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Sustainable Development of Antimicrobial Polyvinyl Chloride Bioplastics Using *Chlamydomonas reinhardtii* Extract

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Abstract

The green microalga *Chlamydomonas reinhardtii* was utilized for bio-based plastic production, focusing on optimizing polyhydroxybutyrate (PHB) synthesis. Optimal cultivation conditions (40 °C, pH 8.8, light intensity 1100 lux, salinity 0.15 M) yielded 102.84 ± 0.5 mg/g of PHB, while phosphorus-free media with 2 g/L sodium acetate achieved a maximum of 201 ± 0.5 mg/g. Bioplastic films were developed by blending polyvinyl chloride (PVC) with *C. reinhardtii* extracts (0-75%). The 50% algal extract/PVC blend exhibited the best mechanical properties, with a tensile stress of 9.41 MPa and 2% elongation. Chloroform extracts of *C. reinhardtii* showed significant antimicrobial activity against bacteria and fungi, with the highest inhibition (31 ± 0.28 mm) against *Staphylococcus aureus*. This study highlights the potential of *C. reinhardtii*-based biodegradable antimicrobial bioplastics as eco-friendly alternatives to conventional plastics.

Keywords: Bioplastic films; Polyhydroxybutyrate (PHB); *Chlamydomonas reinhardtii*; antimicrobial activity.

1. Introduction

Nowadays, plastic is a necessary part of our contemporary lifestyle. At the beginning of the twenty-first century, the average person used 15 kilogram of plastic each year. [1, 2]. The period from 1950 to 2020, global plastic production reached to 9500 million metric tons [3]. The amount of plastic produced worldwide has grown from 245 million metric tons in 2008 to around 359 million metric tons in 2018, and it is predicted to treble by 2050 [4]. The persistence of plastics in various environments has resulted in white pollution, characterized by the leakage and accumulation of plastic waste. The amount of petroleum-based material pollution increases, high risk to the environment, human health as well as marine ecosystem including ocean animals and coral reefs. Because they are more environmentally friendly than petroleum-based polymers, bioplastic made from renewable resources has become essential in recent years. [5,6].

The production of bioplastics often relies on terrestrial crops like potatoes, corn, wheat, soy proteins, milk proteins, gelatine, and collagen. This competition with food sources, coupled with the significant consumption of land, water, and nutrients, makes such bioplastic production unsustainable in the long term [7, 8]. This makes it important to synthesize new bio-based biodegradable plastic polymers. So, there is a rising demand for environmentally friendly polymers, particularly biodegradable plastics, in a wide range of applications including packaging, agriculture, healthcare, and industry. Microalgae are garnering significant attention as a potential future feedstock. Microalgae that can thrive on waste resources and accumulate high levels of lipids offer a promising source of biomass for bioplastic production, as they do not compete with food sources [9, 10]. Rosero-Chasoy [11] concluded that the microalgae biorefinery model can contribute to a more circular bioeconomy, given the rapid growth and renewable nature of microalgae and their potential to produce various bio-based products.

The algal biomass contains a potential bioactive compound such as lipids, protein, carbohydrate, polyhydroxyalkanoate (PHA), fatty acids, starch and cellulose that can be to develop biodegradable plastics [12, 13]. Among these polymers, PHA, is a highly recommended bioplastic due to its enzymatic biodegradability, similar properties to conventional plastics, and resource efficiency. This biodegradable approach offers significant advantages, including energy savings, reduced food waste, and

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lower carbon dioxide emissions [14]**.** Polyhydroxybutyrate (PHB) is a member of the PHA family of microbial polyesters that are made by bioprocessing. Microalgae must manufacture PHAs and lipids to survive in unfavourable environments, such as nutritional limitation or environmental stress, hence they synthesize PHAs from a variety of microalgae species [15].

Chlamydomonas reinhardtii, a single-celled green alga, is a widely recognized model organism for studying various biological processes, including biogenesis and genetics [16]. C. reinhardtii has also emerged as a potential platform for sustainable bioplastic production. Chaogang et al. [17] demonstrated the accumulation of polyhydroxybutyrate (PHB) in a specific strain of this alga, highlighting its potential for producing biodegradable plastics.

Bioplastics can be combined with synthetic polymers to enhance their durability and physical properties [7]. Polyvinyl chloride (PVC) is a versatile thermoplastic polymer used in various applications. Produced by polymerizing vinyl chloride monomer, PVC can be processed into both rigid and flexible materials. Plasticizers can be added to increase its flexibility [18]. PVC's adaptability, combined with its proven safety profile, biocompatibility, and chemical resistance, makes it a valuable material in healthcare settings. Its ability to be processed into both rigid and flexible forms allows for a wide range of applications, from medical devices to pharmaceutical packaging. PVC's biocompatibility ensures minimal risk of adverse reactions when in contact with tissues or bodily fluids.

Composites with promising uses in biomedical domains like tissue engineering, wound dressing, drug administration, and biosensors can be produced by successfully and appropriately combining PVC with biopolymers and carbon-based materials [19]. This study set out to investigate the possible uses of PVC in combination with different concentrations of *Chlamydomonas reinhardtii* chloroform extract in order to create biodegradable bioplastic sheets that might be used for a variety of purposes.

2. Materials and Methods

2.1. Algal strains and growth conditions.

Chlamydomonas reinhardtii was obtained from culture collection of Hydrobiology Lab, Water Pollution Research Department, National Research Centre. BG11 medium Allen [20] was used for the culture of this strain. The growth of algal biomass was determined by optical density according to Adhikary [21].

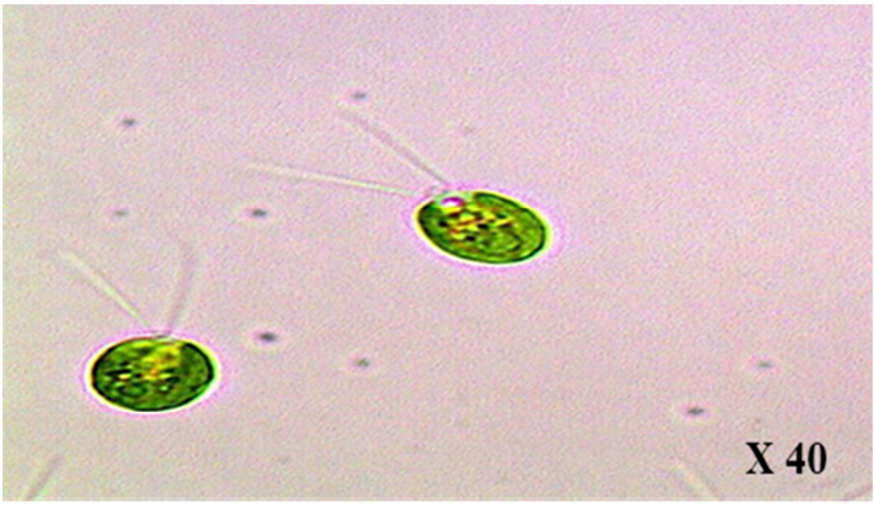

Plate 1: ***Chlamydomonas reinhardtii***

2.2. Application of Response Surface Methodology and Central Composite Design for Optimizing Algal Growth and PHB Production

To maximize *Chlamydomonas reinhardtii* cultivation conditions, Response Surface Methodology (RSM) with a Central Composite Design (CCD) was used. Four critical factors such as temperature, pH, light intensity, and salinity were analysed for their effects on algal growth and PHB production. A full factorial CCD, incorporating multiple levels for each factor, was used to construct a quadratic polynomial model, enabling the prediction of optimal growth conditions. The experimental data were analysed using Minitab® version 18, generating mathematical equations and graphical optimization curves to visualize factor interactions and their impact on growth. Model validation was performed by comparing predicted and actual results from confirmatory experiments Myers *et al.* [22]. The linear, quadratic, and interaction terms were evaluated for statistical

significance using Analysis of Variance (ANOVA). This approach facilitated the precise identification of optimal growth conditions [22], providing a robust statistical framework for enhancing PHB yield.

