The potential of waste chicken feather protein hydrolysate as microalgae biostimulant using organic fertilizer as nutrients source

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Abstract. High costs associated with chemical triggers to promote microalgae productivity when waste-based sources are used as nutrients source has diverted the attention of microalgae growers to seek for sustainable substitute for synthetic triggers. On the other note, vast disposal of chicken feather waste cause severe environmental pollution due to its low decomposition characteristics. Following the call for rigid regulations on its disposal and in attempt to valorize this waste, chicken feathers were subjected to hydrolysis process using 1M sodium hydroxide (NaOH) and precipitated by 1M hydrochloric acid (HCL) to produce chicken feather protein hydrolysate (CFPH). The prepared CFPH was further tested for its feasibility as biostimulant for *Chlorella vulgaris* grown in organic fertilizer as nutrients source. From the data obtained via elemental analysis, the protein content of CFPH was determined as 73.56%. The biomass and lipid productivities of *C. vulgaris* cultures were significantly improved by 30.4 and 34.3 to 44.6%, respectively compared to control cultures. This research work indicated that CFPH may serve as a potential low-cost biostimulant for simultaneous augmentation of microalgae biomass and lipid. Characterization of physicochemical properties of the produced CFPH is an essential step in identifying possible avenues for its application in microalgae cultivation.

1.Introduction

Circa 1460 trillion liters of global diesel consumption by the transportation sector alone was reported and the stipulation is expected to increase by 40% in the coming decade [1]. Moreover, due to greenhouse effects and other climatic issues caused by excessive burning of fossil fuels, there is an urgent need to explore new and sustainable energy sources [2]. Therefore, microalgae (including cyanobacteria) have been suggested by energy researchers as a potential feedstock for biofuel production owing to its lucrative properties. Among a myriad of biofuel products attained from microalgae biomass (i.e., biodiesel, bioethanol, biohydrogen, and biomethane), microalgae biodiesel is the most promising biofuel to replace petroleum-based fuels in the upcoming years [3]. This is because most of the microalgae species can accumulate significantly high amounts of lipids than those of oil-bearing crops or animal fats, which can be potentially explored to address the current issue of fossil

fuel depletion [4]. However, one of the major setbacks for current microalgae biodiesel scheme is the high cost associated with chemical nutrients required for mass scale microalgae cultivation. It was reported that, the cost of chemical nutrients or inorganic fertilizers could account for 10–20% of the entire cultivation cost, turning it a fundamental factor to be considered for commercial microalgae cultivation processes [5].

Accordingly, the utilization of waste resources to formulate culture media could be a viable option to reduce the overall energy input [5]. Various waste-based nutrients that rich in nitrogen and phosphorus have been explored in recent years for microalgae growth, such as animal excreta, raw chicken manure, municipal wastewaters, domestic sewage, etc. [1]. It was reported that the cost of microalgal biodiesel production can be subsided to $\$0.73 kg^{-1}$ dry weight when waste resources are used as cultivation media [6]. Despite being broadly adopted, contamination risk and diverse compositions of the nutrients and adaptability issues of microalgae strains remain as drawbacks to be tackled [7]. Alternatively, organic fertilizer as a nutrient source is a simple way to minimize the pollution level in water as well as for cost-effective microalgae cultivation. Nevertheless, detailed studies reporting on the use of organic fertilizers as nutrients for microalgae cultivation are still scarce.

On the another note, several chemicals, i.e., epigallocatechin gallate, cycloheximide and so on are found to increase lipid productivity of microalgae by more than 200% without hampering growth and biomass production, demonstrating the feasibility of chemicals as practical approach in addressing the issue of low microalgae productivity, but high costs of these chemicals make their applications economically unfeasible for microalgae industry [8]. Accordingly, utilization of waste biomass that are readily available as biostimulants to improve microalgae cell growth and/or lipid accumulation could help to further reduce the cost of microalgae biodiesel production.

