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Potential of Natural Sheep Casings Waste as a Sole Nitrogen Source for the Marine Microalga *Scenedesmus rubescens* MDP19 Growth and Lipid Production

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Abstract: The meat industry is one of the main sources of organic waste in the food processing sector. Due to their high content of biodegradable organic matter, these wastes represent a potentially valuable resource for the development of recycling and valorization processes, particularly with regard to the circular economy and environmental sustainability. The present study aimed at assessing the potential of natural sheep casings waste (NSCW) as a source of nitrogen for promoting the growth and lipid production of *Scenedesmus rubescens* MDP19, a marine microalga isolated from the Mediterranean coastline of northern Morocco. For this purpose, we evaluated the effects of different NSCW concentrations (0.25–5 g L^{−1}) on the microalga growth, its ability to utilize organic waste components (proteins, amino acids, and carbohydrates) as nutrients, and its efficiency in eliminating nitrogen and phosphorus. Lipid and pigment contents were determined using colorimetric methods, and their composition was analyzed by high-performance liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS/MS). The results showed that *S. rubescens* MDP19 achieved the highest biomass production of 1.737 g L^{−1} at an NSCW concentration of 5 g L^{−1}. This strain removed 33.70–47.63% of protein, 71.84–87.62% of amino acids, 41.9–92.97% of carbohydrates, 59.72–99.30% of nitrogen, and 80.74–99.10% of phosphorus. Furthermore, *S. rubescens* MDP19 showed a significantly enhanced lipid content (68.11%) at an NSCW concentration of 0.5 g L^{−1}. At this concentration, the lipid composition of *S. rubescens* MDP19 was particularly complex, including monounsaturated and polyunsaturated fatty acids, digalactosyldiacylglycerols, sulfoquinovosyldiacylglycerols, phosphatidylglycerols, and acylglycerols. The pigment profile includes neoxanthin, canthaxanthin, lutein, chlorophyll a, geranylgeranyl chlorophyll a, chlorophyllide b, hydrochlorophyllide b, and pheophytin a. These results indicate that natural sheep casings waste represents a promising source of nitrogen, reducing the need for nutrient supplementation in microalgae production. This approach not only offers a sustainable and economical alternative for optimizing microalgae cultivation but also contributes to the valorization of organic waste, thus supporting more ecological and responsible practices.



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Keywords: *Scenedesmus rubescens*; marine microalgae; natural casings; organic solid waste valorization; nutrient removal; lipids; carotenoids

1. Introduction

The management of waste from slaughterhouses and meat processing factories continues to present a significant challenge on a global scale. These facilities generate a wide range of waste products, including liquid effluents with high organic content, animal by-products such as blood, bones, gastrointestinal contents, and internal organs, and solid waste.

Natural casings are manufactured from animal intestines [1]. More than 1.5 tons of waste can be generated every day by the various processing operations involved in the production of these products [2]. The generated waste can contain valuable ingredients, such as proteins, cellulose, fats, carbohydrates, and minerals, which can be recycled and reused for a wide range of applications across multiple industries.

Owing to their proteinaceous nature, natural casings waste is highly susceptible to microbial decomposition [3]. This, in turn, can result in the release of toxic gases, including nitrous oxide, ammonia, and hydrogen sulfide [4]. These emissions represent a significant threat to human health, as well as to natural resources and ecosystems. Conventional waste treatment methods, such as landfilling, incineration, anaerobic digestion, and composting, are commonly deployed [5,6]. Nevertheless, these techniques are not particularly appealing to industrial manufacturers due to their lack of sustainability and environmental friendliness. Thus, treating waste with microalgae offers a convincing alternative that is both an environmentally friendly solution and a strategy for meeting economic challenges.

In recent decades, a growing area of research has focused on harnessing the potential of microalgae in an integrated approach to waste treatment and lipid production for biofuel synthesis. This strategy is consistent with global efforts to identify renewable energy sources with minimal environmental impact [7]. The remarkable taxonomic, physiological, and biochemical diversity of microalgae offers promising prospects for the prospecting of new oil-producing strains with high lipid yields and adaptability to substrates derived from organic waste [8]. Compared to other oil-producing crops, microalgae can produce as much as 70% or more of the lipids [9]. Their oil production has been estimated at 136,900 L per hectare [10]. The lipid profiles of microalgae are very rich, with about 135 types of fatty acids identified [11]. These commonly include saturated, monounsaturated, and polyunsaturated fatty acids [12]. Many other structural lipids, such as glycosylglycerides and phosphoglycerides, are also described [10]. While their lipid value is certainly an attractive feature for the biofuel sector, microalgae also offer significant advantages in terms of rapid growth and ease of cultivation, which may be worth considering. They are able to flourish in a number of different environments, including arid regions, and do not conflict with agricultural activities [13,14]. They can reduce gas emissions and efficiently absorb nutrients and pollutants from diverse waste materials [15]. It is estimated that microalgae have the capacity to remove the equivalent of 450 tons of CO₂, 25 tons of nitrogen, and 2.5 tons of phosphorus per hectare annually [16]. Furthermore, they have been demonstrated to be capable of utilizing a diverse array of nitrogen sources, including the proteins and amino acids, inorganic nitrate, ammonium, urea, and aromatic compounds containing amine groups [13]. Consequently, they are particularly well suited to bioremediation.

In this work, a marine strain of microalgae *Scenedesmus rubescens* MDP19 was isolated from northern Morocco. To the best of our knowledge, no study has yet integrated microalgae into the treatment of natural casings waste for lipid production. Accordingly, in

this study, *S. rubescens* MDP19 was cultivated in varying concentrations of natural sheep casings waste as the sole nitrogen source. The potential of *S. rubescens* MDP19 to utilize proteins, amino acids, and carbohydrates as a source of nutrients, and to remove nitrogen and phosphorus from the waste product was examined. The microalgal growth, biomass production, and lipids and pigments contents were monitored, and the composition of lipids and pigments was analyzed by HPLC-APCI-MS.

2. Results and Discussion

2.1. Identification of *S. rubescens* MDP19

Microscopic evaluation of the isolate showed round, globular, slightly elliptical, and non-motile cells. They have a thick wall and can be isolated or form chains of up to four cells. Initially, the cells are green but turn brick-red or orange as they age or undergo stress conditions (Figure 1). Based on partial 18S rRNA sequencing and the results of the Basic Local Alignment Search (BLAST version +2.15.0) in the NCBI gene bank database, the isolated microalga was identified as *Scenedesmus rubescens* IPPAS D-292, also known as *Halochlorella rubescens* IPPAS D-292, with an identity level of 100% (accession number: KU057946.1). The new strain has been designated as *Scenedesmus rubescens* MDP19. This species is a halotolerant microalga in the order Chlorococcales and the family Scenedesmaceae. The species was first recorded on a culture of brown algae near Bordeaux, France, then on the coast of the Buhta Blagopoluchiya Bay in Russia and on the coast of the Solone Salt Lake in Ukraine [17]. However, this is the first time that it has been observed on Morocco's Mediterranean coast.