2.2. *Design of experiments (DOE) models*

The statistical optimization involved enhancing algal growth processes using central composite design (CCD) and verifying the optimization curves' projected parameters. Low and high levels for each factor were determined from preliminary one-factor-at-a-time (OFAT) experiments**.** Thirty-one flasks, each containing 300 ml of algal culture inoculated with 100 ml of algal suspension, were exposed to five levels of temperature each was (12.5, 20, 27.5, 35, and 42.5 °C), pH (5, 6, 7, 8, and 9), light intensity (500, 2000, 3500, 5000, and 6500 lux), and salinity (0, 0.1, 0.2, 0.3, and 0.4 M). The flasks were aerated using plastic fish tank hoses connected to air pumps. The experiment ran for several days, with a 12-hours light/dark cycle, measuring optical density at the end to assess algal growth. Analysis of variance (ANOVA) was used to evaluate the statistical significance of linear, quadratic, and interaction terms.

2.2. Optimization Curves to Identify the Best Conditions for Algal Growth

Design of experiments (DOE) statistical analysis was conducted using the response optimizer tool in the Minitab® software package to generate optimization curves. These curves were employed to determine the optimal combination of process variables (temperature, pH, light intensity, and salinity) for maximizing algal growth. The individual desirability function (d), ranging from 0 to 1, was utilized to evaluate how effectively the predicted settings optimized the response. The optimization process was concluded with confirmatory experiments (n = 10 replicates) to validate the accuracy of the predicted optimal conditions derived from the optimization curves Myers *et al.* [22].

2.3. Quantification of Polyhydroxybutyrate (PHB) in Microalgal Strain

A sample (0.01 g) of commercial PHB is accurately weighed and dissolved in 10 ml of chloroform by heating in a water bath at 65 - 70 °C until the solution becomes clear, creating a 1 mg/ml PHB stock solution. The glass tube is sealed with a stopper during heating. From this stock solution, 1 ml is transferred into a fresh tube containing 9 ml of chloroform, resulting in a 100 µg/ml PHB solution. After that, the mixture is heated to 65–70 °C and vortexed to guarantee thorough mixing. After that, the tubes are sealed with glass stoppers and 10 milliliters of concentrated H_2SO4 are added. To guarantee that PHB is completely converted to crotonic acid, the tubes are next heated in a boiling water bath at 94–96 °C for 20 minutes. A silica cuvette is then filled with 1 ml of the resultant sample for spectrophotometric examination. The spectrophotometer is set to scan the sample between 190 and 800 nm, identifying a peak at 235 nm corresponding to crotonic acid. Concentrated H_2SO_4 is used as the blank (zero). The absorbance at 235 nm is recorded and plotted against the known PHB concentrations to construct a standard curve for quantification [23].

2.4. Extraction of PHB from algal biomass

A 0.01 gram of algal biomass was suspended in sterile water and homogenized then allowed for mixed thoroughly using a vortex. From the resulting, 2 ml of suspension, an equal volume of 2 N HCL was added, and the mixture was heated for 2 hours in a water bath. The sample was then centrifuged at 6000 rpm for 20 min. subsequently , 5 ml of chloroform was added to supernatant , and the mixture was incubated over night at , 28 °C on a shaker at 150 rpm, Afterward the solution was centrifuged at 2000 rpm for 20 minutes , and 1 ml of chloroform was extracted and dried at 40 °C, following the drying process, 5ml of sulfuric acid was added to the tube and the mixture was heated at 100 °C in a water bath for 20 min to convert PHB crystals into crotonic acid. The PHB content was quantified by measuring the absorbance at 235 nm using a UV-spectrophotometer with sulfuric acid blank serving as the reference. The PHB content produced by alga was calculated based on the cell dry weight and the measured PHB content [23].

2.5. Enhancement of PHB Concentration in Selected Algal Species

The highest polyhydroxybutyrate concentration in *Chlamydomonas reinhardtii* under the best growth conditions from statistic data obtained will be used to enhance PHB productivity. PHB production was further enhanced by five concentrations of sodium acetate (0.0, 0.5, 1, 1.5, and 2 g/L) were tested under four different media conditions: normal media, nitrogen-free media, phosphorus-free media, and media lacking both nitrogen and phosphorus. The cells were initially grown under normal photoautotrophic conditions for 16 days to reach maximum biomass production. Subsequently, the cells were exposed to different acetate concentrations, and PHB content was measured in mg/g.

2.6. Bioplastic Production

Bioplastic films were prepared by cultivating *Chlamydomonas reinhardtii* on BG11 medium under heterotrophic conditions, specifically with phosphorus-free media and 2 g/L acetate concentration. After cultivation, the algal biomass was harvested and dried.

2.7. Microalgal Biomass Extraction

One gram of algal biomass was suspended in sterile water, homogenized, and vortexed. To 2 ml of this suspension, 2 ml of 2 N HCl was added, and the mixture was heated in a water bath for 2 hours. The suspension was then centrifuged at 6000 rpm for 20 minutes. Next, 5 ml of chloroform was added, and the mixture was shaken overnight at 28°C at 150 rpm. After centrifuging at 2000 rpm for 20 minutes, the chloroform extract was collected and dried at 40°C. To the dried residue, 5 ml of concentrated sulfuric acid was added, and the mixture was heated at 100°C for 20 minutes to convert PHB crystals to crotonic acid. PHB

content was measured at 235 nm using a UV spectrophotometer, with sulfuric acid as the blank. The percentage of PHB was calculated based on cell dry weight and PHB content [24].

2.8. Preparation of Bioplastic Films

Blends of algae extract and polyvinyl chloride (PVC) were made by dissolving two grams of PVC in one hundred milliliters of tetrahydrofuran (THF) and algal extract in one hundred milliliters of chloroform, then combining the two. To make the algal extract/PVC blends, several amounts of algal extract (25%, 50%, and 75%) were added to the PVC solution. The films were labeled as 25%, 50%, and 75% algal extract/PVC films and were made via the casting method. **Table 1** detailed the chemical makeup of the PVC and algae extract films.

Table (1): Analysis of the Chemical Composition of PVC Films Enhanced with Algal Extract (AE).

Sample code	PVC %	Algal extract weight g	Algal extract %
PVC	2	0	0
PVC/AE 25%	2	0.25	25%
PVC/AE 50%	2	0.5	50%
PVC/AE 75%	2	0.75	75%

2.9. Characterizations of bioplastic films**.**

2.9.1. Mechanical properties of the prepared bioplastic films

In compliance with ASTM Standard D638-91 [25], the mechanical properties of the produced bioplastic sheets were assessed using kN5, a universal testing apparatus. The machine was fixed with a 5 K N load cell and ran at a rate of 10 mm/min

2.9.2. Scanning electron microscopy (SEM)

A scanning electron microscope (JSM 6360LV, JEOL/Noran) running at a voltage of 10-15 kV was used to analyze the morphology of the produced bioplastic films. Before analysis, the bioplastic samples were produced, placed as far away from the target as feasible to avoid damage, and sputter coated with a thin layer of gold at a low deposition rate [23,24].

2.9.3. Fourier transforms infrared (FTIR) spectroscopy

Bioplastic films with varying percentages of algal extract (25%, 50%, and 75%) had their FT-IR spectra recorded on a Shimadzu 8400S in the 500–4000 cm^{-1} range [24].

2.9.4 Biodegradability study

The biodegradability test was performed in a natural environment keeping the synthesized bioplastic films under the soil for 7, 15, and 30 days. The mass of each sample was measured prior to the experiment and measured again at the end of the testing period to determine the weight loss that indicates the biodegradability of the samples [26]. The following formula was used to assess the samples' weight loss:

$$\text{biodegradability}(\%) = \frac{W1-w2}{W1} \times 100$$

Where, W1 = Initial mass weight of the bioplastic sample.

W2 = Final mass weight of the bioplastic sample.

2.9.5 Antimicrobial activity

The antimicrobial activity of the chloroform extract of *Chlamydomonas reinhardtii* was evaluated using 75 % algal extract concentration in bioplastic film. The extract was tested against two Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), two Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and the well diffusion method for one fungus (Candida albicans). The spread plate method was used to inoculate nutrient agar plates with microbial cultures (105–106 CFU/mL of individual bacteria). Three 5 mm-diameter wells were made in each plate, and 20 µL of 75% of the extract, the common antibiotic (ciprofloxacin), and the control (chloroform) were added to each well. After that, the plates were incubated for twenty-four hours at 37°C. The zones of inhibition around the wells were measured and expressed as the diameter of the clear zones. The experiment was conducted in triplicate, and the average values were calculated [27, 24].