A survey by the United States Department of Agriculture revealed that circa 100.5 million tons of meat was generated in year 2020, causing more than 4.7 million tons of chicken feathers waste was produced [9]. Chicken feathers are mostly made up of keratins (90% w/w), which are structural proteins with very inflexible structures that do not readily degrade after landfilling [10]. Besides the economic and environmental constraints associated with current technologies used in managing by-products of poultry processing, inappropriate disposal results in the potential loss of useful biological resources as these feathers can be potentially beneficiated into high-value products comprised of keratin proteins or keratin fibres [11]. Inactivation of reactive oxygen species (ROS), scavenging free radicals and chelating pro-oxidative transition metals by protein hydrolysates have been well described in the literature [12]. To the best of our knowledge, no researchers have valorized CFPH as biostimulant for microalgae cultivation. Hence, in the current study, the biostimulation performance of CFPH on *C. vulgaris* grown in organic fertilizer will be investigated and assessed in terms of biomass and lipid productivities.

2. Methodology

2.1. Pure microalgae strain and culture conditions

C. vulgaris was obtained Universiti Teknologi PETRONAS. The strain was preserved in a 100mL Erlenmeyer flask with 50mL of the Bold's Basal Medium (BBM), at initial pH of 6.8, aerated with compressed air and illuminated continuously with cool-white fluorescent light (Philip TL-D 36 W/865, light intensity of 60–70 $\mu mol/m^2/s$) at a surrounding temperature of 25–28°C [5].

2.2. Microalgae cultivation with organic fertilizer

For subsequent cultivation of *C. vulgaris*, Baja Tani (organic fertilizer) was used as the main nutrient source without any modification. The organic fertilizer was immersed in tap water in a ratio of 1:60 and stirred overnight at room temperature. The fertilizer solution is then filtered using filter paper (Double Rings 101) and the resulted filtrate was stored in Scott bottles in the freezer until further use. Subsequently, a pre-optimized ratio (84: 8: 8% v/v = unsterilized tap water: organic fertilizer: inoculum concentration) was applied with a working volume of 5 L in a photobioreactor system, followed by the pH adjustment to 3–3.5. The photobioreactor is then aerated with compressed air with a flow rate of 4L/min under continuous illumination with the help of cool-white, fluorescent light (Philip TL-D36W/865, light intensity of 60–70$\mu mol/m^2/s$). The well-growth of *C. vulgaris* was monitored by measuring the optical density of the culture at 688 nm by using a UV visible spectrophotometer (Shimadzu UV-2600) [13].

2.3. Preparation of CFPH

Waste chicken feathers were accumulated from the local poultry house in Perak. The feathers were washed with soap liquid followed by sterilizing with 5% hypochlorite solution, as described by Akpor et al. (2019) [14]. Next, the feathers were dried in an oven at 65^0 C overnight and stored in a ziploc bag for subsequent analysis. For hydrolysis, dried chicken feathers were added with 1M NaOH in a ratio of 1:5. The feather-NaOH mixture was stirred vigorously and allowed to stand for 10 hours. Following the hydrolysis, the mixture was filtered with filter paper (Double Rings 101) and the unhydrolyzed fraction was removed. The removed feathers were washed a couple of times with deionized water for complete elimination of the contaminants, followed by drying in an oven at 65^0 C for overnight. Next, protein hydrolysate was precipitated out from the hydrolyzed feather solution using 1M of HCL for 10 minutes at 25^0 C. The precipitated CFPH was then separated from the solution by filtration followed by air-drying and quantification. The CFPH was finally ground into powders using a mini grinder and stored in air-tight vials for further use. Figure 1 shows the simplified schematic flow diagram of CFPH preparation.

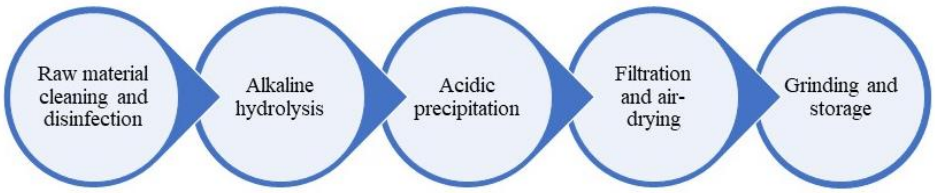

Figure 1. Schematic flow diagram of CFPH preparation.

