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ORIGINAL ARTICLE **OPEN ACCESS**

Chitosan as a Bioflocculant For Harvesting Of Marine Microalgae. *Chlorella marina Butcher 1952*

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ABSTRACT
Microalgae cells are photosynthetic, very tiny structure with the sizes range from 5 - 50 µm and it is a potential source for biofuel, feed and food. Hence the harvesting method is an important and major challenge to make it cost efficient. Consequently, Chitoson is a biopolymer used to flocculate the microalgae instead of chemical flocculent to avoid negative effects on human and animal health. The focus of this research is to document on the microalgae Chlorella marina harvesting by utilizing chitosan prepared from crab shells (Scylla serrata) as a flocculent agent. In this investigation, the chitosan generation is carried out by deproteination, demineralization and deacetylation of crab shell waste. Then it was allowed to characterize the content of moisture, ash, solubility and deacetylation degree (DDA). In addition, the chitosan dosage for microalgae harvesting (Chlorella marina) is determined by performing Jar test. At various chitosan concentrations the recovery efficiency of C. marina was tested. According to the findings of this study, moisture content is 5.70%, Ash is 4.30% while chitosan solubility reached up to 65.45%. The DDA value obtained was high, 71%. In a flocculation test, 40 ppm of chitosan demonstrated the greatest performance (89.4%) in a period of 30 minutes at a pH of 9.8.
Keywords: *Chitosan, Chlorella marina, Flocculation, Harvesting efficiency, Degree of Deacetylation.*

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INTRODUCTION

Microalgae biomass has generally been known as a potential aquaculture and biofuel source [1]. Microalgae have been successfully commercialized in the manufacture of food additives, cosmetic products, antioxidants and organic dyes. A microalgae strain must consider many factors in order to be used in aquaculture, including sufficient speed of cultivation, non – toxicity and Protein, vitamins, and polyunsaturated fatty acids are the most significant elements [2].

The collection of biomass from growth media is a critical step, representing between 20–30% of the overall biomass production costs [3], due to the diluting nature of microalgae culture. Due to the small size of microalgae cells (3–30m diameter), cost-effective harvesting is became challenging.

Microalgae harvesting strategies include centrifugation, flocculation, sonication, coagulation, filtration and air floatation. Among these, flocculation stands out as the most appealing choice in the view of its ease of use and cheap cost [4].

Flocculation is the process of aggregating dispersed particles into spacious particles for settling [7]. Flocculation can be accomplished in a variety of ways, and proceed toward microalgae have been investigated in recent decades. Among these strategies of biomass collection standard flocculation method, is frequently applied in different emerging and industrial sectors. Inorganic substance was also used to flocculate the microalgae at the low enough pH [8].

For example, Ferric and aluminum salts can combine with microbial cells, inclusive of microalgae, to form flocs. In a previous experiment, Ca(OH), Mg(OH), KOH and NaOH were applied as flocculants at pH 8.5 – 11.5 [9].

Despite of their high efficiency, such chemicals frequent utilization can corrupt microalgae biomass, resulting in negative consequences for future applications such as human and animal feed [4]. Polymers derived from nature like chitosan, may offer a hopeful solution in relation to these problems [5]. Chitosan is a linear poly-amino-saccharide synthesised from the alkaline deacetylation of chitin [6]. Several factors influence microalgae harvesting effectiveness including flocculant dose, flocculant type, culture pH and settling time [10].

The pH is also important in the flocculation process [1] that can pH influences the combination of microalgae and flocculant and make resulting impact on harvesting proficiency [3]. We investigated the efficiency of chitosan as a bioflocculant employed for harvesting of microalgae in this experiment. Also demonstrated that the influence of bioflocculant dose and its pH on the harvesting proficiency.