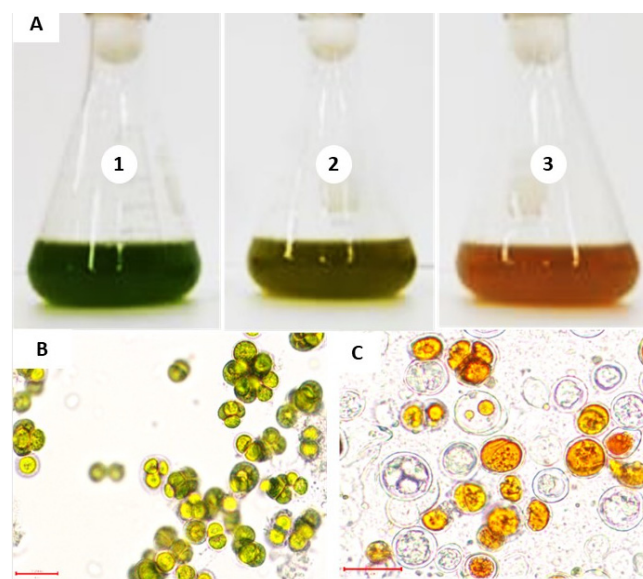


Figure 1. (A) Variations in culture color of *S. rubescens* MDP19 cells during cultivation. (A1) Green cells. (A2) Green-brown cells. (A3) Brick-red/orange cells. (B,C) Micrographs of green and orange *S. rubescens* MDP19 cells, respectively. Scale bars: 10 µm.

2.2. Effect of NSCW on *S. rubescens* MDP19 Growth and Biomass Production

The effect of NSCW as a sole nitrogen source on the growth of *S. rubescens* MDP19 was evaluated by testing a range of concentrations from 0.25 to 5 g L⁻¹. The growth curves obtained over 25 days of cultivation are represented in Figure 2. Cell densities, specific growth rates, biomass production, and daily biomass productivities are plotted in Figure 3. According to Figure 2, *S. rubescens* MDP19 growth was strongly influenced by the waste concentration. The application of elevated concentrations of NSCW (2–5 gL⁻¹) resulted in a significant deceleration of microalgal growth. The observed inhibition at this stage can be

attributed to a phase of cellular adaptation necessary to cope with the increased substrate levels in the medium. The maximum cell density ($7.8 \text{ cells} \times 10^6 \text{ mL}^{-1}$) and the minimum cell density ($1.56 \text{ cells} \times 10^6 \text{ mL}^{-1}$) were achieved in cultures grown at 5 g L^{-1} NSCW and in the control, respectively.

As demonstrated in Figure 3, upon completion of the cultivation period (25th day), the highest biomass production of 1.737 g L^{-1} was achieved at an NSCW concentration of 5 g L^{-1} , representing a 4.73-fold increase over the values obtained in the control. NSCW at 3 g L^{-1} showed the second highest biomass production of about 1.486 g L^{-1} . This was followed by NSCW concentrations of 2 g L^{-1} (1.361 g L^{-1}), 1 g L^{-1} (1.295 g L^{-1}), and 0.75 g L^{-1} (1.146 g L^{-1}). The lowest biomass productions (0.878 ± 0.04 and 0.511 ± 0.05) were observed at NSCW concentrations of 0.5 g L^{-1} and 0.25 g L^{-1} , respectively. The decline in the biomass production observed under low concentrations of NSCW is attributable to nutrient scarcity and nitrogen limitation.

In this study, the growth rates varied between 0.9072 d^{-1} and 0.9781 d^{-1} , and the daily biomass productivities were between 18.32 and $86.87 \text{ mg L}^{-1} \text{ day}^{-1}$. Overall, all of the cultures that were supplemented with NSCW exhibited enhanced growth and biomass production compared to the control. The enhancing effect of NSCW on the productivity of *S. rubescens* MDP19 is most likely due to the provision of appropriate amounts of essential nutrients needed to support its growth and functioning [18].

Consistent with our findings, *Dunaliella salina* grew on a hydrolyzed waste of sheep gastrointestinal wall as a substitute for the nitrogen source in the growth medium and demonstrated a 20% increase in biomass productivity [19]. *Spirulina platensis* was also successfully grown using a protein hydrolysate derived from fish waste as the sole source of nitrogen in its growth medium, producing a 39% gain in biomass accumulation [20]. It must be noted that in our study, natural sheep casings waste was used to culture *S. rubescens* MDP19 without any hydrolysis pre-treatment, which is profitable for market-scale production. Indeed, by avoiding the often energy-intensive and costly physico-chemical pretreatment steps, this method promotes a more sustainable and economically viable process. However, the effectiveness of this approach is highly dependent on the bioavailability of the organic compounds present in the raw waste, as well as the metabolic capacity of the algal strains to assimilate them without inhibition.

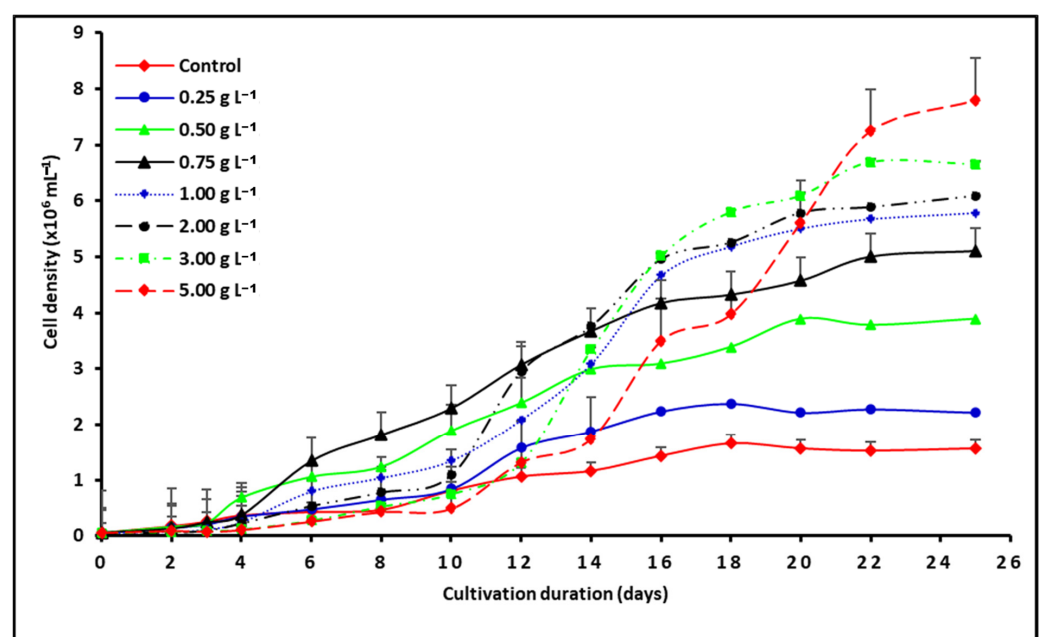


Figure 2. Growth curves for *S. rubescens* MDP19 using different concentrations of NSCW.