2.4. Screening for potential biostimulation effects of CFPH

The biostimulation effects of CFPH were tested using very low *C. vulgaris* cell inoculums (2 and 4% v/v, respectively) and compared with the control cultures (2 and 4% v/v) without CFPH supplementations. Similar cultivation methodology as in sub section 2.2 was repeated but in a smaller scale with a total working volume of 400mL in 600mL conical flasks. Approximately, 0.5g of CFPH was supplemented to respective cultures on day 0 and the cell growth was monitored. The biomass concentration (N, g/L), specific growth rate (μ, 1/day), biomass productivity (P_{max}, g/L/day), lipid content (Y, %) and productivity (P_{lipid}, g/L/day) were calculated according to methods described by Lam and Lee (2012) [7]. All the experiments were conducted in triplicates.

2.5. Harvesting of microalgae biomass

After 14 days of cultivation, the culture was left to settle by gravitational sedimentation and the supernatant was decanted while the bottom layer consisting of microalgae biomass was collected and dried in oven at 105.5°C for 24 hours. The dried microalgae biomass was then stored in an air-tight container and sealed with parafilm for lipid extraction [5].

2.6. Extraction of lipid

A 0.1g of dried *C. vulgaris* biomass was mixed with 30mL of methanol-chloroform solution in the ratio of 2:1. The mixture was then shake in an incubator shaker with a speed of 200rpm and room temperature for 1 hour for, followed by filtration using filter paper (Double Rings 101) and the filtrate is air-purged and oven-dried at 105.5°C until all the solvent was evaporated. The steps above were repeated for second cycle of lipid extraction using the remaining residue after filtration [5].

2.7. Elemental analysis of CFPH

The nitrogen (N) content as well as other average elemental composition of the prepared CFPH such as carbon (C), hydrogen (H), sulphur (S) was determined with the help of CHNS analyzer (Perkin-Elmer Model 2400, CHNS elemental analyzer). The obtained nitrogen content was then used to quantify the percentage of protein in the CFPH sample by using the factor is 6.25 since most protein contains 16% of nitrogen [15].

2.8. Quantification of CFPH

The hydrolysis rate of chicken feather was calculated using the Equation 1 below:

$$Hydrolysis\ rate\ (\%) = 100 \times (B - A) / A \quad (1)$$

where *B* is the dry weight of the feathers before hydrolysis, and *A* is the dry weight of the feathers after hydrolysis [14]. Meanwhile, the protein content (*Z*, %) in CFPH was estimated based on Equation 2 below:

$$Z\ (\%) = N\ (\%) \times 6.25 \quad (2)$$

where *N* (%) is the total nitrogen content determined using elemental analyzer (Perkin-Elmer Model 2400) and 6.25 is the constant value of nitrogen-to-crude protein conversion factor [15].

2.9. Statistical analysis

Statistical analysis was performed using Excel 2010 (Version 14.3.2 e, Microsoft, USA). Values are presented as statistical means. Differences of the separating means were evaluated by t-test. One-way analysis of variance (ANOVA) based on triplicate data was used to test the significant difference of the results ($p<0.05$ considered to be statistically significant).

3. Results and discussion

3.1. Quantitative analysis of CFPH

According to Equation 1, the hydrolysis rate of chicken feather was calculated to be 93.44%. It was found the tested CFPH contains 42.53% of C, 6.21% H, 11.77% of N, and 2.44% of S as detected by CHNS analyzer. Present elemental analysis on CFPH are comparable to the ones previously reported by Olajumoke et al. (2020) [16]. Substituting the percentage value of N into Eq. (7), the amount of protein, Z recovered by hydrolysis of chicken feather was calculated to be 73.56%.

3.2. Potential of CFPH to enhance growth of C. vulgaris

The growth curve of *C. vulgaris* corresponding to variation in inoculum concentrations (2 and 4% v/v) using CFPH as biostimulant is illustrated in Figure 2. During the cultivation process using Tani organic fertilizer, similar patterns of lag phases were observed in the control cultures and the ones supplied with CFPH, which clearly indicating that the microalgae cells could adapt well to the tested biostimulant. On 6th day, the biomass concentration of cultures with CFPH supplementations exceeded the control cultures in linear trend until 14th day, proving the efficiency of CFPH as biostimulant to promote the growth of low inoculum dosages of *C. vulgaris* cells. Interestingly, there were no stationary phases observed for both cultures (2 and 4% v/v of inoculum dosages) that supplied with CFPH whereas obvious stationary phases were detected in control cultures after 13th day of cultivation. Even though the initial biomass concentrations of cultures supplied with CFPH were nearly the same as control cultures, the overall biomass concentration of *C. vulgaris* when supplemented with CFPH at both inoculum dosages (2 and 4% v/v) were higher than of control cultures by 25.8 and 8.1%, respectively at day 14.

