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Physicochemical Characterization of Oil and Chitosan Extracted from Cross Breed and Bivoltine Hybrid Silkworm Pupae: A Comparative Analysis

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The oil and chitosan were extracted from silkworm pupae and analysed its physicochemical properties. Oil content in silkworm pupae ranged from 26 to 28 % on a dry weight basis. Notably, female pupae had a higher oil content (27.99 %) compared to males (27.03%). Further, bivoltine hybrids exhibited a slightly higher oil content (27.65 %) than cross breeds (27.30 %). In comparison

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to edible oils like groundnut oil and sunflower oil, the pupal oil showed favourable results in terms of moisture content, iodine value and saponification values, indicating quality similar to edible oils. However, acid value of silkworm pupal oil (1.570 mg KOH/g oil) was lower than that of groundnut oil (3.125 mg KOH/g oil). The acid value and peroxide value were found to be lower than three in pupal oil samples, revealed that pupal oil is good for edible purposes and indicates good oxidative stability. Among the samples, Chitosan content was higher 2.526% in the cross breed (PM × CSR2) than bivoltine hybrid 2.308%. Male silkworm pupae had a higher chitin content (3.242%) compared to female pupae (3.013%) and chitosan content was also higher (2.430%) in male pupae than in female pupae (2.345%). Pupal chitosan shows better solubility (99 %), degree of deacetylation (>85%) and ash content (<1%). These physicochemical properties were achieved the desired level for utilizing the silkworm pupal oil and chitosan as commercial utilization.

Keywords: Silkworm pupae; chitosan; pupal oil; degree of deacetylation.

1. INTRODUCTION

Silkworm cocoons are composed with outer silk layer which is commercially important for textile industry and it has been reeled by the reeling process. Inside the cocoon, it contains pupae which constitutes 60 % of cocoon (dry wt. basis). These spent pupae are the major by-product produced in large quantities after reeling process. For every one kg of raw silk, eight kg of wet pupae (2 kg of dry pupae) are produced. Around 40,000 metric tons of pupae, measured by dry weight, are annually generated and regarded as waste material. Considering the worldwide silk production of approximately 1.60 million tons, it's inferred that at least three million tons of pupae are accessible each year [1].

The silkworm pupae are reported to possess a high nutritive value in terms of protein, fat, glycogen, chitin, good quantities of vitamins (such as pyridoxal, riboflavin, thiamin, ascorbic acid, folic acid and nicotinic acid), minerals, fibre and lipids [2]. Silkworm pupae boast high protein and fat content. The oil obtained from silkworm pupae contains over seventy percent unsaturated fatty acids, notably α-linolenic acid and oleic acid. The usage of this oil extends to various applications, including its utilization in food, pharmaceuticals and cosmetics [3,4]. After the oil is taken out, the leftover material without the fat, known as defatted material contains chitin. Chitin is usually obtained from shrimps and crustaceans on a large scale in industries. But, an alternative source of chitin is found in silkworm pupae. This alternative source provides chitosan, a substance derived from chitin after a specific process called deacetylation [5,6,7].

One significant drawback of the silkworm pupae collected after silk reeling is its high moisture content, typically ranging from 70 to 75 %. This excessive moisture content makes pupae susceptible to microbial activity leading to substantial environmental concerns. Thus, there is a need for utilization of the leftover pupae as reeling waste, these valuable resources are simply being discarded as waste or under-utilized [8]. Considering all these aspects and to convert them into valuable products, presently attempt was made to know the recovery percentage of oil and chitosan from the silkworm pupae. The present study was intended to understand further the physicochemical properties of extracted chitosan and pupal oil.

2. MATERIALS AND METHODS

2.1 Materials

For this experiment, the cross breed (PM × CSR2) and bivoltine hybrid (FC1×FC2) silkworm pupae were obtained from the commercial reeling unit, College of Sericulture, Chintamani - 563 125 and Wahid reeling unit, Kolar, respectively. Pupae were cleaned and dried for oil extraction, commercial sunflower oil and groundnut oil were used to compare the physicochemical properties with pupal oil. The hexane was used as solvent for oil extraction. The Sodium hydroxide (NaOH) and Hydrochloric acid (HCl) were used to extract the chitin and chitosan.