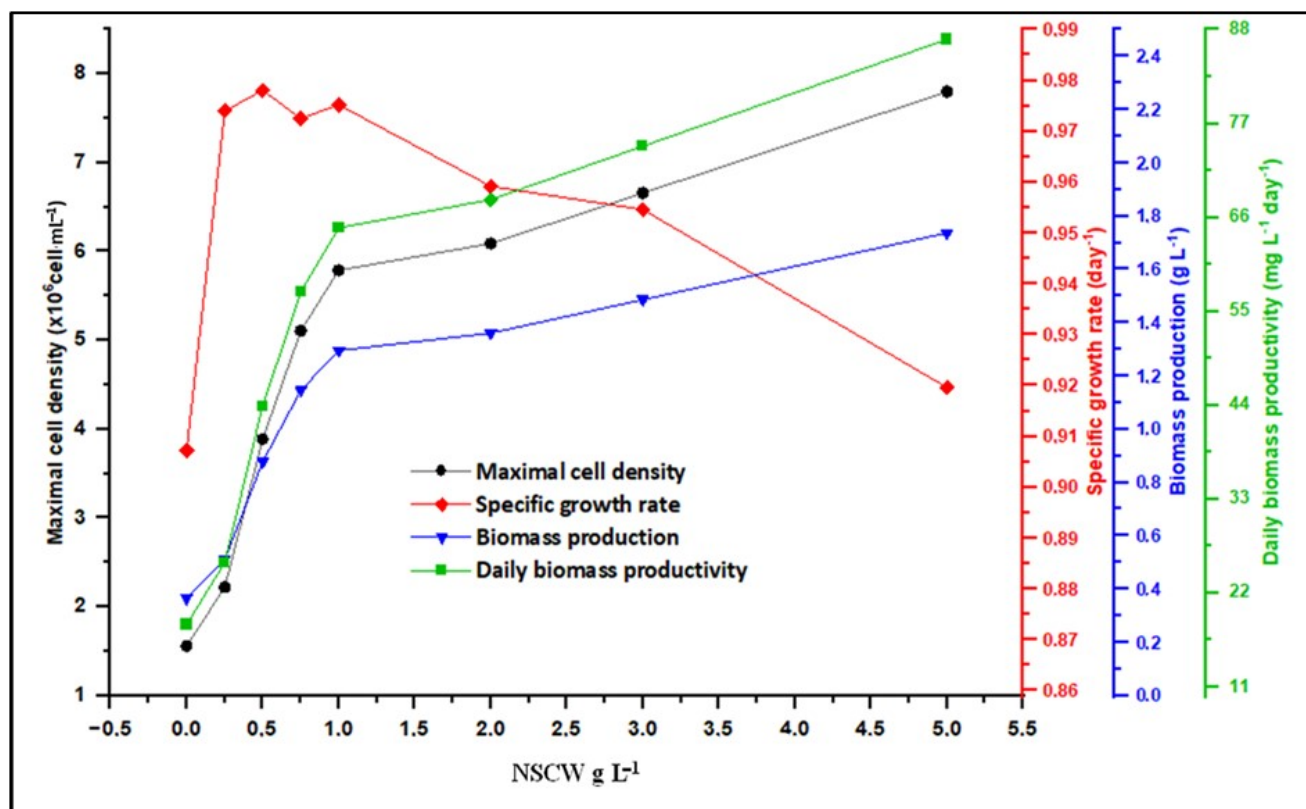


Figure 3. Cell density, specific growth rate, biomass production, and daily biomass productivity of *S. rubescens* MDP19 in different NSCW concentrations.

2.3. Utilization of Protein, Amino Acids, and Carbohydrates from NSCW by *S. rubescens* MDP19

The NSCW composition in this study indicates no ammonium in the waste (Table 1). Thus, the nitrogen available in the waste may be present in organic form, particularly in the form of proteins and amino acids. Figure 4A,B show the percentages of amino acids and proteins removed by *S. rubescens* MDP19 from the various NSCW-based growth media. The findings show that the percentage of protein removed ranged from 33.70% to 47.63%, with the highest percentage observed in cultures operated with 0.25 g L⁻¹ NSCW. A similar trend was observed in the percentage of amino acids removed, which varied from 71.84% to 87.62%, with the highest percentage also observed in cultures supplemented with 0.25 g L⁻¹ NSCW. The least percentages of protein and amino acid removal were observed in cultures grown at 5 g L⁻¹ NSCW.

Table 1. Chemical and biochemical composition of the NSCW.

Parameter	Unit	Amount
Total organic carbon (TOC)	(mg g ⁻¹ L ⁻¹)	2960
Total nitrogen (TN)	(mg g ⁻¹ L ⁻¹)	480
Total phosphorus (TP)	(mg g ⁻¹ L ⁻¹)	80
Ammonia	(mg g ⁻¹ L ⁻¹)	0
Total proteins	(%)	57.0
Total carbohydrates	(%)	32.3
Total amino acids	(%)	52.17

In light of the obtained results, it can be inferred that *S. rubescens* MDP19 was actively assimilating proteins and amino acids from NSCW. Indeed, the genus *Scenedesmus* has been demonstrated to produce proteolytic enzymes with the capacity to degrade proteins

into amino acids [21]. In response to nitrogen deprivation, algae cells use and convert amino acids for the production of enzymes involved in the synthesis of energy-storing compounds such as lipids [22]. This may explain the significant reduction in protein and amino acid content observed in the different NSCW-based growth media at the end of the cultivation period. Another reason for the reduction of amino acids in the growth medium is that when amino acids are taken up by microalgal cells, they are deaminated by L-amino acid oxidase, which produces ammonia and other products (oxo acids and H_2O_2). Ammonia can therefore be used by microalgal cells as a nitrogen source [23]. The ability of several microalgal species to assimilate ammonia as a preferred organic nitrogen source has been demonstrated in previous studies. These include *Phaeodactylum tricornutum* [24], *Chlamydomonas reinhardtii* [23], and *Scenedesmus acutus* [25].

With regard to carbohydrate components, the results showed that even in cultures supplemented with elevated concentrations of NSCW (5 g L^{-1}), the carbohydrate removal efficiency remained remarkably high, reaching 92.97% (Figure 4C). Therefore, *S. rubescens* MDP19 was also capable of utilizing carbohydrates from NSCW. This may be due to the heterotrophic property of this microalga, which implies a high capacity to assimilate organic carbon [26]. Carbohydrates can supply the energy needed for the metabolism of the microalgae and allow them to temporarily survive in the dark when needed [27,28].