As for cultures with and without CFPH supplementations, higher biomass concentrations, growth rates and biomass productivities were observed when a higher inoculum dosage (4% v/v) was used which corresponded with the fact that a relatively high initial cell density is needed to promote higher biomass productivity and decreased the chances of photodamage in microalgae cells due to over-exposure to light source [17]. As can be seen from Figure 3, the specific growth rate was improved from 0.233/day (control culture with 4% v/v inoculum dosage) to 0.272/day when CFPH was supplied to culture with 4% v/v inoculum dosage whereas there was a minor reduction about 0.01% recorded when CFPH was added in comparison to control culture with 2% v/v inoculum dosage. This could be due to availability more microalgae cells at early cultivation stage to utilize the supplied biostimulant for cell replication when 4% v/v of inoculum dosage was used. Even though the specific growth rate of CFPH-supplemented culture with 4% v/v inoculum was the higher than that with control culture, yet it exhibited a very close biomass productivity (0.055 g/L/day) to the control with biomass

productivity of 0.056 g/L/day, as shown in Figure 3. On the other hand, there was a significant increase in biomass productivity about 30.4% compared to control culture when CFPH was added to culture with 2% v/v inoculum dosage. Hence, it is evident that CFPH did not augment the biomass productivity of *C. vulgaris* when 4% v/v inoculum dosage was used which can be caused by biostimulant limitation over the cultivation days.

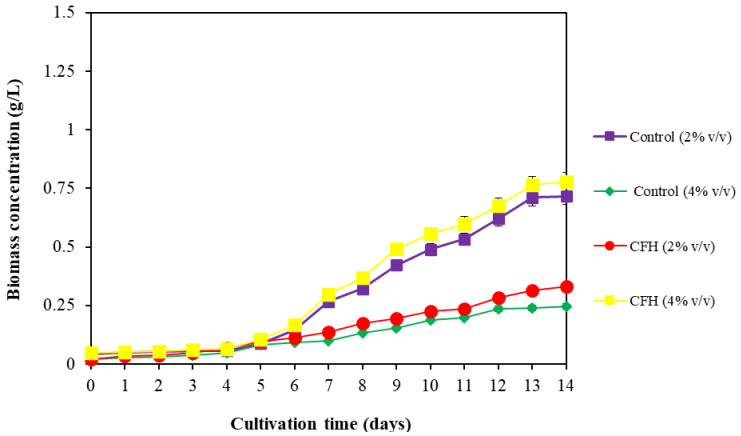

Figure 2. Effects of inoculum dosage (% v/v) on the growth behaviour of *C. vulgaris* with CFPH as biostimulants.

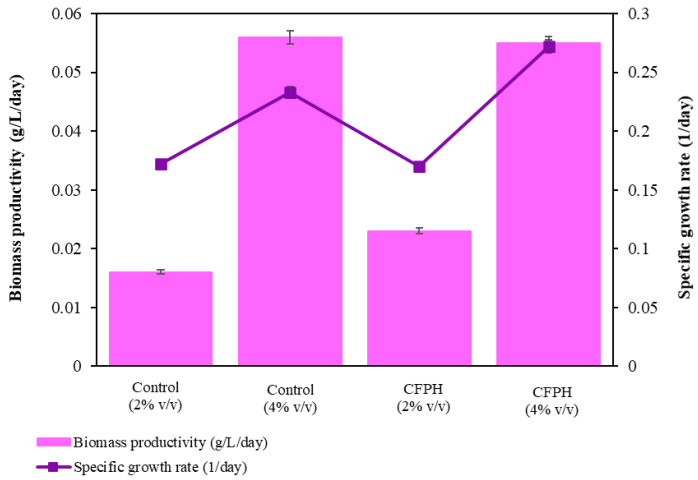

Figure 3. Effects of inoculum dosage (% v/v) on biomass productivities and growth rates of *C. vulgaris* with CFPH as biostimulants.