3. Results and Discussion

3.1. The Central Composite Design (CCD)

the central composite design successfully optimized the growth conditions of Chlamydomonas reinhardtii for maximum biomass production. Statistical analysis of the 31 experimental runs revealed the significant impact and interactions of temperature, pH, light intensity, and salinity on optical density. Results in Table (2) presents the experimental design matrix and the corresponding OD values for different factor combinations. Additionally, the ANOVA results (Table 3) revealed that the used factors significantly affected OD values, with p-values < 0.05 and a strong correlation coefficient (R^2).

Evaluating the growth conditions of *Chlamydomonas reinhardtii* (Table 2) .The most promising conditions for maximizing Chlamydomonas reinhardtii biomass production were identified as 20°C, pH 8, 5000 lux light intensity, and 0.1 M salinity (0.70 OD). Good growth was also observed at 27.5°C, pH 7, 3500 lux, and 0 M salinity (0.53 OD), and promising growth at 35°C, pH 8, 2000 lux, and 0.1 M salinity (0.47 OD). Overall, *Chlamydomonas reinhardtii* thrives under moderate to high light intensity, low salinity, a temperature range of 20-35°C, and a pH range of 7-8, these results were agreed with Singh and Singh [28] showed that optimum temperature range 20OC to 30OC was observed for growth of different algae species. Kornaros and Sakarika [29] found optimal heterotrophic growth of *C. vulgaris* at pH 7.5-8.0 (range 5.0-8.0). Bialevich *et a*l. [30] observed that higher light intensity increased maximum cell size and daughter cell division in *C. reinhardtii, D. quadricauda*, and *P. kessleri*. Studies [31, 32] indicate that low salinity during growth maximizes biomass in freshwater microalgae.

Table (2): Design matrix of 25 full factorial CCD and results of *C. reinhardtii*; OD (nm) in response to all combinations of low and high levels of interacted factors

Design points				Factors				Response and error estimation*		
Std Order	Run Order	Pt Type	Blocks	Temp.	pH	LI	Salinity	OD	Fits	Residual
1	1	1	1	20.0	6	2000	0.1	0.10	0.108812	-0.0088121
2	2	1	1	35.0	6	2000	0.1	0.22	0.169645	0.0503546
3	3	1	1	20.0	8	2000	0.1	0.39	0.397145	-0.0071454
4	4	1	1	35.0	8	2000	0.1	0.47	0.457979	0.0120213
5	5	1	1	20.0	6	5000	0.1	0.27	0.327979	-0.0579787
6	6	1	1	35.0	6	5000	0.1	0.17	0.153812	0.0161879
7	7	1	1	20.0	8	5000	0.1	0.70	0.616312	0.0836879
8	8	1	1	35.0	8	5000	0.1	0.43	0.442145	-0.0121454
9	9	1	1	20.0	6	2000	0.3	0.17	0.188812	-0.0188121
10	10	1	1	35.0	6	2000	0.3	0.20	0.249645	-0.0496454
11	11	1	1	20.0	8	2000	0.3	0.26	0.177145	0.0828546
12	12	1	1	35.0	8	2000	0.3	0.29	0.237979	0.0520213
13	13	1	1	20.0	6	5000	0.3	0.46	0.407979	0.0520213
14	14	1	1	35.0	6	5000	0.3	0.27	0.233812	0.0361879
15	15	1	1	20.0	8	5000	0.3	0.35	0.396312	-0.0463121
16	16	1	1	35.0	8	5000	0.3	0.23	0.222145	0.0078546
17	17	-1	1	12.5	7	3500	0.2	0.24	0.255709	-0.0157092
18	18	-1	1	42.5	7	3500	0.2	0.11	0.142376	-0.0323759
19	19	-1	1	27.5	5	3500	0.2	0.25	0.231454	0.0185461
20	20	-1	1	27.5	9	3500	0.2	0.45	0.508121	-0.0581206
21	21	-1	1	27.5	7	500	0.2	0.02	0.052376	-0.0323759
22	22	-1	1	27.5	7	6500	0.2	0.24	0.255709	-0.0157092
23	23	-1	1	27.5	7	3500	0.0	0.53	0.544043	-0.0140426
24	24	-1	1	27.5	7	3500	0.4	0.37	0.404043	-0.0340426
25	25	0	1	27.5	7	3500	0.2	0.38	0.369787	0.0102128
26	26	0	1	27.5	7	3500	0.2	0.37	0.369787	0.0002128
27	27	0	1	27.5	7	3500	0.2	0.38	0.369787	0.0102128
28	28	0	1	27.5	7	3500	0.2	0.34	0.369787	-0.0297872
29	29	0	1	27.5	7	3500	0.2	0.37	0.369787	0.0002128
30	30	0	1	27.5	7	3500	0.2	0.35	0.369787	-0.0197872
31	31	0	1	27.5	7	3500	0.2	0.39	0.369787	0.0202128

*OD (nm) = the response; Fits = are the value of point estimates of the mean optical density for given values of the factors; Residuals = are the difference between the observed value and its corresponding fitted value.

3.1.1. The main (linear) and square (non-linear) effects

The results (Table 3, and Fig. 1) demonstrated a significant variation in optical density based on temperature and pH levels. Specifically, higher pH and light intensity led to increased optical density, while higher temperature and salinity resulted in a decrease in optical density.

Table (3): **Results of ANOVA model to test for differences in *C. reinhardtii* OD (nm) in response to different factors**.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	0.531015	0.059002	29.67	0.000
Linear	4	0.225500	0.056375	28.35	0.000
Temp.	1	0.019267	0.019267	9.69	0.005
pH	1	0.114817	0.114817	57.74	0.000
LI	1	0.062017	0.062017	31.19	0.000
Salinity	1	0.029400	0.029400	14.78	0.001
Square	3	0.160290	0.053430	26.87	0.000
Temp.*Temp.	1	0.052701	0.052701	26.50	0.000
LI*LI	1	0.084140	0.084140	42.31	0.000
Salinity*Salinity	1	0.019648	0.019648	9.88	0.005
2-Way Interaction	2	0.145225	0.072612	36.52	0.000
Temp.*LI	1	0.055225	0.055225	27.77	0.000
pH*Salinity	1	0.090000	0.090000	45.26	0.000
Error	21	0.041760	0.001989	-	-
Lack-of-Fit	15	0.039874	0.002658	8.46	0.007
Pure Error	6	0.001886	0.000314	-	-

Significant differences are denoted in bold (R^2 = 92.71%), p-values < 0.05.

3.1.2. The interaction effects

When a factor's response changes from low to high levels based on the levels of another factor, the interaction is effective. The ANOVA model was also used to examine the effects of the interaction. The optical density of *C. reinhardtii* (Table 3, Fig. 1).

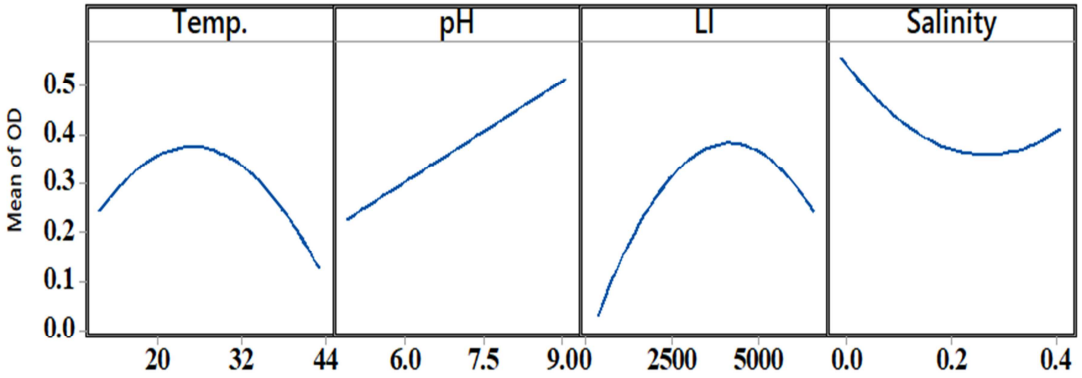

Fig. (1): Main effects plots explain the changes in *C. reinhardtii* OD between low and high levels of each factor

The Pareto chart and Normal probability plots.