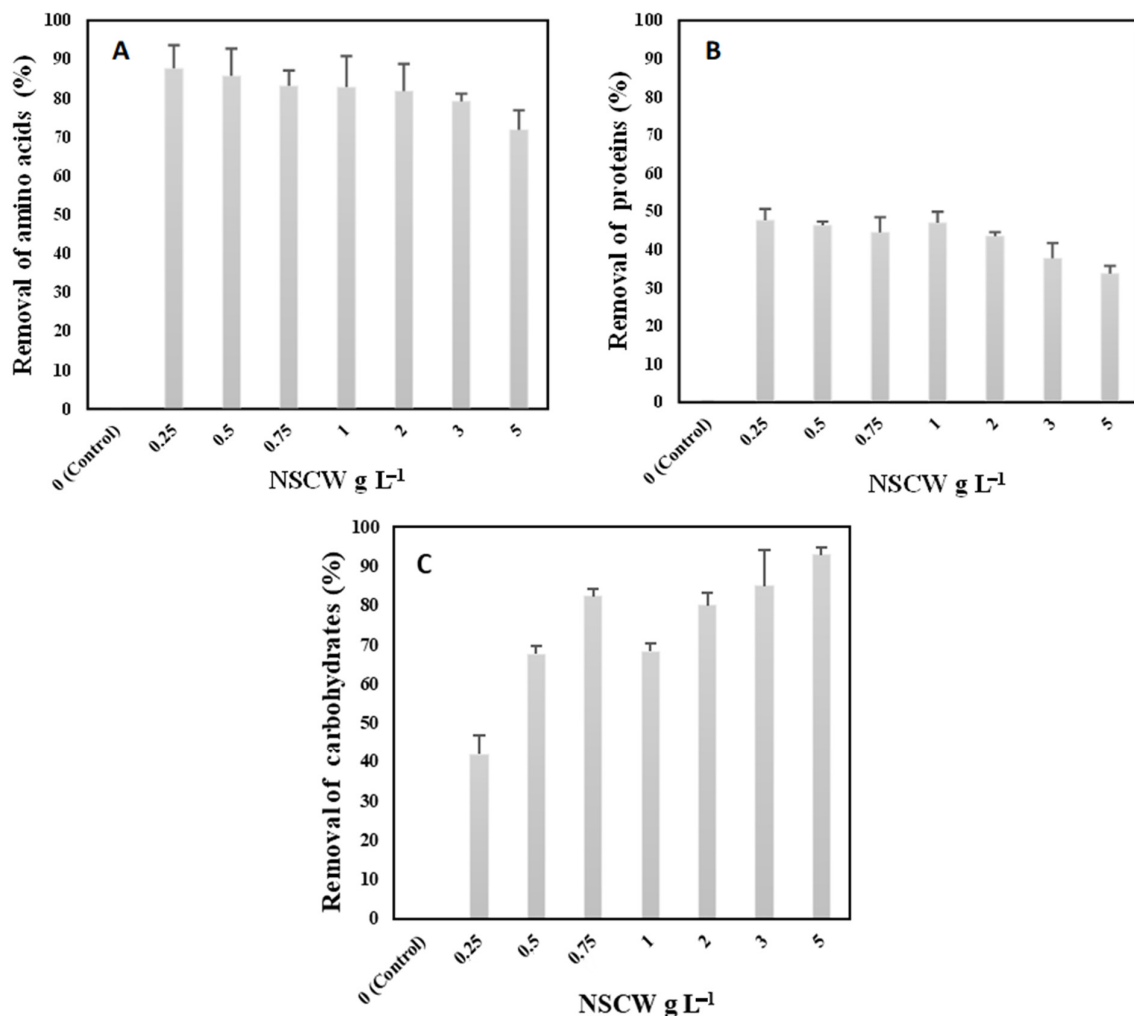


Figure 4. Removal percentage of total amino acids (A), total proteins (B), and total carbohydrates (C) from various NSCW-based growth media.

2.4. Removal Efficiency of Total Nitrogen and Total Phosphorus from NSCW by *S. rubescens* MDP19

To investigate the ability of *S. rubescens* MDP19 to reduce TN and TP load, the levels of these elements in the culture media were monitored at the onset and at the end of the cultivation period. Data in Figure 5A indicate that *S. rubescens* MDP19 was highly effective in removing TN, but only up to a certain concentration threshold, achieving the highest percentage removal (99.30%) in medium with an NSCW concentration of 0.5 g L⁻¹. The TN removal efficiency of *S. rubescens* MDP19 was found to drop as waste concentration increased. The removal percentage of TN was only 59.72% at an NSCW concentration of 5 g L⁻¹. This finding suggests that *S. rubescens* MDP19 exhibited limited efficiency in removing nitrogen from elevated concentrations of NSCW. It is conceivable that the nitrogen load at these levels has surpassed the removal capacity of *S. rubescens* MDP19. Corroborating our findings, it has been established that the capacity of microalgae to remove nutrients from wastewater increases when dilution is applied [29].

With regard to TP, the data in Figure 5B show that the removal efficiency of *S. rubescens* MDP19 remained high regardless of the concentration of NSCW, with TP removal percentages ranging from 80.74% to 99.10%. These outcomes corroborate those reported in previous studies showing that microalgae are more effective at removing phosphorus than nitrogen from wastewater [30,31]. It has been found that microalgae tend to assimilate large quantities of phosphorus and accumulate it in the form of intracellular polyphosphate during the early stages of their development, even though they only need small amounts of this nutrient at the start of their growth [32]. Microalgae use stored polyphosphate under phosphorus-deficient conditions to synthesize cellular phosphate compounds to ensure survival [33]. Little research has been done on the cultivation of microalgae on solid waste from meat processing factories and slaughterhouses, but there have been numerous studies on their wastewater and effluents. For instance, *Scenedesmus* sp. was observed to remove 70.67% and 60.91% of the TN and TP, respectively, from a pig slaughterhouse effluent [29]. The implementation of *Scenedesmus* sp. in an anaerobically digested slaughterhouse effluent resulted in the elimination of 33.6% of nitrogen and 85.2% of phosphate [34]. In addition, *Neochloris* sp. SK57 exhibited a 95% nitrite removal efficiency and a 79.3% phosphate removal efficiency from poultry slaughterhouse wastewater, while *Chlorella* sp. SL7A achieved 93.5% nitrite removal efficiency and 64.5% phosphate removal efficiency from the same wastewater [18]. In this study, we successfully demonstrated that *S. rubescens* MDP19 has a greater capacity to remove nitrogen and phosphorus from NSCW, making it a promising candidate for the treatment of solid waste from meat processing companies and slaughterhouses. It is worth mentioning that the reduction of nitrogen and phosphorus is of crucial importance in the treatment of organic waste, as it not only helps to significantly reduce the volume of waste to be landfilled, but also to optimize recovery processes and limit their environmental impact.