2.2 Methods

Oil extraction: For extraction of oil, solvent extraction method given by Shanker et al. [9] was followed (pupae: hexane ratio of 1: 4).

Chitosan extraction: The chitin and chitosan extraction involved mainly three steps *viz*., Deproteinization, Demineralization and Deacetylation [7].

Deproteinization: After oil extraction defatted pupal powder was washed with distilled water to remove solvent residues and dried defatted pupal powder was treated for 4 h with 4 % NaOH at 70 °C with 1:10 ratio (material to liquid).

Demineralization: Deproteinized powder was treated with 3 % HCL (1:10, material to liquid ratio) heated at 25 °C to remove the mineral. After demineralization chitin was formed.

Deacetylation: Chitin was boiled with 45 % aqueous NaOH (1:12 ratio) at 90-95°C for 3 h to remove acetyl group resulting chitosan.

Physicochemical properties of oil: The specific gravity was examined at 25° C using a specific gravity bottle. Moisture content, acid value, peroxide value, iodine value, free fatty acids and saponification value of the extracted oil were examined by using standard methods [10] and density was tested by A.S.T.M. [11] method.

Iodine value (ppm): Silkworm pupal oil (0.3 to 0.4 g) was mixed with 25 ml carbon tetrachloride and 25 ml Wijs solution in a glass bottle. After standing in the dark for 30 min, 15 ml potassium iodide solution was added and the mixture was titrated with sodium thiosulphate ($Na_2S_2O_3$) solution (0.1N). Starch solution was used to detect the end point of the titration.

$$\text{Iodine value (ppm)} = \frac{12.69\ \{\text{Titre value (Blank)} - \text{Titre value (Sample)}\}\text{N of std. } Na_2S_2O_3}{\text{Weight pupal oil (g)}}$$

Free fatty acid (%): Five grams of silkworm pupal oil were mixed with a 50 ml combination of 95% alcohol and ether (1:1) in a 250 ml conical flask. After adding one ml of phenolphthalein indicator, it was titrated with 0.1N KOH until a constant pink colour appeared.

$$\text{Free fatty acid (\%)} = \frac{\text{Titre value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample(g)}}$$

Saponification value (mg KOH/g oil): Two grams of pupal oil mixed with 25 ml of 4% alcoholic KOH in a flask and heated until completely saponified. After cooling, it was titrated with 0.5N HCL using phenolphthalein indicator.

$$\text{Saponification value}\left(\text{mg}\frac{\text{KOH}}{\text{g}}\text{oil}\right) = \frac{56.1(\text{B} - \text{S})\ \text{N}}{\text{W}}$$

Where,

B = Volume ml of standard hydrochloric acid required for the blank

S = Volume in ml of standard hydrochloric acid required for the sample

N = Normality of the standard hydrochloric acid, and

W = Weight of the silkworm pupal oil taken for the test

Acid value (mg KOH/g oil): Two grams of silkworm pupal oil were measured in a dry 200 ml flask. 50 ml of neutralized hot ethyl alcohol and 1 ml of phenolphthalein were added. Boiled for 5 minutes, then titrated with 0.5 N standard potassium hydroxide solution.

$$\text{Acid value (mg KOH /g oil)} = \frac{56.1 \times \text{Titre value} \times \text{N of Std. KI solution}}{\text{Weight of oil (g)}}$$

Peroxide value (ppm): Silkworm pupal oil (5g) was added to a boiling tube with 1g of potassium iodide (KI) and a solvent mix (20ml, glacial acetic acid and chloroform in 2:1 ratio). Boiled for 30 seconds in a water bath. The contents were transferred to a flask with 20ml of 5% KI solution, washed twice with 25ml water, then titrated with 0.002M sodium sulphate using 1% starch solution [12].

$$\text{Peroxide value} = \frac{\text{Titre value (Sample value} - \text{Blank value)} \times \text{M of } Na_2S_2O \times 1000}{\text{W}}$$

2.3 Physicochemical Properties of Silkworm Chitosan

Moisture Content (%): Moisture content of the chitosan was determined by the gravimetric method [13].

$$\text{Moisture content (\%)} = \frac{\text{Wet weight(g)} - \text{Dry weight(g)}}{\text{Wet weight(g)}} \times 100$$

Ash (%): Two grams of chitosan were put into a clean crucible and heated in a furnace at 500°C for 2 hours. After cooling, the crucible and its contents were weighed A.O.A.C. [14].