MATERIAL AND METHODS

Microalgae cultivation

Microalgal culture at laboratory condition

Pure isolate of *Chlorella marina* was obtained from Marine science department, Bharathidasan University, Tiruchirappalli. The culture medium used for cultivation was Conway medium (Fig. 1). The algal biomass was cultured under the laboratory and exposed to suitable condition of physical parameter. Such as, light (16:8 hour light: dark), temperature (25°C), Salinity (28o/oo) and aeration. The microalgal culture was monitored by using spectrophotometer UV- Vis spectrophotometer (UV- 1800 Shimadzu) for the whole growth period [11].

Preparation of chitoson

Chitin was made from *Scylla serrata* carapaces (Fig. 2) obtained from a local landing center in Cuddalore, Tamil Nadu. The carapaces (50g) were washed to remove impurities before being oven dried. Finally, the dried shells were crushed in a 0.1 mm sieve fitted blender. The powder was then Demineralized with 2 M hydrochloric acid at 60˚ C for 150 minutes [12] afterwards Deproteinization contacted at 80˚ C by using 3 M sodium hydroxide (NaOH) for 120 minutes while stirring.

Finally, the sample was decolored by passing it through a 1:2:4 mixtures of choloroform, methanol and distilled water, followed by a rinse with distilled water. After 24 hrs in an oven at 60˚C, the chitin extraction process was completed [13].

The acetyl groups of chitin was removed by employ a 50 percent NaOH concentration at the temperature of 65˚ C and a solid to solvent proportion of 1:10 (w/v) for 20 hours. Formalised Tap water was used to wash the residue until it reached a pH of neutral. The obtained chitosan (Fig.3) was allowed to dry for 4 hours at 65˚C in a hot air oven before being prepared for characterization [14].

Chitosan characterization

The contents of Yield and Ash

The final yield of chitin was measured via considering the mass of the raw material to the mass of the treated chitin; Gravimetric analysis was used to determine the ash content after the chitin sample (0.5-0.6 g) was incinerated for at least 4 hrs in a muffle furnace at 650˚ C Using the following equation, the ash content was estimated as the proportion of the mass of the residue to the sample mass:

W2/W1× 100 = Ash %

Where W1 and W2 are the initial sample weights (in gram) if chitin and residue, respectively [16].

Moisture content

Gravimetric analysis was used to determine the content of moisture in chitins derived from the waste of crab shell. The water mass was determined in this method, the chitosan sample was allowed to drying by using oven at 110˚ C for 24 hours.

(W1 – W2)/W1 100 = Moisture content %

W1 and W2 are the moist and oven dried sample weights, respectively [19].

Degree of acetylation measurement

The 0.25 g of isolated chitosan was dissolved in 30 ml of prepared 0.1 mol/l HCL aqueous solution at room temperature. For about 50 minutes, the solution was stirred up to the chitin was completely dissolved. After cooling to room temperature, in addition of 5-6 drops of methyl orange; the red colour changed chitin solution turned into orange after being titrated with prepared 0.1 mol/l NaOH solution. The following formula was applied to calculate the DA [20].

$NaoH + HCL \rightarrow Nacl + H_2O$

After the reaction with chitin, the molarity of HCL remains unchanged.

$C_1 = V_2 \times C_2 / V_1$

Chitin treated with conc.HCl

The number of moles of HCL that reacted with chitin C1 V1 per 100 ml.

Chitin mass = number of moles chitin × molar mass chitin

DA chitin percentage = MC/MS 100

The concentration of standard HCL aqueous solution (mol/l) is C_1, the concentration of standard NaoH (mol/l) is C_2; During titration, consumed standard NaoH solution (ml) volume is V_2, the weight of chitin (g) is expressed as M, the mass of chitin (g) is indicated as M_c and finally the mass of the sample(g) is M_s

Solubility

In a centrifuge tube, 0.1g of samples with the addition of 40% acetic acid at the volume of 10 ml was kept in incubation shaker set to 240rpm at 25˚C about 30 minutes. The obtained findings was immersed in a water bath at 90˚c for about 10 minutes after cooling to ambient temperature, centrifuged at 10,000 rpm for about 10 minutes, and the supernatant was discarded. Before centrifuging, by using distilled water, washed the undissolved particles. The separated supernatant and undissolved pellets were dried at 60˚C for 24hrs. And at last, the particles were measured, and the solubility percentage of chitin was determined by the following formula [21]:

Solubility percentage = (Initial weight of tube + chitin) - (Final weight of tube + chitin) /(Initial weight of tube + chitin)- (Initial weight of tube) 100

Analysis of Fourier- transform infrared spectroscopy analysis (FT-IR)

1 mg of chitins was studied with an IR Prestige 21, Shimadu FTIR spectrometer to evaluate the occurrence of chitin-specific IR bands. The absorbance values vary between 4000 cm-1.

Scanning Electron Microscopy analysis (SEM)

Scanning electron microscope A JEOL JEM 6390 was employed to study the surface morphologies of crustacean chitins.

Flocculation test

Preparation of the chitosan solution

To prepare 1 % of stock solution, 100ml of 1 percent acetic acid solution (Merck) was used to dissolve the 100mg of chitosan. Then, 40ppm, 80ppm, 120ppm, 160ppm and 200ppm of five set of experiment prepared by using the above stock solution.

5.2 Jar test

Jar tests with 100 mL glass beakers were used for the experiments. The 60 mL of microalgae cells were placed in the beakers. According to the experimental design, five sets of microalgae-containing beakers were each supplemented with 40ppm, 80ppm, 120ppm, 160ppm, and 200ppm of chitosan solution (Fig.4). The microalgae culture and chitosan was merged only at 100 rpm for 2 minutes. After being mixed, the microalgal cells were rest to settle down. A 0.5 mL of sample was taken from the middle of the experimenting jar to monitor optical density. At 600nm, the OD of the sample was recorded by using spectrophotometer (UV – 1800 shimadzu, Japan). The sedimentation time was measured in this experiment to determine the efficiency of flocculation. Biomass recovery efficiency was measured as below [22]:

Percentage of cells removed (%) = $\frac{I_{blank} - I_{Sample}}{I_{blank}} \times 100\%$

Where, the intensity absorbance of the microalgae culture prior to chitosan addition is denoted as I blank and the intensity absorbance of the sample after coagulation is I sample.

RESULTS AND DISCUSSION

Yield of chitosan

In this report, crab has a chitosan content of 22.75 percent by dry weight (Table 1). Chitosan ingredients of organisms used to produce commercial chitosan, such as crab, are known to range between 20% and 31%. [23], [24], [25]; Chitin content ranged from 31.11 percent to 69.65 percent in various marine seashell wastes [26]. Based on the species, the chitosan content of crustacean shells vary from 7% to 40% [27].

Physico- chemical properties of isolated chitosan

Ash and moisture contents

Crab shell waste chitosan samples were found to have 4.30 % ash content (Table 1). Chitosan from crab shell waste had a1.08 % ash content in previous study [28].Viscosity and average molecular weight are reduced when there is a high ash content. 5.70% moisture content was found in chitosan obtained from crab shell waste. These findings were lesser than the moisture content of mussel shell chitosan (12.90%). [29]

Measurement of degree of acetylation (DA)

The degree of acetylation of the chitin product is serving a significant role in the influences all physiochemical characteristics (molecular weight, viscosity, solubility, etc.). In degree of acetylation, the concentration of NaOH has a significant impact. It is difficult to remove the acetyl group from chitin without using high temperatures and a large amount of sodium hydroxide. The percentage of N-acetylation founding of this study are displayed in Table 2. In the current study, crab shell waste chitin had the great degree of acetylation value of 71% (Table 1) when compared to the other chitins. Chitins isolated from marine seashells have DA values that range from 51.61 to 91.0%, depending on species and isolation method [26].

Solubility

According to the findings, chitosan isolated from crab has an excellent and good solubility of 65.45 percent (Table 1). Solubility ranges from 58.83% to 85.71% for seashell waste chitosan including pang, oysters, mussels etc, according to earlier studies [26].

