fig1(b): Bioflocculation of *Dunaliella* sp (A) Control (B) *Delonix regia* leaf protein at pH **2**

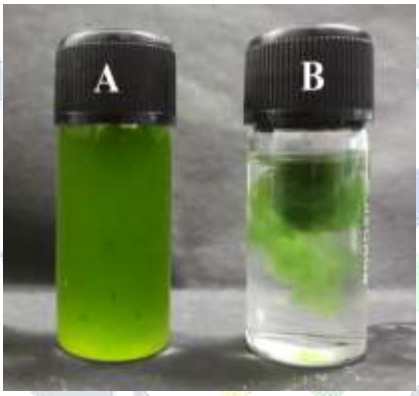

Fig1(c): Bioflocculation of *Chlorella* sp (A) Control (B) *Delonix regia* leaf protein at pH 2

Mechanism of microalgae flocculation via leaf protein

The negative charges found in the microalgal cell wall primarily originate from the carboxylic (COOH) and amino (–NH2) functional groups (Pushparaj et al. 1993). Frequently, the introduction of a polyelectrolyte with a robust positive charge becomes indispensable. Therefore, the harvesting of co-cultivated microalgal biomass is facilitated using specific flocculants based on cationic protein polymers. Ideally, these flocculants are economically viable, environmentally friendly, and demonstrate superior effectiveness compared to others, even when used in low concentrations (Lucie et al. 2017). In this context, the mechanisms involved in floc formation destabilization rely on polymeric adsorption and are greatly influenced by pH. In the process of inducing floc formation for flocculation, the presence of a negative charge on microalgal cells impedes cell aggregation, highlighting the crucial role of charge neutralization (Esser et al. 1983). Cationic flocculants neutralize the surface charge of negatively charged microalgal cells through electrolytic attraction, thereby decreasing the zeta potential and promoting the flocculation process. Collisions and adsorption of the microflocs lead to their growth, facilitated by the presence of flocculants covering nearly all microalgal cell surfaces, thereby enabling cellular interactions. The small, stabilized algal cell particles (micro flocs) coalesce into larger flocs through a combination of charge equilibrium and particle bridging. After flocculation, subjecting the larger flocs to low-energy centrifugation or gravity filtration results in an additional increase in the concentration of the harvested biomass. The economical recycling process of clear, non-toxic growth media, and algal cell harvesting, plays a beneficial role in industrial applications.

Table 1. A comparison of microalgal harvesting of *Delonix regia* leaf protein as compared to other bioflocculants.

Flocculant used	**Microalgae species**	**Microalgae**	**Harvesting efficiencies (%)**	**References**
Modified Tannin	*Scendesmus* sp	Freshwater	97	Wang et al. 2013
Guar gum	*Chlamydomonas* sp.	Freshwater	84	Banerjee et al. 2014
Cationic inulin	*Botryococcus* sp	Freshwater	89	Rahul et al. 2015
Strychnos potatorum	*Chlorella vulgaris*	Freshwater	99.7	Razack et al. 2015
Poly-γ-glutamic acid	*Chlorella vulgaris, Nannochloropsis oculata,* and *Phaeodactylum tricornutum*	Marine	>90	Kandasamy and Shaleh (2016)
Chitosan	*Spirulina*	Freshwater	99.57	Rokhati et al. 2021
Delonix regia leaf protein	*Chlorella, Synechococcus, Dunaliella*	Marine	75, 76, and 84 respectively	Current study