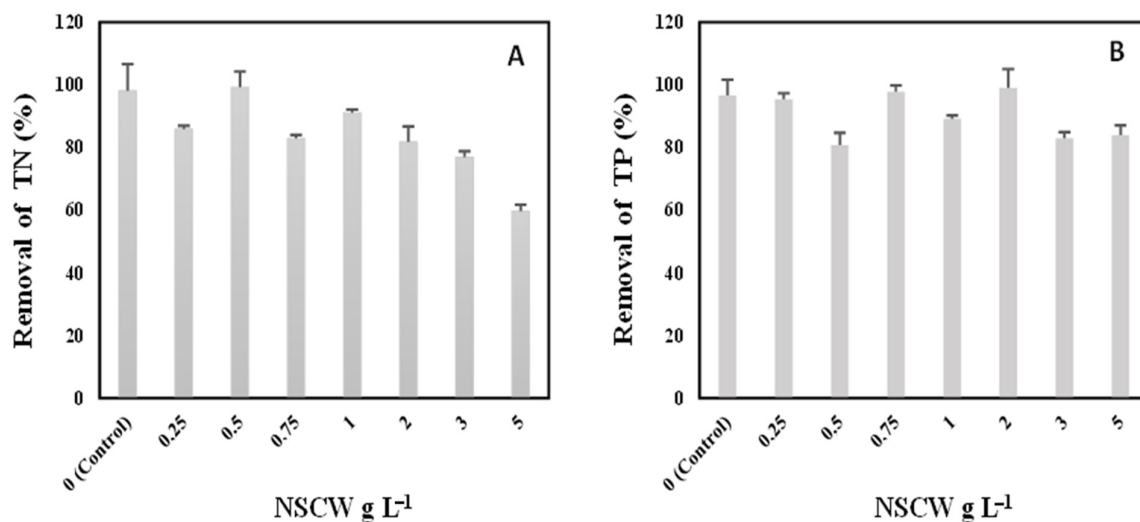


Figure 5. Removal percentage of total nitrogen (A) and total phosphorus (B) from various NSCW-based growth media.

2.5. Effect of NSCW on Lipid Content and Production

The lipid content and production obtained for *S. rubescens* MDP19 on different concentrations of NSCW are shown in Figure 6. The highest lipid content (68.11%) was observed at a concentration of 0.5 g L⁻¹. This content was significantly higher than that of the control, exhibiting an increase of 24.38%. However, at higher concentrations of NSCW, a notable reduction in lipid content was observed. At concentrations exceeding 1 g L⁻¹, the lipid content was diminished to a range of 32.63 to 42.28%. With regard to lipid production, the highest contents were observed in cultures grown with 0.5 and 0.75 g L⁻¹ NSCW, resulting in concentrations of 373.45 and 386.44 mg L⁻¹, respectively. Nevertheless, high concentrations of NSCW also resulted in elevated lipid production. This is due to the fact that lipid production is closely correlated with biomass production. In this study, the lipid content of *S. rubescens* MDP19 was found to exceed that of other documented strains of *Scenedesmus rubescens*. According to previous studies, a lipid content of 38.14% was found in *S. rubescens* KNUA042 grown in nitrogen-limited medium [35], 25.80% in *S. rubescens* SX grown in ammonium-rich wastewater [36], and only 14.91% in *S. rubescens* SAG 5.95 grown in synthetic wastewater [37]. Our results indicate that low concentrations of NSCW are optimal for lipid enhancement. These outcomes are in line with the conclusions drawn by other authors, who have reported that lipogenesis increases under nitrogen-limited conditions [38]. In this context, an inverse relationship between lipid accumulation and urea concentration as the sole nitrogen source was observed in a strain of *S. rubescens* [39]. Similarly, *Scenedesmus obliquus* and *Scenedesmus* sp. LX1 showed improved lipid content under nitrogen starvation, exhibiting lipid contents of 58% and 53%, respectively [40,41].

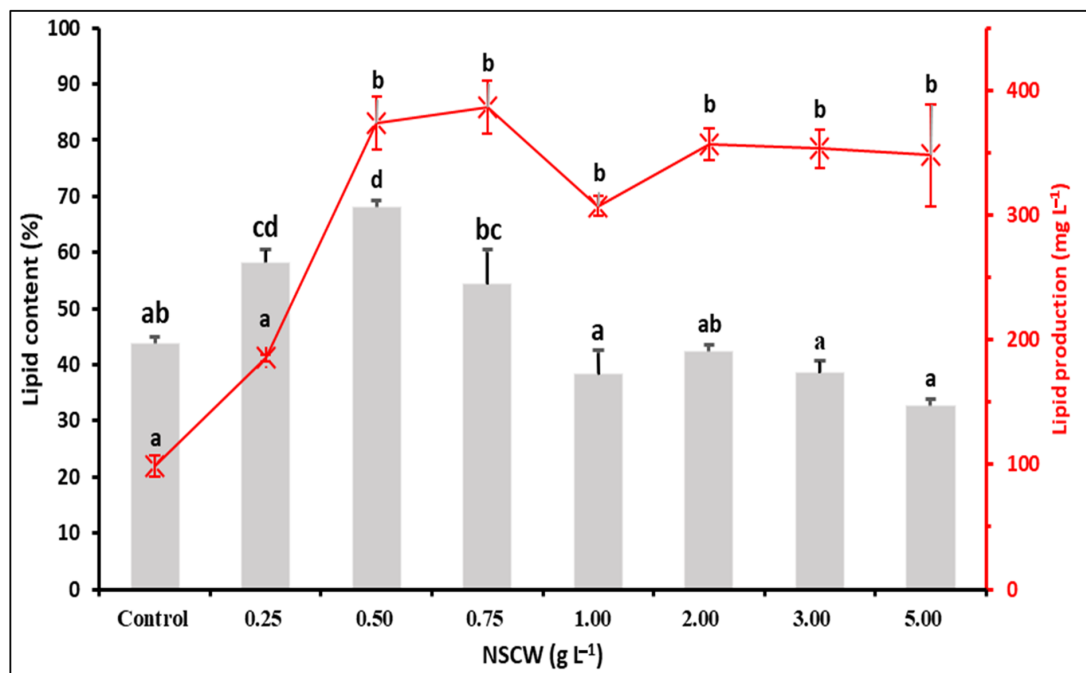


Figure 6. Lipid content and production of *S. rubescens* MDP19 at different NSCW concentrations. Different letters indicate significant differences between groups (Tukey test, $p < 0.05$).

2.6. Effect of NSCW on Chlorophyll Content and Total Carotenoid Content

Figure 7 indicates that the accumulation of chlorophyll a in *S. rubescens* MDP19 was directly proportional to the concentration of NSCW, reaching a maximum of 20.16 mg g^{-1} for cultures maintained in an NSCW concentration of 5 g L^{-1} . This content was found to be 7.93 times higher than that of the control samples, which exhibited a value of 2.54 mg g^{-1} . However, the content of chlorophyll b appeared to be less influenced by the concentration of NSCW in the culture medium. The chlorophyll b content ranged from 6.93 to 11.70 mg g^{-1} . Concerning the total carotenoids, a significant enhancement of their content was only observed at low NSCW concentrations. The highest total carotenoid contents were found to be 4.65 , 1.51 , 0.86 , and 0.23 mg g^{-1} in the control and in cells grown at 0.25 , 0.5 and 0.75 g L^{-1} NSCW, respectively. Drawing from these results, it can be proposed that the observed increase in chlorophyll content at higher NSCW concentrations may be due to the high availability of nitrogen [42]. In opposition, the enhancement of carotenoid levels at minimal NSCW concentrations can be attributed to the scarcity of nitrogen at such low concentrations, which prompts the biosynthesis of carotenoids as a means of survival and adaptation in algal cells [43].