The Pareto chart (Fig. 2A) determined the relative importance of main, squared, and interaction effects using a t-test. Effects with t-values above 2.080 significantly impacted optical density.

Normal probability plots illustrate the direction of an effect on the response. Points near the fitted line represent insignificant factors, while points far from it indicate significant factors, with the direction (positive or negative) shown by the point's position relative to the line. In a positive effect, a rise in the levels results in a rise in the response, and in a negative effect, the opposite is true., as shown in Fig. (2B).

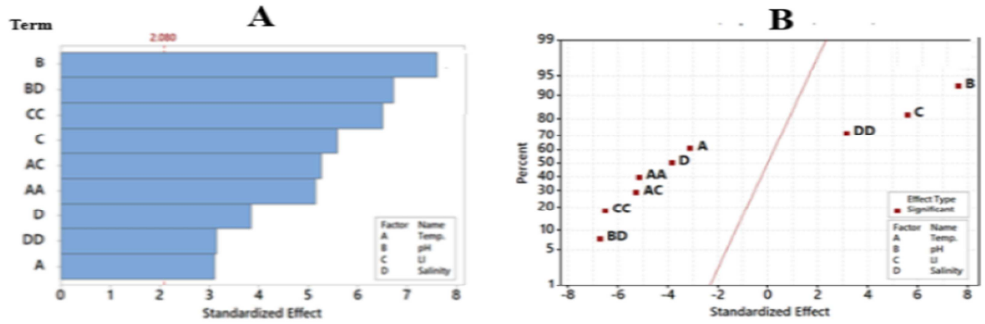

Fig. (2): Pareto chart (A) and Normal probability plot (B) of standardized effects on *C. reinhardtii* OD (nm)

Based on the Pareto chart and normal probability plot, the key factors affecting the optical density (OD) of *Chlamydomonas reinhardtii* were analyzed. The analysis reveals that pH (B) had the most significant impact on optical density (OD), with a p-value of 0.000, confirmed by the Pareto chart. Light intensity (LI) (C) also showed a strong effect with a p-value of 0.000, where higher light intensity increased OD. Salinity (D) was significant (p-value = 0.001), and its squared effect (DD) also influenced OD, showing a negative effect in some cases. Temperature (Temp.) had a moderate impact (p-value = 0.005), with a significant interaction with light intensity (Temp × LI, p-value = 0.000), showing a negative effect on OD. Lastly, the interaction between pH and Salinity (BD) was significant (p-value = 0.000), emphasizing the dependency of OD on both factors. In conclusion, pH, temperature, light intensity, and salinity are key factors influencing the OD of *C. reinhardtii*, with significant main, square, and interaction effects. Precise control of these variables is crucial for optimizing algae growth. These results align with previous studies that highlight the crucial role of these environmental factors in microalgal cultivation [33, 34, 35].

3.1.3. The optimization curves

The response optimizer tool was used to generate the optimization curves (Figs. 3, 4, 5) to determine the final optimal settings of the factors that showed the three cases of growth (maximum, medium and minimum) of C. *reinhardtii* according to the value of optical density as the way of growth determination.

3.1.3.1. Maximum Growth

- Optimal Settings:
  - Temperature: 19.4 ^{O}C
  - pH: 9
  - Light Intensity (LI): 5106 Lux
  - Salinity: 0 M
- Predicted Outcome:

The optical density (OD) increases to 1.02 absorbance units (AU), achieving individual desirability of 100% as in fig. (3)

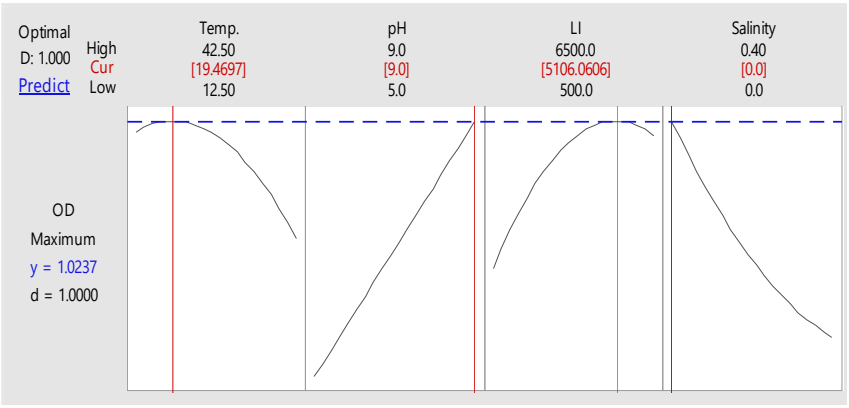

Fig. (3): The optimization curves of *C. reinhardtii* show how the factors affect the predicted responses (y) including maximum OD, the optimum factor settings (Cur) were predicted with composite desirability (D) = 1.0 (100%)

The results showed that to achieve maximum growth at a composite desirability (D) = 1.0 (100%) was achieved by increasing light intensity and pH, decreasing temperature, and eliminating salinity. This aligns with previous research Singh and Singh [28] found optimal algal growth between 20-30^{O}C; Liu *et al*. [36] reported pH 7-9. suitable for most algae, including *Chlamydomonas* sp.; Bialevich *et al*. [37] linked increased light intensity to larger cell size and more daughter cells; and Borowitzka *et al*. [32] found low salinity maximized biomass in freshwater microalgae.

3.1.3.2. Medium Growth

- Optimal Settings:
  - Temperature: 40.4 ^{O}C
  - pH: 8.7
  - Light Intensity (LI): 1136.9 Lux
  - Salinity: 0.15 M
- Predicted Outcome:

The optical density (OD) reaches a medium value of 0.34 AU, achieving individual desirability of 99% as in fig. (4).

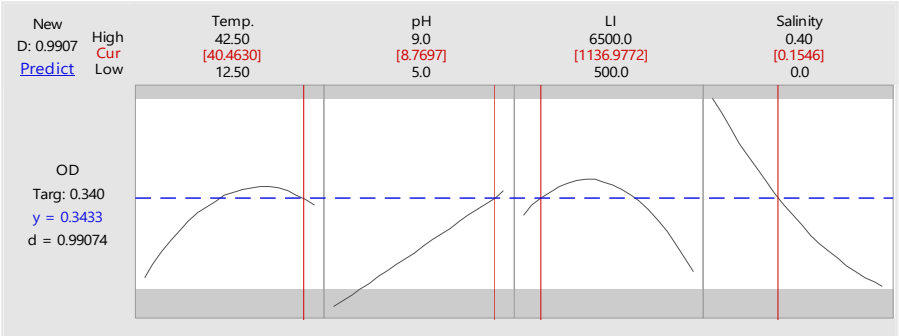

Fig. (4): The optimization curves of *C. reinhardtii* show how the factors affect the predicted responses (y) including giving medium value of OD, the optimum factor settings (Cur) were predicted with composite desirability (D) = 0.99 (99%).

The results showed that to achieve medium growth at a composite desirability (D) = 1.0 (100%) occurred with increased temperature (40^{O}C) and pH (8.7), but decreased light intensity and salinity. This reduced growth compared to optimal conditions may be due to the combined influence of light and temperature on photosynthesis [38], consistent with Machida *et al*. [39] who reported synergistic negative effects of high temperature and low light on macroalgae.

3.1.3.3. Minimum Growth

- Optimal Settings:
  - Temperature: 12.5 ^{O}C
  - pH: 5
  - Light Intensity (LI): 500 Lux
  - Salinity: 0 M

Predicted Outcome:

The optical density (OD) decreases to 0 AU, achieving individual desirability of 100% as shown in Fig(5).