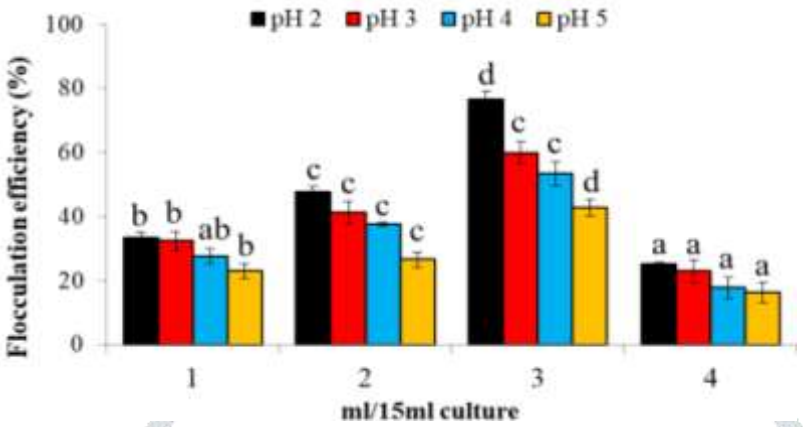

Fig 1 (b): Effect of *Delonix regia* leaf protein dose and pH on the bioflocculation efficiency of Synechococcus sp.

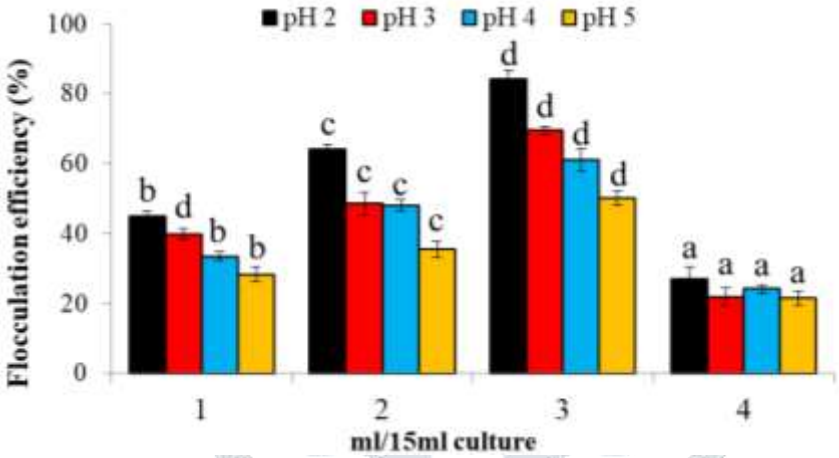

Fig 1(c): Effect of *Delonix regia* leaf protein dose and pH on the bioflocculation efficiency of Dunaliella sp.

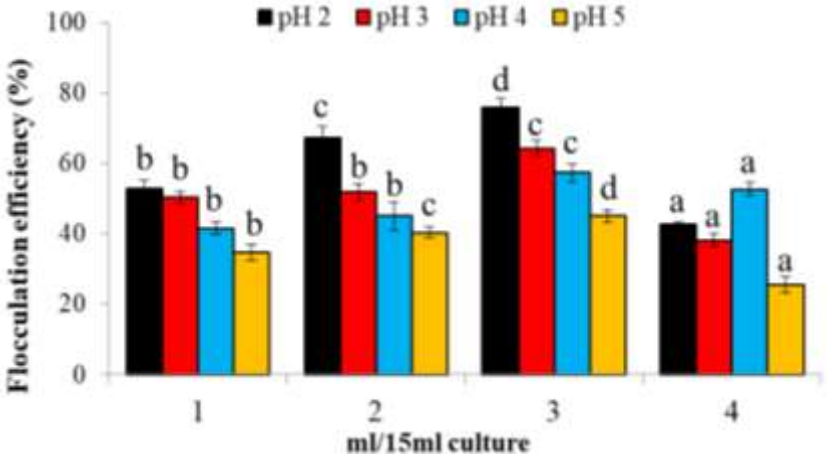

Fig1(a): Effect of *Delonix regia* leaf protein dose and pH on the bioflocculation efficiency of Chlorella sp.

Conclusion

Our study findings suggest that utilizing *Delonix regia* leaf protein for microalgal harvesting is promising, environmentally friendly, and cost-effective as minimal flocculant dose is required. A flocculation efficiency exceeding 84% could be observed for the microalgal biomass at optimal conditions, using an 80 ml/ L bioflocculant dose.

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