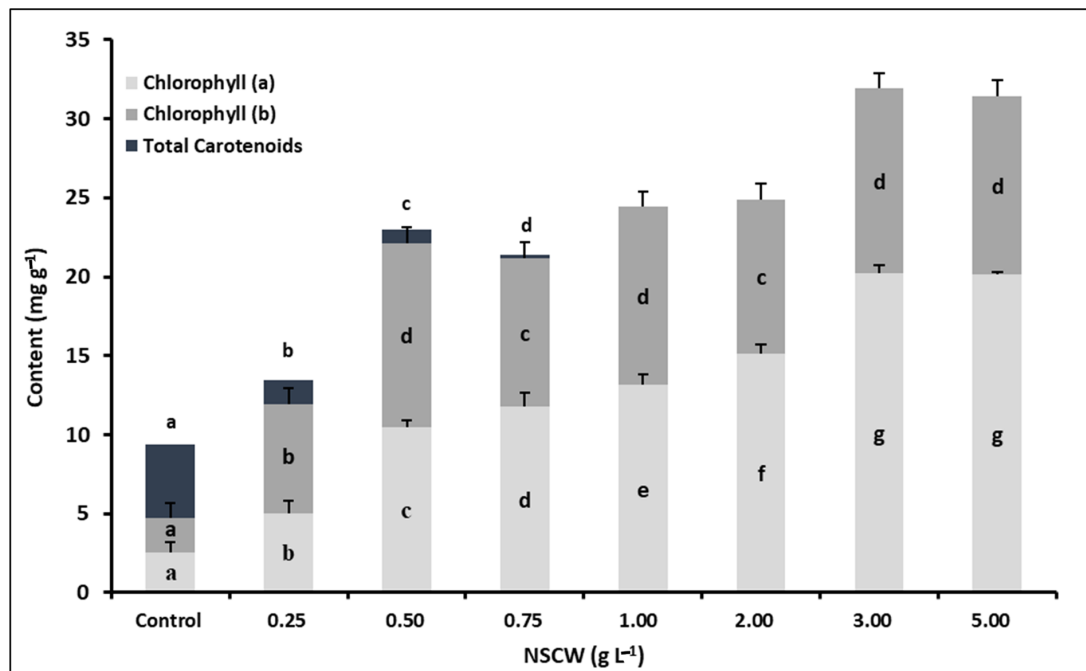


Figure 7. Chlorophyll a, chlorophyll b, and total carotenoid content of *S. rubescens* MDP19 at different NSCW concentrations. Different letters indicate significant differences between groups (Tukey test, $p < 0.05$).

2.7. Lipid Analysis by HPLC-APCI-MS

In the present study, 17 distinct lipid compounds were identified in *S. rubescens* MDP19 grown with 0.5 g L^{-1} NSCW, as detailed in Table 2. The detected compounds included four fatty acids: monounsaturated (palmitoleic acid (C16:1) and oleic acid (C18:1)) and polyunsaturated (linolenic acid (C18:3) and linoleic acid (C18:2)); seven glycolipids, comprising three digalactosyldiacylglycerols (DGDG (34:1), DGDG (34:2) and DGDG (36:6)) and four sulphoquinovosyldiacylglycerols (SQDG (32:1), SQDG (32:2), SQDG (34:1), and SQDG (34:2)); three phospholipids, including two phosphatidylethanolamines (PE (34:2) and PE (34:3)); and one phosphatidylglycerol (PG (36:2)). In addition, three acylglycerols (di- and triglycerides) were identified, specifically, DG (32:5), TG (SOO/SSL/PLA), and TG (OOMo/LnLn18:4). These results demonstrate the remarkable diversity of lipid composition in the isolated *S. rubescens* MDP19 strain. In comparison, the fatty acids identified in this study have been similarly reported in other strains of *S. rubescens* [44–46]. Nevertheless, to the best of our knowledge, this is the first investigation of the polar lipid profile of *S. rubescens*. It must be stressed that the lipid profile determined in this study is of great importance for the biodiesel industry. In particular, the presence of triacylglycerols is crucial due to their high energy density [47]. These lipids are known for their ability to be converted into methyl esters via the transesterification process, a fundamental step in the manufacture of biodiesel [48].

Table 2. Lipid profile of *S. rubescens* MDP19 by HPLC-APCI-MS.

Compounds	[M-H] [−]	%
C18:3	277.3	8.5 ± 1.3
C16:1	253.1	7.8 ± 0.9
C18:2	279.0	7.2 ± 0.8
C18:1	281.2	4.1 ± 0.4
DGDG (36:6)	935.6	3.4 ± 0.3

Table 2. Cont.

Compounds	[M-H] [−]	%
SQDG (32:2)	788.4	2.9 ± 0.5
DGDG (34:1)	937.5	9.5 ± 1.7
PE (34:3)	460.3	2.2 ± 0.2
SQDG (32:1)	790.5	1.9 ± 0.1
SQDG (34:2)	816.5	1.5 ± 0.1
PG (36:2)	792.5	5.8 ± 1.9
PE (34:2)	714.5	6.3 ± 2.1
SQDG (34:1)	818.5	7.1 ± 1.8
DGDG (34:2)	915.6	7.8 ± 1.9
DG (32:5)	581.5	8.2 ± 1.7
TG (SOO/SSL/PLA)	885.5	8.5 ± 1.9
TG (OOMo/LnLn18:4)	870.4	8.5 ± 1.8

2.8. Pigment Analysis by HPLC-APCI-MS

The pigment composition of *S. rubescens* MDP19 samples from cultures grown at a concentration of 0.5 g L^{−1} NSCW was analyzed by HPLC-APCI-MS, and the resulting data are summarized in Table 3. A total of eight pigments were identified, including three xanthophylls and five compounds related to chlorophylls and their derivatives. Neoxanthin was identified as the predominant xanthophyll, accounting for 58.0 ± 1.5% of the total carotenoid content. It was followed by canthaxanthin (21.5 ± 2.3%) and lutein (20.5 ± 2.1%) as the main carotenoids in this biomass. As for the chlorophyll content, it was found to be predominantly composed of geranylgeranyl-chlorophyll a (63.5 ± 2.5%), with comparatively smaller percentages attributed to chlorophyllide b (12.5 ± 1.8%), hydrochlorophyllide b (8.5 ± 2.6%), pheophytin a (8.0 ± 2.1%), and chlorophyll a (7.5 ± 1.9%). The composition obtained shows some agreement with literature data. In particular, chlorophyll a, lutein, and canthaxanthin were identified in a strain of *S. rubescens* grown under nitrogen deficiency stress [35]. However, neoxanthin, although identified in other species of the same genus such as *Scenedesmus obliquus* and *Scenedesmus armatus*, has not yet been reported in *S. rubescens* [49,50]. With regard to the other compounds identified, their presence has already been reported in some species of microalgae, but until now, they had never been detected in the studied species. For instance, chlorophyllide b has been reported in *Scenedesmus obliquus* [51]. Furthermore, geranylgeranyl chlorophyll a has been identified in the microalga *Picochlorum* sp. [52]. Additionally, the presence of pheophytin a has been identified in the microalga *Chlorella vulgaris* [53]. It is worthy of note that this analysis constitutes the first documented occurrence of hydrochlorophyllide b being identified in algae.