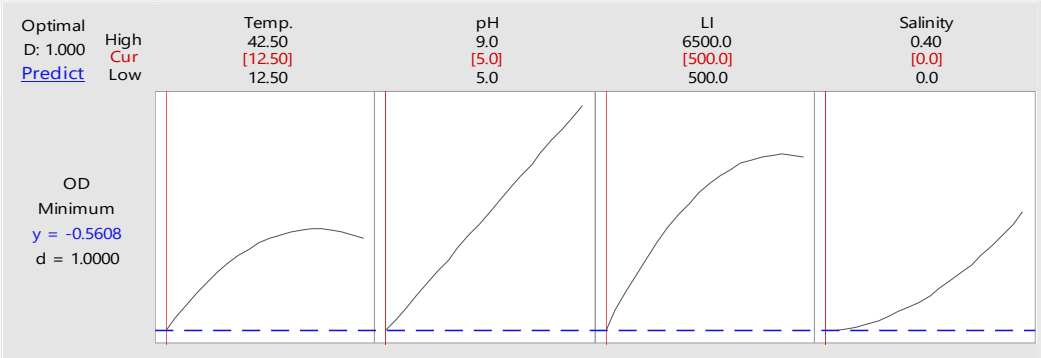

Fig. (5): The optimization curves of *C. reinhardtii* show how the factors affect the predicted responses (y) including minimize the OD, the optimum factor settings (Cur) were predicted with composite desirability (D) = 0.99 (99%).

The results showed the negative impact of these optimal settings on OD, achieving the minimum growth with a composite desirability (D) = 0.99 (99%). Lowering temperature, pH, light intensity, and salinity negatively impacts algal growth (OD). This is due to the synergistic effects of these interconnected environmental factors on vital biological processes. Specifically, Renaud *et al.* [40] demonstrated temperature's influence on metabolic rates, enzyme activity, and membrane fluidity, with lower temperatures reducing growth. Cai *et al.* [41] showed that extreme pH whereas low or high inhibits algal growth and can cause cell death. Richardson *et al*. [42] established that insufficient light limits photosynthesis and, consequently, growth.

3.2. Detection of Polyhydroxybutyrate concentration

This study highlights the relationship between algal growth conditions and the production of PHB, a biodegradable plastic with potential as a sustainable alternative to traditional plastics. The results (Table 4) show significant differences in PHB production under maximum and medium growth conditions for *Chlamydomonas reinhardtii*.

The results shown that Polyhydroxybutyrate concentration under growth conditions can be summarized as following:

- Maximum Growth:

The results showed that the concentration of PHB averaged 58.24 ± 0.59 mg/g, with individual values ranging from 57.61 mg/g to 58.76 mg/g. Which, the optimal growth supports biomass production but not necessarily the accumulation of PHAs. In this state, microalgae prioritize cellular division and growth over the synthesis of storage compounds.

- Medium Growth:

On the other hand, the concentration of PHB was significantly higher, averaging 102.84 ± 0.5 mg/g, with values ranging from 102.32 mg/g to 103.37 mg/g indicates that moderate stress optimizes PHB yield. Meanwhile, stressful but non-lethal conditions favour the metabolic shift towards PHB production.

- Minimum Growth:

Algal strains could not survive under minimum growth conditions, and no PHB production was detected.

Table (4): Results of Polyhydroxybutyrate concentration under maximum and medium growth conditions for *Chlamydomonas reinhardtii*

Algal species	Growth Condition	Absorbance	Concentration of PHB (µg/ml)	Concentration of PHB (mg/g)	Mean Concentration of PHB (mg/g)
C. reinhardtii	Maximum growth	1.1227	576.15	57.61	58.24 ± 0.59
		1.2552	583.89	58.38	
		1.2611	587.00	58.76	
	Medium growth	2.0991	1023.26	102.32	102.84 ± 0.5
		2.1103	1033.78	103.37	
		2.1032	1033.78	102.85	
		0.9982	555.30	55.53	
		1.1895	549.31	54.93	

The results showed that medium growth conditions, which often involve some degree of stress (e.g., nutrient limitations or suboptimal light intensity), promote higher PHB production compared to optimal (maximum) growth conditions. This aligns with previous findings by Perez-Rivero *et al*. [16] which suggest that microalgae synthesize polyhydroxyalkanoates (PHAs), including PHB, as a response to stress. This is because of under stress, microalgae channel their metabolic activities towards survival strategies, including the accumulation of energy-rich storage compounds like PHAs. This phenomenon can be attributed to limited nitrogen availability or excess carbon, which diverts resources away from growth and towards PHA synthesis [43] PHB is created in microorganisms' cells as a byproduct of secondary metabolism Rao *et al* [44] This typically occurs when the cells are under nutrient stress or in an unfavorable environment, such as one with high carbon levels and few nutrients [45].

The industrial implications findings suggest that industrial production of PHB can benefit from deliberately inducing stress conditions rather than maximizing algal biomass. Strategies such as nutrient limitation, controlled light intensity, and precise temperature settings could enhance PHB yields while maintaining economic viability. The productivity of PHA production directly impacts the final cost. Higher PHA productivity leads to lower production costs. Therefore, to reduce the overall cost of PHA biopolymer production, it is essential to develop production methods that yield high quantities of PHA with enhanced efficiency Kumar *et al*. [46]

Significance for Sustainability the study reinforces the potential of microalgae as a sustainable source for bioplastics. By optimizing growth conditions to maximize PHB production, industries can reduce the reliance on fossil-based plastics, contributing to environmental conservation and waste reduction [47].

3.3. Enhancement of Polyhydroxybutyrate concentration.

The results of the enhancement of Polyhydroxybutyrate (PHB) concentration for *Chlamydomonas reinhardtii* is shown in Table (5), Figure (6).

Plate 2: Biomass production after optimization of PHB in *Chlamydomonas reinhardtii*

Table (5): The PHB content of *Chlamydomonas reinhardtii* under different nutrient condition

Nutrient Condition	Acetate concentration (g/L)	OD	Concentration of PHB (µg/ml)	Concentration of PHB (mg/g)	Mean Concentration of PHB (mg/g)
Normal	0	2.09	1023.26	102.32	102.48±0.5
		2.11	1033.78	103.37	
		2.11	1033.78	102.85	
	0.5	2.68	1333.78	133.37	149.68±0.9
		2.69	1349.57	134.95	
		2.71	1339.05	133.90	
	1	2.81	1402.21	140.22	155.83±0.6
		2.80	1396.94	139.69	
		2.76	1386.42	138.64	
	1.5	3.11	1560.10	140.22	155.83±0.6
		3.15	1581.15	139.69	
		3.13	1570.63	138.64	
	2	3.31	1665.36	156.01	161.62±0.3
		3.31	1665.36	158.11	
		3.13	1660.10	157.06	
- N	0	3.11	1544.31	154.43	155.48±0.9
		3.08	1560.10	156.01	
		3.11	1644.31	164.43	
	0.5	3.27	1623.26	162.32	163.37±1
		3.23	1633.78	163.37	
		3.25	1665.36	166.53	
	1	3.31	1649.57	164.95	166±1.61
		3.28	1681.15	168.11	
		3.34	1802.21	180.22	
	1.5	3.57	1791.68	179.16	179.34±0.8
		3.55	1786.42	178.64	
		3.54	1870.63	187.06	
	2	3.70	1865.36	186.53	187.07±0.5
		3.69	1875.89	154.43	
		3.71	1544.31	156.01	
-P	0	3.41	1718.00	171.80	163.11±0.3
		3.40	1712.73	171.27	
		3.38	1702.21	170.22	
	0.5	3.57	1802.21	180.22	171.09±0.8
		3.54	1786.42	178.64	
		3.58	1807.00	180.70	
	1	3.61	1823.26	182.32	179.34±1
		3.61	1823.26	182.32	
		3.60	1818.00	181.80	
	1.5	3.99	2023.26	202.32	182.14±1
		3.97	2012.73	201.27	
		3.98	2018.00	201.80	
	2	3.41	1718.00	171.80	201±0.5
		3.40	1712.73	171.27	
		3.38	1702.21	170.22	
-N ,- P	0	2.79	1391.68	139.16	139.68±0.5
		2.80	1396.68	139.66	
		2.81	1402.21	140.22	
	0.5	2.98	1491.68	149.16	149.68±0.9
		2.98	1491.68	149.16	
		3.01	1507.40	150.74	
	1	3.12	1565.36	156.53	155.83±0.6
		3.10	1554.84	155.48	
		3.10	1554.84	155.48	
	1.5	3.22	1618.00	161.80	161.62±0.3
		2.79	1391.68	139.16	
		2.80	1396.68	139.66	
	2	3.22	1718.00	171.80	171.44±1
		3.21	1702.21	170.22	
		3.41	1723.26	172.32	

The cells of *C. reinhardtii* was cultured in normal nutrient (Normal), nitrogen limiting media (-N), phosphorus limiting media (-P), nitrogen and phosphorus limiting media (-N, -P) , with the addition of various acetate concentration (0, 0.5, 1, 1.5, 2 g/L) . Data are the average ±1 SD from three independent culture.