SEM analysis of chitin made by crab shell wastes

SEM was used to examine the morphology of chitosan, which revealed that it had a long thin crystal structure on a smooth surface (Fig. 8).

FT-IR analysis of chitin made by crab shell wastes

The main absorption peak in the range at 3443.93 cm-1 in the FTIR profile of chitin extracted from crab shells could be attributed to N=H stretching and 1633.65 cm-1 N-H bend of amide bonds. The N=C=S strech was assigned at 2074.43 cm-1 and acid chlorides stretch was found in 684.01 cm-1. Fig.7

Chitin was successfully isolated from crab, according to the FT-IR analysis. The observed FT-IR bands are quite closely related to those reported in the literature [30]; [31]. At 3436 cm-1, [32] discovered hydroxyl group bonds. The band spectrum for hydroxyl group and –NH2 at 3438 cm-1[33], which have been in close agreement with the band spectrums found in earlier experiment are similar findings to this study.

Furthermore, the FTIR bands of the chitin for this experiment corresponded to the FTIR bands of alpha chitins isolated from various organisms in prior reports [13]; [34]; [35]; [23]. When the FT-IR results of isolated chitin are particularly in comparison to those of commercial chitin, the findings show that crab shell wastes are great resources of chitin, and the isolated chitin is really similar to that of marketing chitin. According to the above mentioned findings, the chitins isolated from crab shell waste are of the alpha type.

Chitosan dose effect on culture

The chitosan dose effect on the efficiency of *C. marina* harvesting was examined. For that purpose, C. marina was cultured for upto 30 days and the growth was monitored and recorded in chart 1. Chitosan was conducted in a variety of concentrations (40ppm, 80ppm, 120ppm, 160ppm and 200ppm). There was a significant decrease in optical density after only 30 minutes of settling time. As the chitosan dose increased, the optical density decreased. In terms of harvesting efficiency, this study shows a decrease in optical density. The greatest slow down in optical density was observed at 40 ppm chitosan solution and the smallest decrease at 200 ppm chitosan solution. The highest harvesting efficiency (89.73 0.4 percent) was obtained at 40 ppm (Fig. 5). The efficiency lowered as the flocculant dose increased, with 200 ppm reducing efficiency (86.34 0.5 percent). The harvesting was nearly finished in 30 minutes. Chitosan has a distinct advantage in that it can be harvested quickly, even at low doses. With *C. vulgaris*, only 60% flocculation performance was found using a considerably large dose of chemical salts, i.e., 1000 mg/L Al2(SO4)3 and a 6-hour incubation time [18]. Chart 2 compares efficiencies of harvesting are varying by chitosan dosages. chitosan at 30mg/l to work on microalgae flocculation[36]. The flocculation of microalgae *C. marina* was examined under microscope at different time interval and showed in Fig.6 (i), (ii), (iii).

Table 1: Physiochemical properties of chitosan

S. no	Physiochemical properties of chitosan	Percentage (%)
1.	Ash	4.30
2.	Moisure	5.70
3.	Degree of deacetylation	71
4.	Solubility	65.45

Table .2 Effect on chitosan on microalgae flocculation

Set	Before flocculation		After flocculation		Percentage of removal
	OD value	pH	OD value	pH	
I	0.476	7.6	0.046	8.9	89.73 %
II	0.473	7.6	0.049	9.2	89.01 %
III	0.458	7.7	0.055	9.4	87.99 %
IV	0.446	7.5	0.062	9.6	86.89 %
V	0.448	7.6	0.065	9.8	86.34 %