Table 3. Pigment profile of *S. rubescens* MDP19 by HPLC-APCI-MS.

Compounds	[M-H] [−]	[M+H] ⁺	λ _{max} (nm)	% (Carotenoids)	% (Chlorophylls)
Lutein	568.4 *		330–438–465	20.5 ± 2.1	
Canthaxanthin	564.4 *	565.4	472	21.5 ± 2.3	
Neoxanthin	599.1	601.2	418–438–465	58.0 ± 1.5	
Hydrochlorophyllide b	643.1	645.1	470–660		8.5 ± 2.6
Chlorophyll a	891.2	894.4	430–666		7.5 ± 1.9
Chlorophyllide b	-	629.2 #	469–654		12.5 ± 1.8

Table 3. Cont.

Compounds	[M-H] [−]	[M+H] ⁺	$\lambda_{\max}$ (nm)	% (Carotenoids)	% (Chlorophylls)
Geranylgeranyl-chlorophyll a	886.4	887.4 #	425–665		63.5 ± 2.5
Pheophytin a	869.4	871.4 #	406–665		8.0 ± 2.1

* corresponds to a radical anion (M[−]); # corresponds to a radical cation (M⁺)

3. Materials and Methods

3.1. Isolation and Identification of the Microalgal Strain

The seawater samples were taken from M'diq beach on the Mediterranean coast in northern Morocco. They were enriched in Guillard F/2 medium and cultivated in Guillard F/2 medium and maintained at a temperature of 23 ± 1 °C, under a 16:8 h photoperiod, with an intensity of 3000 lux. The cultures showing growth were purified by serial decimal dilution followed by plating onto Guillard F/2 agar. Individual colonies from the plates were picked and repeatedly plated on new F/2 agar plates until purification. The purity and morphological appearance of the isolated strain were evaluated by performing a microscopic examination. DNA extraction, PCR amplification of 18S rRNA, and partial sequencing of the 18S rRNA gene were carried out at SECUGEN, S.L., Moncloa-Ciudad Universitaria, Madrid, Spain.

3.2. Waste Characterization

The natural sheep casings waste (NSCW) used in this study was provided by a local industrial agri-food company. This waste consists of natural casings used in the production of sausages, made from the submucosa of sheep intestines. Following acquisition, the samples were subjected to multiple rinses with water, drained, and subsequently subjected to drying at 47 °C for a period of 48 h. Afterwards, the samples were rigorously ground, sieved, and stored in a dry, opaque container until use. The composition of the waste was examined in terms of total organic carbon (LCK TOC test Kits (Hach, Germany)), total nitrogen [54], total phosphorus [55], and ammonia [56], as well as total proteins [57], total amino acids [58], and total carbohydrates [59]. The main characteristics of NSCW are given in Table 1.

3.3. Testing the Effect of NSCW on Microalgae Growth and Biomass Production

3.3.1. Cultivation Experiments

The Guillard F/2 medium devoid of the nitrogen source (NaNO₃) was first prepared according to the standard formula [60] and then supplemented with different concentrations of NSCW (0.25 to 5 g L^{−1}) as a substitute for the nitrogen source. Each concentration tested was prepared separately, and the pH was adjusted to 7.2. The controls were prepared using Guillard's F/2 medium supplemented with NaNO₃ as a nitrogen source. Experiments were carried out with 50 mL of an exponential culture of the microalgal strain (approx. 5 × 10⁶ cells mL^{−1}), grown in Guillard's F/2 medium without any nitrogen source to deplete nitrogen reserves in the cells, and inoculated into 1 L conical flasks that each contained 450 mL of the appropriate growth medium. All cultures were performed in triplicate. Incubation was performed in batch mode with constant shaking at 23 ± 1 °C under a light intensity of 3000 lux and a light/dark cycle of 16.8 h.

3.3.2. Microalgal Growth, Biomass Production and Productivity

Cell density in each culture was assessed by daily microscopic counting using an improved Neubauer counting chamber.

Specific growth rate (μ) was estimated using Equation (1) as follows:

$$\mu(\text{day}^{-1}) = \text{Ln}(N_2/N_1)/(t_2 - t_1) \quad (1)$$

where N_1 and N_2 represent the cell densities achieved at time points t_1 and t_2 , respectively.

Biomass production (BP) was calculated using a calibration curve plotting cell density (OD at 680 nm) against dry cell weight (mg mL^{-1}) and expressed in g L^{-1} .

The daily biomass productivity (DBP) was calculated using Equation (2):

$$\text{DBP}(\text{mgL}^{-1}\text{day}^{-1}) = \text{BP}/\text{CV}/\text{CP} \quad (2)$$

where BP is the biomass production on dry weight (mg L^{-1}), CV is the culture volume (L), and CP is the cultivation period (days).

3.4. Testing the NSCW Utilization Capacity and Nutrient Removal Efficiency of the Microalgal Strain

To evaluate the efficacy of the algal strain in removing nitrogen and phosphorus from NSCW and to utilize its components (proteins, amino acids, carbohydrates) as nutrients, algal cultures were sampled at the beginning and end of the cultivation period. Analyses were conducted on cell-free supernatants. To separate the supernatants from the biomass, the samples were subjected to centrifugation at 5000 rpm for 10 min.

3.4.1. Total Nitrogen (TN) and Total Phosphorus (TP)

To determine TN and TP content, the samples were digested and analyzed using the standard Kjeldahl method [54] and the molybdate blue–ascorbic acid method [55], respectively.

3.4.2. Total Proteins

The protein contents were assayed by Lowry's Folin–Ciocalteu method [57]. A volume of 1 mL of the sample was reacted with 2 mL of a reaction mixture consisting of 10 mL sodium carbonate in water (2%, v/v), 100 μL potassium tartrate in water (1%, v/v), and 100 μL copper sulfate in water (0.5%, v/v), followed by 200 μL twice-diluted Folin–Ciocalteu reagent. The resulting mixture was then incubated in the dark at room temperature for a period of 30 min. The absorbance was measured at a wavelength of 760 nm. Protein contents were determined using a standard calibration curve prepared with bovine serum albumin.

3.4.3. Total Amino Acids

The total amino acids content was estimated using the ninhydrin assay, with minor modifications to the standard procedure [58]. In this method, an aliquot of 1 mL from the sample was combined with 0.5 mL of phosphate buffer solution (0.07 mol L^{-1} ; pH 8.04) and 0.5 mL of 2% ninhydrin solution made in ethanol. The resulting mixtures were boiled for 15 min, then diluted to 25 mL with sterile distilled water and incubated for 10 min. The absorbance of the resulting blue-violet color mixtures was determined by measuring the absorbance at a wavelength of 550 nm. A calibration curve was constructed using glycine as the reference amino acid, and this was employed to derive the amino acid contents.