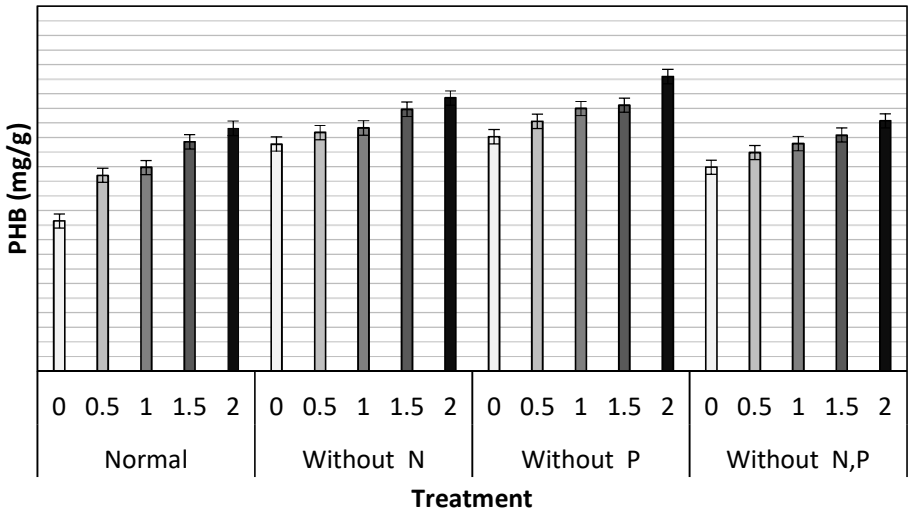

Fig. (6): Polyhydroxybutyrate concentration of *Chlamydomonas reinhardtii* under different nutrient condition

The results indicated that the cells cultured in media with the absence of N ; P alone and (N, P) together recorded increase in the polyhydroxybutyrate concentration than that recorded in the normal media, on the other hand the increase in the acetate concentration causing increase in the PHB concentration in all different treatments, the highest concentration of PHB content was found in the cells that cultured in media without P, and at 2 g/L of acetate concentration with value (201±0.5 mg/g).

The addition of sodium acetate to the growth medium of the selected algal strain was found to positively influence polyhydroxybutyrate production. This finding is supported by previous research by Carr [48] who measured PHB production in *Chlorogloea fritschii* using chemical analysis in the presence of sodium acetate and found PHB production was increased. Also, together De Philippis *et al.* [49] found that addition of sodium acetate to the culture medium increased the PHB content in the algal cells to 3% of their dry weight.

This study found that algal cells grown in media lacking nitrogen or phosphorus produced higher concentrations of polyhydroxybutyrate compared to those grown in normal media. These results align with previous research by Deschoenmaeker *et al*. [50], which reported a fourfold increase in PHB concentration under nitrogen-limited conditions. Additionally, *Arthrospira platensis* grown under phosphate-limited conditions reached a PHB content of 3.5% of cell dry weight [48]. Physiological stresses, such as nutrient limitations (particularly phosphorus or nitrogen), can redirect cellular metabolism towards the accumulation of polyhydroxyalkanoates in photosynthetically growing organisms. This phenomenon has been observed in various studies [51, 52, 53, 54] The highest PHB content was achieved in algal cells cultured in a media lacking phosphorus (P) and supplemented with 2 g/L of sodium acetate. This result aligns with the findings of Monshupanee *et al.* [55] who investigated the production of PHB by *Chlorogloea fritschii* under heterotrophic conditions. They observed that *C. fritschii* cells grown in a media deficient in phosphorus and nitrogen (-P or -N-P) exhibited a significant increase in both the precursor and enzyme activity involved in PHB production. Additionally, the highest PHB concentration was obtained in cells cultured in a media without P and supplemented with 4 g/L of acetate. Generally, nutrient limitation, is more favorable for PHB accumulation [56, 57].

3.3. Bioplastic production.

The coded algal extract/PVC films were prepared as mentioned before at (blank, 25%, 50%, and 75% concentration) as it appeared in Figure (7).

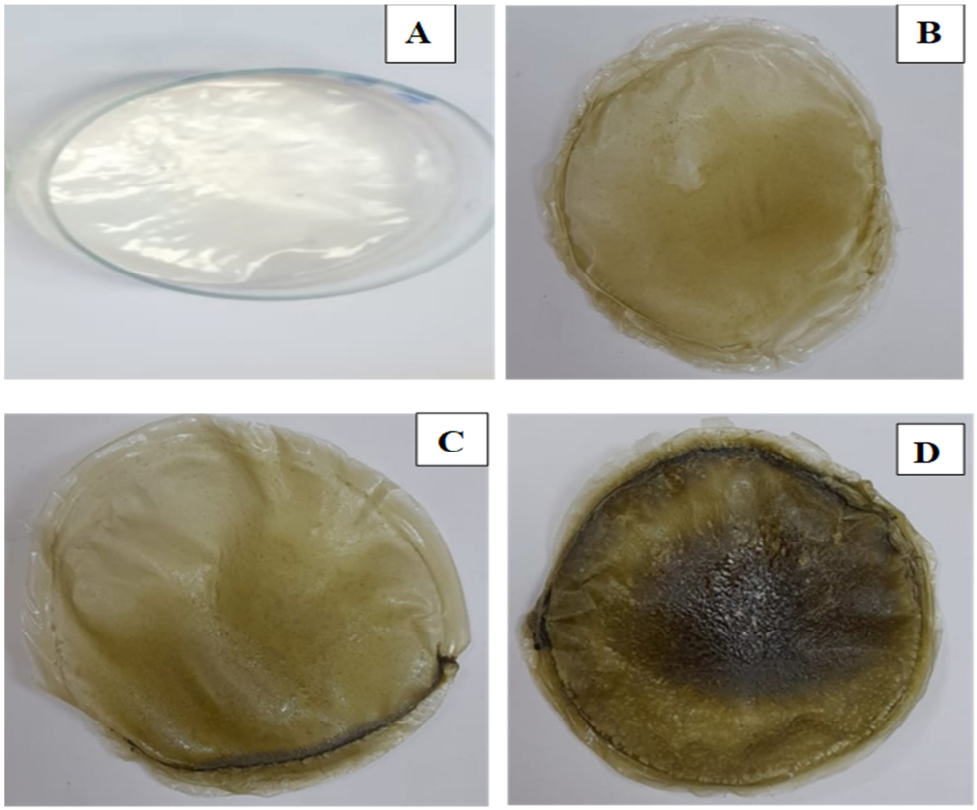

Fig. (7): The algal extract/PVC films: (A): Blank plastic film; (B): 25% algal extract/PVC; (C): 50% algal extract/PVC; (D): 75% algal extract/PVC

3.5. Mechanical properties

As indicated in Table (6), the mechanical characteristics of PVC and algal extracts/PVC films were determined by comparing their tensile strength and elongation at maximum load. The results showed that the best tensile stress detected in 50% algal extract/PVC was (9.41 Mpa) with elongation strength 2%. At the same time, the tensile stress detected for blank (7.29 Mpa) but it is not the one of the best results because the elongation stress is weak 0.74 %. Also, the two other algal extract/PVC films consider as a moderate results as shown in table (6). The results confirm the role of algal extract to enhance the mechanical properties and it is obvious that the algal bio plastic has good plasticizing capacity..