$$\text{Ash (\%)} = \frac{\text{Weight of residue (g)}}{\text{Sample weight (g)}} \times 100$$

Viscosity (cp): Chitosan viscosity was measured using an Ostwald viscometer. 0.5g of

chitosan was dissolved in a mix of 10 ml 0.5M acetic acid and 20 ml 0.25M sodium chloride, then stirred for 10 mins in a vortex mixer [15]. A vertical viscometer held on a stand filled with solution up to mark A. Solution flow time from mark A to B was measured thrice. Then, compared the flow time of the test liquid with a known viscosity liquid.

$$\text{Viscosity (cp)} = \frac{f_1 t_1}{f_2 t_2} \times \eta_2$$

Where,

f_1=Density of chitosan solution
t_1 =Time of flow of chitosan liquid
f_2 =Density of standard liquid
t_2 =Time of flow of standard liquid
η_2=Viscosity of standard liquid

Solubility (%): Chitosan powder (0.1g) dissolved in 10ml of 1% acetic acid for 30 mins at 25°C using an incubator shaker (240 rpm). The solution was boiled for 10 mins, cooled and centrifuged at 10,000 rpm for 10 mins. Supernatant was removed. Undissolved particles were washed with 25ml distilled water, centrifuged again at 10,000 rpm and dried at 60°C for 12h [16].

$$\text{Solubility (\%)} = \frac{(\text{Initial weight of tube + chitosan}) - (\text{Final weight of tube + chitosan})}{(\text{Initial weight of tube + chitosan}) - (\text{Initial weight of tube})} \times 100$$

Determination of degree of deacetylation (DD): Potentiometric titration assessed to measure DD [17]. Chitosan (200 mg) dissolved in 20 ml of 0.1 M hydrochloric acid was mixed with 25 ml of distilled water and stirred for 30 min. Then, another 25 ml of water was added and stirring continued for another 30 min until complete dissolution. The resulting solution was titrated against 0.1 M sodium hydroxide. Degree of deacetylation of chitosan was calculated using Eq. [18]

$$\text{DD (\%)} = 2.03 \frac{V_2 - V_1}{m + 0.0042(V_2 - V_1)}$$

Where,

m - Weight of the sample
V_1, V_2- Initial and final burette reading.
2.03 - Coefficient resulting from the molecular weight of chitin monomer unit
0.0042- Coefficient resulting from the difference between molecular weights of chitin and chitosan monomer unit

Nitrogen (%): Nitrogen content was determined using Micro-kjeldhal method A.O.A.C [19].

pH: Chitosan of 0.5g was dissolved with 50 ml of distilled water and used to measuring the pH by using a Digital pH meter.

3. RESULTS AND DISCUSSION

3.1 Silkworm Pupal Oil Yield

The silkworm pupal oil was quantified and expressed in percentage (Table 1 and Plate 1). A noteworthy disparity was reflected in pupal oil content among the different breeds. Bivoltine hybrid female pupae exhibited the highest oil yield (28.14%), followed by bivoltine hybrid male pupae (27.24%) and the lowest oil content was observed in cross breed male pupae (26.83%). Whereas, the oil yield from cross breed female pupae (27.78%) was on par with bivoltine hybrid female pupae (28.14%).

In the current study, oil yield was in the range of 26.83 to 28.14 %, align with the results of Supanida et al*.* [20], who documented oil contents ranging from 24 to 29% in five native varieties of *B. mori*. Similar findings were also reported by Longvah et al. [21], Heo et al*.* [22] and Thirupathaiah et al*.* [23], all of whom noted that the oil content in silkworm pupae ranged from 23 to 34%.

Table 1. Oil yield extracted from cross breed and bivoltine hybrid silkworm pupae

Samples	Oil yield (%)
S_1: Bivoltine hybrid male pupae	27.24[b]
S_2: Bivoltine hybrid female pupae	28.14[a]
S_3: Cross breed male pupae	26.83[c]
S_4: Cross breed female pupae	27.78[a]
F - test	*
SEm ±	0.123
CD @ 1 %	0.373

***Note**: * Significant; NS- Non-significant*

Plate 1. Silkworm pupal oil extracted from (A). Bivoltine hybrid female pupae (B). Cross breed female pupae (C). Bivoltine hybrid male pupae (D). Cross breed male pupae

In the current study, it was observed that female have a higher content of pupal oil as compared to males. In *B. mori*, Kotake et al. [4] found 9.0% oil in females and 4.8% in males. Ray and Gangopadhyay [24] noted 26.21% oil in female eri silkworms versus 24.13% in males. Anno. [25] also reported higher oil content in female *B. mori* (26.11%) compared to males (21.44% This increment in oil content of the pupae may be attributed to the greater overall lipid content in female pupae.