3.3. Potential of CFPH to enhance lipid content of C. vulgaris

The lipid accumulation efficiencies by *C. vulgaris* in control and with supplementation of CFPH are displayed in Figure 4. It was found that, significantly higher amounts of lipid were accumulated in cultures supplemented with CFPH than in the control cultures under continuous illumination for 14 days of cultivation. Approximately, 29.44 and 30.02% of lipid content by weight percentages were detected in microalgae cultures (2 and 4% v/v, respectively) that supplied with CFPH, which were 8.06 and 8.66% higher than that produced by both control cultures. The difference in lipid contents when higher inoculum dosage (4% v/v) was used could be due to ability of CFPH to induce the de novo accumulation of excess lipids in microalgae cells which subsequently led to higher ratio of lipid-rich cells in proportion to specific growth rate and biomass productivity. As lipid production is the function of lipid content and biomass concentration, the total lipid productivities of *C. vulgaris* cultures (2 and 4% v/v) supplemented with CFPH were 0.694 and 1.662 g/L/day, respectively, which

were 44.6 and 34.3% greater than that produced by both control cultures during 14 days of growth. Besides the lipid induction capability of CFPH, nutrients limitation and/or depletion, mainly nitrogen source over the cultivation days creates a stress environment in which microalgae synthesize more lipids and/or fatty acids as a means of energy storage [18]. Moreover, it was reported that upon nitrogen depletion in culture media, microalgae cells start to utilize the intracellular nitrogen pool such as chlorophyll to aid the synthesis of cell materials for further cell divisions. Since chlorophyll is the key unit of the photosynthetic mechanism of green microalgae that responsible for capturing solar energy and carbon dioxide (CO_2) to generate the metabolic flux for both cell growth and lipid secretion, a significant drop in chlorophyll content will impede the overall biomass and lipid productivities [19]. Present results suggest that there was an adequate trade-off between maximizing biomass and lipid contents when nitrogen-rich CFPH was added as biostimulant to alleviate any nutritional deficiencies, mainly nitrogen source besides being a potential photosynthesis precursor under stress conditions owing to its documented antioxidant properties in literatures. All in all, this study proved the efficiency of CFPH as state-of-the-art biostimulant to promote the growth of *C. vulgaris* cells even at very low cell densities along with lipid induction effects.

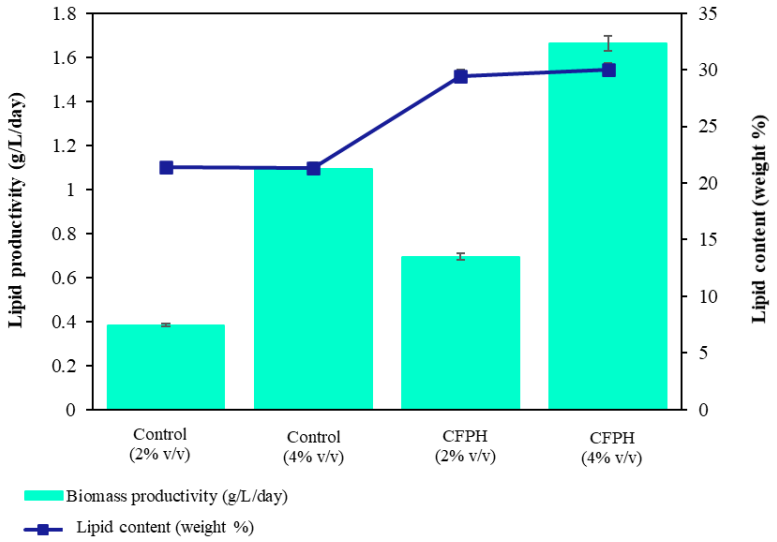

Figure 4. Effects of inoculum dosage (% v/v) on lipid productivities and lipid contents of *C. vulgaris* with CFPH as biostimulants.

4. Conclusion

This preliminary study on the potential of CFPH as biostimulant resulted in positive impacts on biomass and lipid productivities of microalgae cultures with very low inoculum dosages (2 and 4% v/v). Current work serves as precursor for valorization of chicken feather waste to high value biotechnological materials. Identification of chemical, physical and morphological properties of CFPH and its related potential biostimulation effects on microalgae cultures are crucial in further optimization process and in realizing economic microalgae biodiesel production.

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