Table (6): Mechanical properties of bioplastic produced from different concentrations of microalgal biomass

Sample	Tensile strength MPa	Elongation at tensile strength %
Blank	7.29	0.74
25% algal/PVC	5.82	2.82
50% algal/PVC	9.41	2
75% algal/PVC	2	1.84

Polyvinyl chloride is a widely used plastic with applications in the automotive industry and electrical cable sheathing. PVC is known for its resistance to chemicals and solvents, as well as its flame-retardant properties due to the presence of chlorine in its molecular structure, and due to its vinyl content, that gives good tensile strength [58].

The morphological structure and chemical makeup of materials have an impact on their mechanical characteristics. According to Mathiot *et al*., [59] changes to PVC structure by the addition of algal extract were assessed in order to enhance PVC's mechanical qualities. The property of tensile strength is defined as the maximum stress a particular material can withstand when being stretched or pulled without breaking [60]. In addition, elongation is a mechanical property that absorbs energy by

plastic deformation concurrent to determine the maximum bending and shaping of the particular material [61]. In order to improve the strength of plastic material, both tensile strength and elongation parameters are needed.

3.6. SEM Characterization.

The bioplastic film surface microstructure micrographs are displayed in Fig. (8). The control film (PVC) surface characteristics revealed that the surface is covered in nano-porous material. The roughness and nanopores of bioplastic films containing algal extract were reduced by increasing the algal extract percentage especially at algal concentration 50% algal extract /PVC. As can be observed in Fig. (8). The accumulated algal extract produced a more uniform, smoother microstructure in films compared to the irregular, broken surface of polyvinyl chloride films. Increased microalgal extract content allows partial cell plasticization with sufficient extrusion shearing [62].

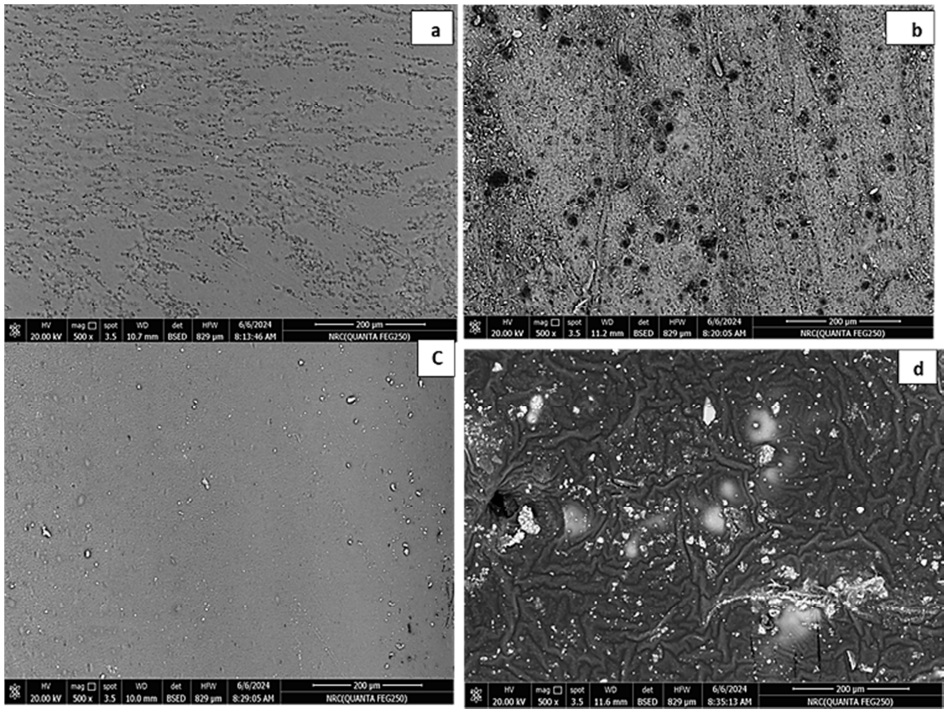

Fig. (8): SEM photograph of algal bioplastic (8000 X): a) control; b) 25% algal extract /PVC; c) 50% algal extract /PVC; d) 75% algal extract /PVC

3.7. FTIR spectroscopy of the prepared algal extract/PVC films

FTIR was used to analyze the chemical interaction between algal extract and PVC-containing films at various mass ratios of algal extract (25%, 50%, and 75%). The scan was conducted between 500 and 4000 cm-1. The FTIR spectrum of the extracted polymer with a different concentration were observed as seen in Fig. (9). Where, the peaks at 3669- 3289 cm^{-1}, 3694- 3399 cm^{-1}, 3644- 3241 cm^{-1} indicated strong H bond created by the terminal OH groups of alcohol. Similar results have been reported in other works [63, 64]. The peaks at 2923/2933 and cm^{-1} are corresponding to C–H stretching of alkene, methyl and methylene groups. These were agreed with results mentioned by Kumalaningsih *et al.* [65] (2925.81 cm^{-1}) and Anish *et al.* [66] (2932 cm^{-1}). The absorption bands of 1737 and 1722 cm^{-1} are corresponding to C=O bond, also the ester bond that consider the marker for PHB were located at series of peaks 1197, 1162, 1164, 1170 cm^{-1}. Thus, result was correlated with values obtained by Kumalaningsih *et al.* [65]. The peaks found at 1328, 1251, 1378 cm^{-1} were representing C-N of aromatic amine, other peaks (960- 541, 875- 543, ,966-534, 823-532, cm^{-1}) correspond to the presence of Alkyl halides the same results were found by Selvakumar *et al.* [67]. All these prominent absorption bands confirm that the polymer extracted was poly-β-hydroxybutyrate.

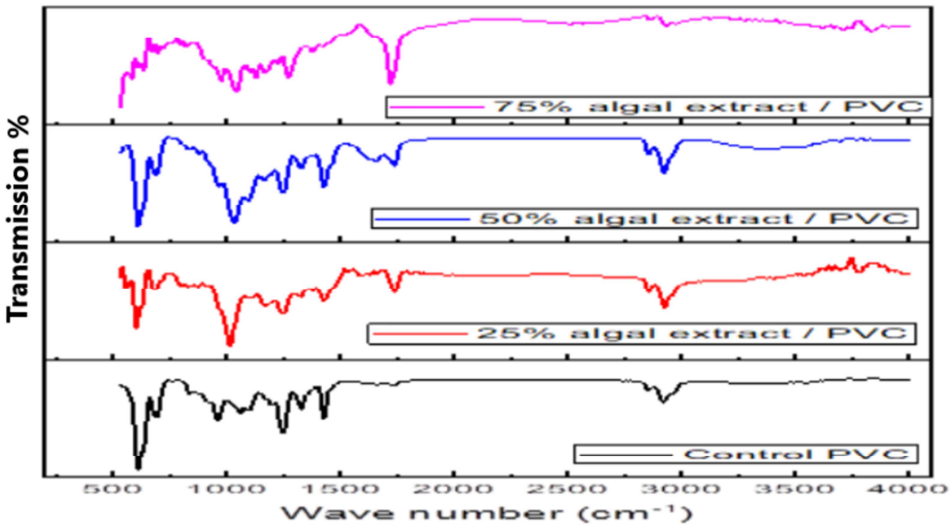

Fig. (9): The FTIR of PVC films as well as the algal extract/PVC films containing different ratios of algal extract

3.8. Antimicrobial activity

The antimicrobial activities of chloroform extract of *C. reinhardtii* at concentration, 50% was tested for antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and fungus (*Candida albicans*) using agar well diffusion. The extract exhibited activity against all tested microorganisms, with inhibition zones ranging from 18 to 31 mm. The highest activity was against *S. aureus* (31±0.28 mm), while the weakest was against *B. subtilis* (18±0.15 mm). Antifungal activity against *C. albican*s was also observed (25±0.11) Table (7) and Fig. (10).

Table (7): Diameters of inhibition zones (mm) of chloroform extract of *C. reinhardtii* at 50 % concentrations against the tested microorganisms by agar well diffusion method.