3.2 Physicochemical Properties of Pupal Oil

There were no significant differences observed in the moisture content, specific gravity, density, saponification value, acid value, peroxide value, viscosity and free fatty acid among different types of silkworm pupal oils (Table 2). However, significantly highest iodine value was noticed in bivoltine hybrid female pupal oil. In contrast, bivoltine hybrid male pupal oil had the lowest iodine value.

The physicochemical properties of pupal oil presented in Table 2. In pupal oil samples, the moisture content was found to be <0.066 % which was lower than the moisture content (0.203%) in ground nut oil and (0.280 %) in sunflower oil. The oil containing higher moisture (> 0.3%) leads to fungal mycelium growth specially *Aspergillus niger* and *Mucor sp.* [codex standard]. Regarding this, our extracted silkworm pupal oil appears to be favourable. In current study, the the density ranged from 0.903 to 0.913 g/ml which was similar to the density value of eri silkworm pupae oil reported by Ravinder et al. [26]. Furthermore, specific gravity varied between 0.911 and 0.916 g/ml consistent with the previous studies [27,28]. The saponification value analysed of silkworm pupal oil sample indicated similar values and the average value was 175.648 mg KOH/g oil which was similar to eri and mulberry pupal oil 187.24 [29]. In comparison to edible oils, groundnut oil and sunflower oil significantly surpassed the saponification value of pupal oils, with values of 192.83 and 183.86 mg KOH/g oil, respectively. Furthermore, the oil exhibited an acid value ranging from 1.480 to 1.655 mg KOH/g oil, indicating a low level of rancidity. The peroxide values of the oil were within the range of 2.3 to 2.5 ppm. It was worth noting that both the acid value and peroxide value, which were found to be less than three in the current study, can be considered indicative of good quality oil [27]. These results are consistent with previous studies [29,30,16] reported similarly low acid and peroxide values, both of which were less than three, thereby suggesting excellent oxidative stability in silkworm pupal oil. A low peroxide value indicated that the oil is relatively fresh and has not undergone significant oxidative changes. The iodine value of pupal oil samples not shown much difference. However, the bivoltine hybrid female pupal oil had the highest value at 112.25 ppm and least was found in the bivoltine hybrid male pupal oil at 109.71 ppm. Several studies confirm varying iodine values (100-128 ppm) in silkworm pupal oil [27,28,20, 31].

Table 2. Physicochemical properties of pupal oil extracted from cross breed and bivoltine hybrid silkworm.

Parameters	Moisture (%)	Specific Gravity (g/ml)	Density (g/ml)	Sap. value (mg KOH/g oil)	Acid value (mg KOH/g oil)	Peroxide value (ppm)	Iodine value (ppm)	Viscosity (cP)	Free Fatty Acids (%)
Bivoltine hybrid male pupal oil	0.040^{c}	0.912^{a}	0.903^{a}	175.88^{c}	1.558^{b}	2.401^{a}	109.71^{d}	33.523^{a}	4.120^{a}
Bivoltine hybrid female pupal oil	0.066^{c}	0.916^{a}	0.913^{a}	177.06^{c}	1.655^{b}	2.453^{a}	112.25^{ab}	33.585^{a}	4.215^{a}
Cross breed male pupal oil	0.034^{c}	0.911^{a}	0.900^{a}	174.25^{c}	1.480^{b}	2.340^{a}	110.25^{cd}	33.510^{a}	4.108^{a}
Cross breed female pupal oil	0.054^{c}	0.914^{a}	0.906^{a}	175.66^{c}	1.586^{b}	2.388^{a}	111.34^{bc}	33.288^{a}	4.190^{a}
Groundnut oil	0.203^{b}	0.914^{a}	0.905^{a}	192.83^{a}	3.125^{a}	1.17^{b}	84.88^{e}	30.018^{b}	1.445^{b}
Sunflower oil	0.280^{a}	0.914^{a}	0.906^{a}	183.86^{b}	1.04^{c}	1.12^{b}	121.81^{a}	28.173^{c}	0.813^{c}
F – test	*	NS	NS	*	*	*	*	*	*
SEm ±	0.011	-	-	1.053	0.128	0.036	0.374	0.330	0.096
CD @ 1%	0.032	-	-	3.153	0.384	0.107	1.12	0.988	0.288
CV%	19.155	-	-	1.17	14.751	3.627	0.747	2.061	6.106