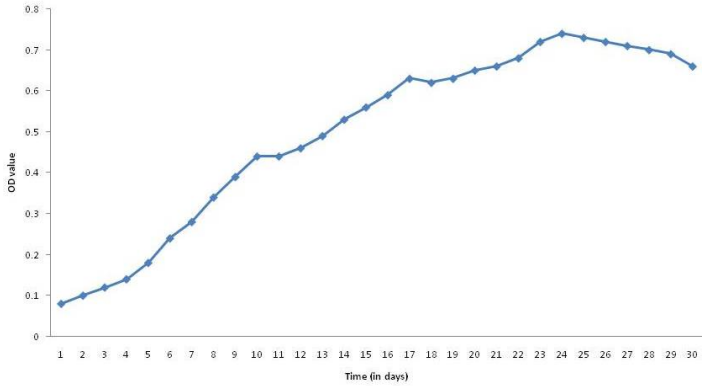

Chart 1. Growth rate of *Chlorella marina*

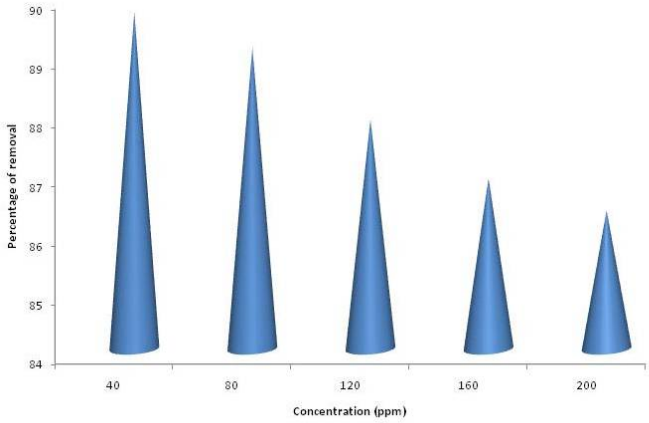

Chart 2. The influence of chitosan dosage on microalgal cell flocculation

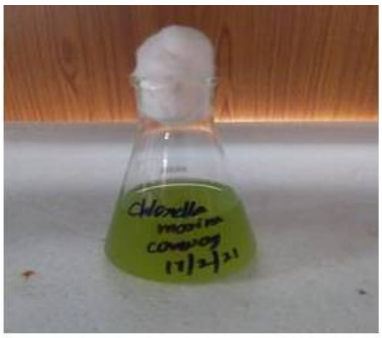

Fig. 1 Subculture of *C. Marina*

Fig. 2 Collected carapaces from *Scylla serrata*

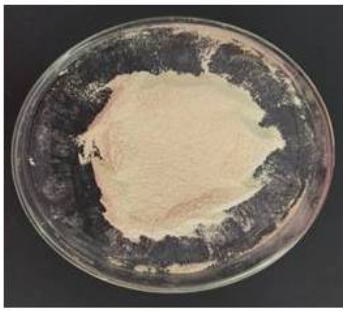

Fig. 3 Prepared Chitosan

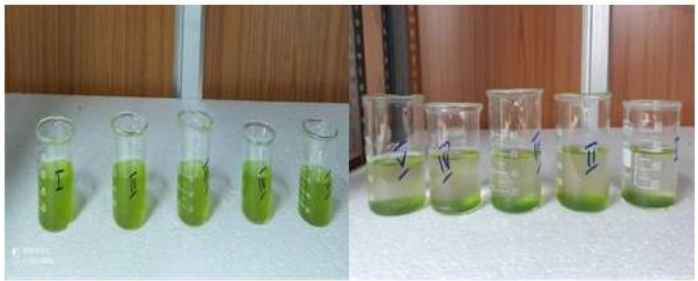

Fig. 4 Jar test – Before flocculation Fig. 5 After flocculation

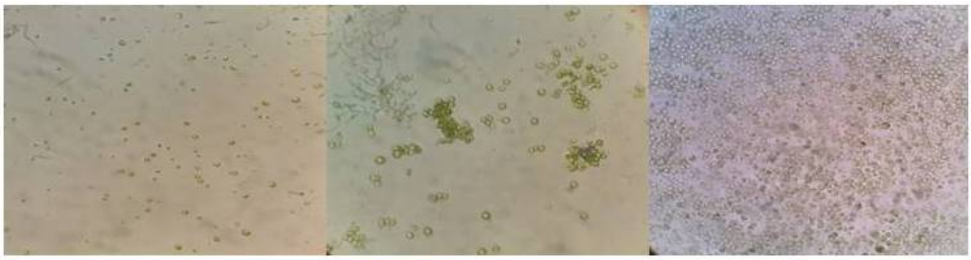

Fig. 6 Microscopic Image of *C. marina* (i) Before addition of chitosan, (ii) After 15 minutes of chitosan addition and (iii) After 30 minutes of chitosan addition

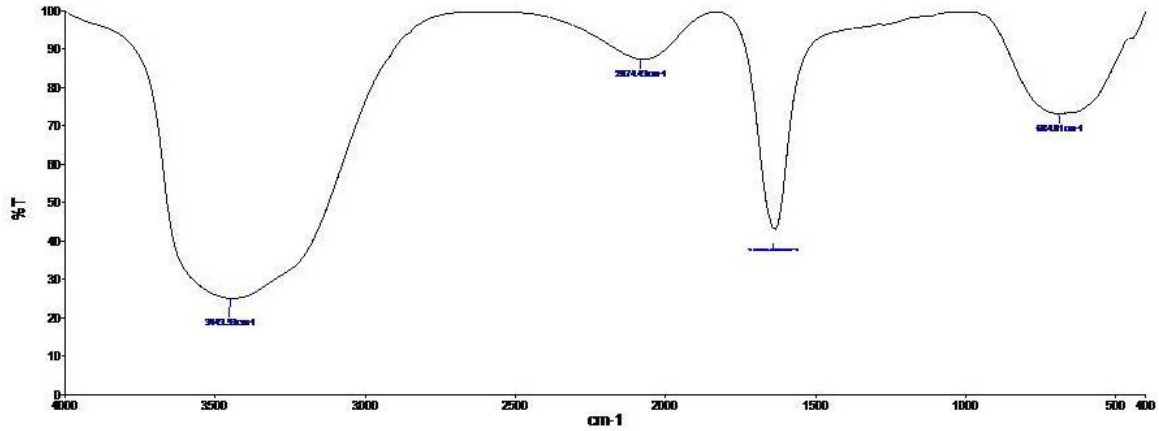

Fig. 7 FTIR analysis of chitosan prepared from carapace of *Scylla serrata*

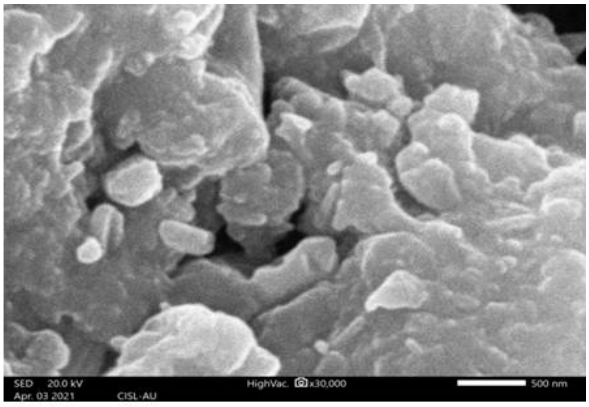

Fig. 8 SEM image of chitosan

CONCLUSION

The flocculation process is caused by inorganic or polymer - based flocculants, depending on their composition. These flocculants, despite their wide range of applications, have some limitations in terms of their use. For example, inorganic flocculants such as aluminium and ferric chloride is adverse and causes a lot of sludge. Consequently, Biopolymers are inexpensive, efficient, and eco-friendly. Generally chose chitosan for its notable molecular weight and excessive charge density. The NH3+ and NH2+ are positively charged aminogroups presented in chitosan that have bind to negatively charged microorganisms, including microalgae. The physiochemical characteristics and dosage of chitosan make influence on the harvesting efficiency.

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ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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