Treatments	Inhibition zone (mm) by agar well diffusion method				
	Gram-negative		Gram-positive		Fungi
	E. coli	*P. aeruginosa*	*S. aureus*	*B. subtilis*	*C. albicans*
Positive control	32±0.17	29±0.05	36±0.17	19±0.17	31±0.15
Negative control	0	0	0	0	0
50 % algal extract	29±0.15	27±0.25	31±0.28	18±0.15	25±0.11

Renukadevi *et al*. [68] reported that *Chlamydomonas* sp. have antibacterial activity in vitro against both Gram-positive and Gram- negative bacteria. Also, Kuda *et al*. [69] reported that a wide range of antifungal activities were obtained from extracts of green microalgae. Ghaidaa *et al*. [70] studied the antimicrobial activity of the methanolic extract of *C. reinhardtii* against pathogenic microorganisms and the results showed the best inhibition zone 32mm was obtained against *Candida albicans*, followed by *S. aureus* with 15mm and *E. coli* 9mm. Also, the same report indicated that the *C. reinhardtii* algal extract showed higher reduction of the existing *Staphylococcus aureus* biofilm. These findings suggest that the algal species may contain promising compounds that can be used to combat biofilms. All these results were agreed with the previous reports that confirmed that *C. reinhardtii* possess antimicrobial effects [71]. The results of this study demonstrate that the bioplastic film possesses remarkable antimicrobial properties without the addition of any external antimicrobial agents. This makes it a cost-effective and environmentally friendly alternative to traditional plastic materials in various applications.

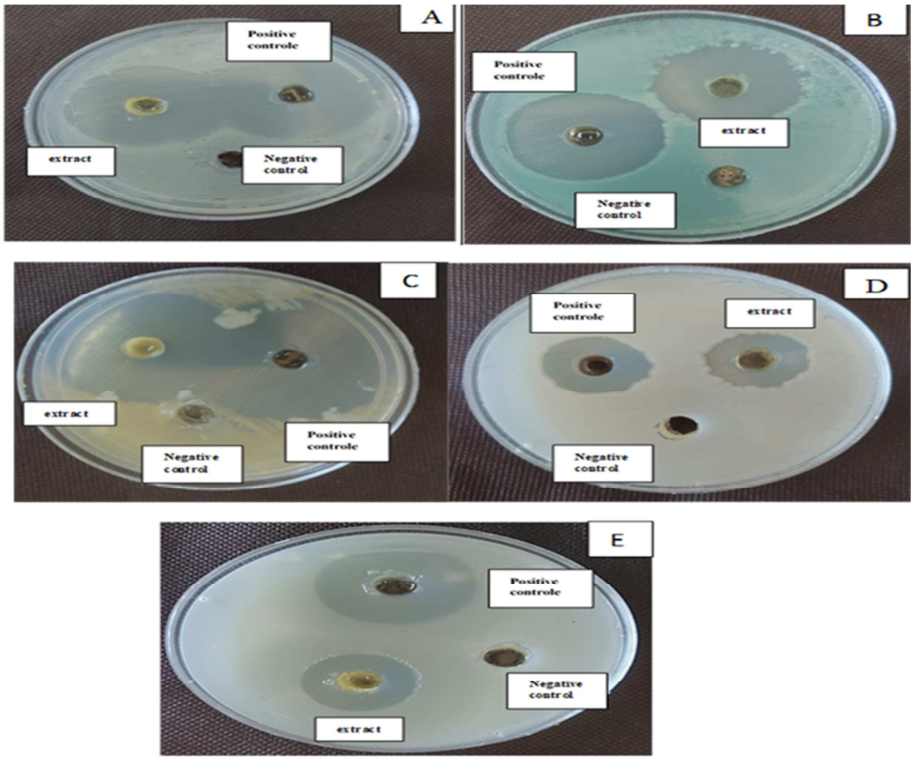

Fig. (10): Inhibition zones of chloroform extract of *C. reinhardtii* against the tested microorganisms by disk diffusion method. (A): *E. coli*; (B): *P. aeruginosa*; (C): *S. aureus*.; (D): *B. subtilis*; (E): *Candida albicans*

3.9. Biodegradability study.

The assessment method used to measure the biodegradability of bioplastic films was mass loss as a function of the length of time they were buried in the soil (Fig. 11).

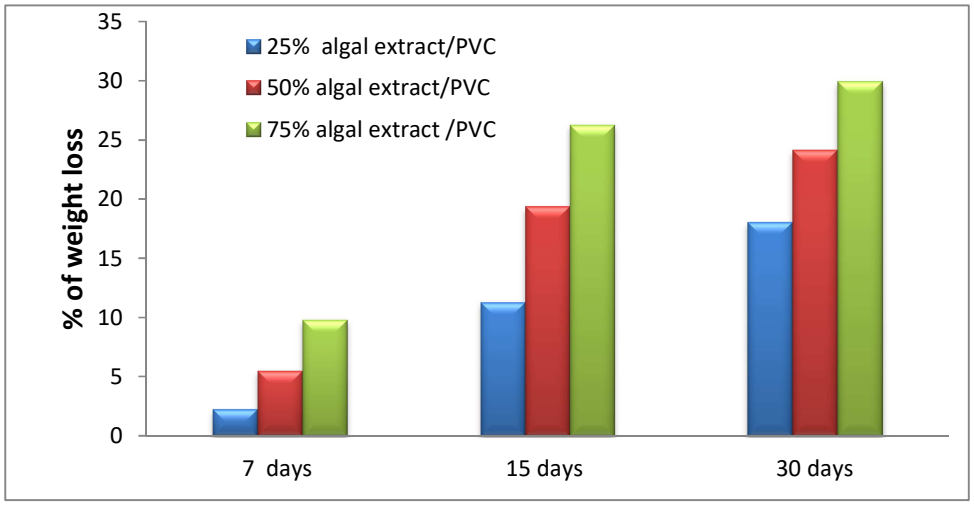

Fig. (11): Biodegradability of bioplastic films containing different percentages of algal extract

The results showed that the biodegradation was very low in all algal extract films after 7 days with value ranges between from 2.27%- 9.8%, after 15 days there are remarkable increase in the biodegradation percentage varies between 11.3% - 26.7 %, while its percentage after 30 days recorded the following values (11.3%, 24%, 30.4%) for 25%, 50%, and 75% algal extract /PVC receptively.

Also, the results showed the increase in the algal extract concentration causes increase in the biodegradation percentage. The biodegradation process depends on the environment's factors, microorganisms and its associated enzymes, and the polymer properties, resulting in a number of parameters that create a complex process affect in biodegradation times and rates can vary from one type to another [72].

Day 1 showed a mass loss value of 0 for all sample. After a week, no discernible mass loss was seen in any of the bioplastics that were analysed. The bacteria in the soil started to multiply in their new surroundings after they adapted. Then, due to increased biodegradation, significant microbial activity, and their strong development, all of the bioplastics lost weight faster [73] Furthermore, it has been observed that the samples' biodegradability rises with a higher percentage of algal extract. According to Nishida and Tokiwa [74] and Yutaka and Buenaventurada[75], PHBs are broken down into water-soluble forms by microbial processes. According to Lee and Choi [76], intracellular depolymerase enzymes carry out the degrading process of PHAs. PHAs are biodegradable bioplastics that completely break down through aerobic biodegradation in 5–6 weeks after undergoing enzymatic depolymerization [77] also, Kim *et al*. [78] reported complete degradation of PHB films within two weeks.

4. Conclusion

This research explores using microalgae, specifically *Chlamydomonas reinhardtii*, for sustainable bioplastic production. Nutrient deprivation encourages the algae to produce significant quantities of PHB, a bioplastic precursor. Extracts from *C. reinhardtii* also showed antimicrobial activity against several bacteria and fungi, suggesting their use in antimicrobial packaging. Blending algal extracts with PVC resulted in bioplastic films with promising mechanical properties. These results highlight the potential of algae-derived bioplastics as environmentally friendly replacements for conventional plastics. Promoting these sustainable materials could help reduce plastic pollution and create a more sustainable future.

5. Conflicts of interest

There are no conflicts to declare.

6. Acknowledgments

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