3.4.4. Total Carbohydrates

The determination of total carbohydrates was conducted in accordance with the phenol-sulphuric acid method, as described by DuBois et al. [59]. The following procedure was employed: samples (1 mL) were treated with a 5% (v/v) phenol solution (0.5 mL) and concentrated sulfuric acid (1 mL), and the resulting mixtures were vortexed thoroughly.

Following a 20 min incubation period at 30 °C, the optical density was assessed at a wavelength of 485 nm. To estimate the carbohydrate content, a glucose calibration curve was utilized.

3.5. Testing the Effect of NSCW on Lipid and Pigment Content

3.5.1. Lipid Extraction and Quantification

The total lipid extraction was conducted in accordance with the protocols provided by Ren et al. [61]. Briefly, samples of 100 mg dried and ground biomass were treated with 7.5 mL chloroform/methanol solution (2:1, *v/v*) under ultrasonication for 20 min. The resulting mixtures were centrifuged at 7000 rpm for 5 min. To the collected supernatants, 1.88 mL of distilled water was added, and the resulting mixtures were centrifuged again at 5000 rpm for 5 min. Two distinct phases (aqueous and organic) were then observed. The organic phase of chloroform with the extracted lipids was collected and transferred to pre-weighed petri dishes. The petri dishes were permitted to dry at room temperature until the solvent had evaporated in its entirety. The weight of the petri dishes containing extracted lipids was again measured. The lipid content was derived using the following formula [Equation (3)]:

$$\text{Lipid content(\%)} = \frac{\text{weight of plates with extracted lipid} - \text{weight of empty plates}}{\text{Weight of extracted biomass}} \times 100 \quad (3)$$

Lipid production was assessed as follows Equation (4):

$$\text{Lipid production}(\text{mg L}^{-1}) = \text{Biomass production}(\text{mg L}^{-1}) \times \text{lipid content(\%)} \quad (4)$$

3.5.2. Pigments Extraction and Quantification

Chlorophylls and carotenoids were extracted from samples of 10 mg dried biomass using an acetone and water (80:20, *v/v*) solvent solution under ultrasonication for 5 min. This was followed by centrifugation at 5000 rpm for 5 min. The absorbance of the resulting supernatants was recorded at three wavelengths: 470 nm, 652.4 nm, and 665.2 nm. The contents of chlorophylls (a and b) and total carotenoids were calculated using Linchtenthaler's Equations (5)–(7) [62], and the results are given in milligrams per gram (mg g^{-1}) on a dry matter basis.

$$\text{Chlorophyll (a)} = (12.25 A_{663.2}) - (2.79 A_{646.8}) \quad (5)$$

$$\text{Chlorophyll (b)} = (21.5 A_{646.8}) - (5.10 A_{663.2}) \quad (6)$$

$$\text{Total Carotenoids} = (1000 A_{470}) - (1.82 \text{ Chlorophyll (a)}) - (85.02 \text{ Chlorophyll (b)}) / 198 \quad (7)$$

3.6. Analysis of Lipids and Pigments by HPLC-APCI-MS

3.6.1. Lipid Extraction

The lipid extraction was carried out using the Folch method [63]. An amount of 100 mg of lyophilized biomass was extracted with 2 mL of chloroform/methanol/water (2:1:0.75, *v/v*) and centrifuged at 3000 RCF for 15 min. This procedure was repeated three times. Subsequently, the extract was evaporated with nitrogen gas, removing the organic solvents. After drying, the final extract was redissolved in 300 μL of 2-propanol and 100 μL of a chloroform:methanol:water mixture.

3.6.2. Pigments Extraction

To identify chlorophyll and carotenoid compounds, 200 mg of lyophilized biomass was extracted with 2.5 mL acetone/methanol (7:3, *v/v*) in an ultrasonic bath for 20 min. The extraction procedure was carried out three times. The EZ2 system was used to evaporate the

resulting solution at a temperature of 35 °C. The concentrated extracts were then dissolved in a solution of methanol and methyl-tert-butyl ether (1:1, *v/v*) and filtered through a 0.22 µm polytetrafluoroethylene (PTFE) syringe filter.

3.6.3. HPLC-APCI-MS Analysis

Lipids and pigments analysis was conducted using a Shimadzu Nexera LC-30A system (Shimadzu, Kyoto, Japan), consisting of a CBM-20A controller, two LC-30AD parallel dual-piston pumps, a DGU-20A5 degasser, a CTO-20A oven, a SIL-30AC autosampler, and an SPD-M20A photodiode array (PDA) detector. The HPLC system was connected to a single-quadrupole LCMS-2020 mass spectrometer equipped with an APCI interface (Shimadzu, Kyoto, Japan). Two Ascentis Express C18 columns, each measuring 10 cm × 2.1 mm; 2.7 µm (Merck Life Science, Merck KGaA, Darmstadt, Germany), were coupled in series for chromatographic separation. A linear gradient of acetonitrile/water (ACN/water) (80:20, *v/v*) (A) and 2-propanol (B) was applied at a mobile phase flow rate of 500 µL/min: 0–103 min, 0–85% B (held for 3 min). The injection volume was 10 µL. The following settings were applied during mass spectrometry: *m/z* range, 150–1200; ion accumulation time, 0.6 s; nebulizing gas flow rate (N₂), 2 L/min; detector voltage, 4.5 kV; interface temperature, 450 °C; CDL temperature, 250 °C; and block temperature, 300 °C. PDA detection was employed in the range 220–700 nm with a sampling frequency of 12.5 Hz and a time constant of 0.080 s. Chromatograms were elaborated at 450 nm and 650 nm to confirm the presence of carotenoids and chlorophylls, respectively.

3.7. Statistical Analysis

Statistical analysis was conducted using a one-way analysis of variance (ANOVA) test with SPSS software version 20.0 (*p* < 0.05). All assays and measurements were conducted in triplicate. The findings are reported as mean ± standard deviation (SD).

4. Conclusions

This study demonstrates the feasibility of using natural sheep casings waste (NSCW) as a nitrogen source for the culture of the marine microalga *S. rubescens* MDP19, isolated from the M'diq Sea (northern Morocco). *S. rubescens* MDP19 was found to exhibit rapid growth and high biomass productivity across a range of NSCW concentration levels, accompanied by substantial nutrient removal. A lipid content of up to 68.11% was achieved by *S. rubescens* MDP19 at an NSCW concentration of 0.5 g L^{−1}. The lipid composition of *S. rubescens* MDP19 was notably diverse, suggesting its substantial potential for biofuel production. Additionally, the findings indicated an enhancement in carotenoid accumulation in the presence of merely minimal NSCW concentrations. In summary, the results of this study demonstrate the viability of *S. rubescens* MDP19 as a promising candidate for the treatment of solid organic waste from the meat processing industry. This approach offers numerous benefits. It would contribute to a reduction in the costs and environmental impact of treating such wastes, while concomitantly enhancing the sustainability and efficiency of industrial processes. However, future research should concentrate on optimizing the cultivation parameters and treatments used to improve both biomass production and lipid content.

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