*Note: * Significant; NS - Non-significant; Sap. value: saponification value*

Table 3. Chitosan yield from silkworm pupae of cross breed and bivoltine hybrid

Samples	Chitin (%) produced over dry wt. pupae	Chitosan (%) produced over dry wt. of pupae	Chitosan (%) produced over dry wt. of chitin
DP_1: Bivoltine hybrid male pupae	3.071^{c}	2.406^{c}	78.333
DP_2: Bivoltine hybrid female pupae	2.847^{d}	2.210^{d}	77.620
DP_3: Cross breed male pupae	3.413^{a}	2.667^{a}	78.128
DP_4: Cross breed female pupae	3.220^{b}	2.486^{b}	77.212
F - test	*	*	NS
SEm ±	0.016	0.02	-
CD at 1 %	0.049	0.06	-
CV%	1.145	1.808	-

*Note: * Significant; NS: Non-significant; DP: Defatted pupae.*

Table 4. Physicochemical properties of pupal chitosan.

Samples	Moisture (%)	N (%)	Ash (%)	DD (%)	Solubility (%)	Viscosity (cp)	pH
DP_1: Bivoltine hybrid male pupae	7.28^{a}	3.27^{b}	0.30^{b}	95.91^{a}	99.52^{a}	44.67^{c}	7.13^{a}
DP_2: Bivoltine hybrid female pupae	7.30^{a}	3.28^{b}	0.38^{b}	96.49^{a}	99.40^{a}	45.22^{bc}	7.16^{a}
DP_3: Cross breed male pupae	7.30^{a}	3.36^{b}	0.32^{b}	96.05^{a}	99.22^{a}	45.58^{b}	7.19^{a}
DP_4: Cross breed female pupae	7.24^{a}	3.28^{b}	0.37^{b}	96.86^{a}	99.25^{a}	45.31^{bc}	7.15^{a}
DP_5: Commercial chitosan (Control)	5.80^{b}	6.86^{a}	1.16^{a}	92.88^{b}	94.25^{b}	160.02^{a}	6.72^{b}
F - test	*	*	*	*	*	*	*
SEm ±	0.127	0.122	0.027	0.355	0.323	0.196	0.033
CD at 1%	0.385	0.370	0.082	1.079	0.982	0.597	0.100
C.V.	3.623	6.068	10.589	0.742	0.656	0.576	0.93

*Note: * Significant; NS: non-significant, N: Nitrogen, DD: Degree of deacetylation, DP: Defatted pupa*

The pH of pupal chitosan differed significantly in comparison with commercial chitosan (Table 4).

The oil's viscosity refers to a property that resists oil flow. Silkworm pupal oils varied slightly in viscosity. However, the pupal oil (33.585 cP) has slightly more viscous than sunflower (28.173 cP) and g. nut oil (30.018 cP). More viscosity of oil was due to high polyunsaturated free fatty acids content [32]. Free fatty acid (FFA) is considered edible oil's most important quality parameter. In this study, the FFA value was similar to the value of eri silkworm pupal oil reported in the literature [26], but higher than G. nut oil (1.445%) and sunflower oil (0.813%).

3.3 Chitin and Chitosan Yield of Silkworm Pupae

The data pertaining of % chitin and chitosan yield over silkworm pupae and % chitosan yield over chitin among the male and female pupae of cross breed and bivoltine hybrid are presented in Table 3.

Chitin yield (%): A significant difference in chitin (%) was found among the sexes of two different breeds of silkworm (Table 3). The chitin yield was significantly higher in cross breed male pupae (3.413 %), while lowest was in bivoltine hybrid female pupae (2.847 %). The pupal chitin yield of current findings was found to be higher in males than females and ranged from 2.8 to 3.4 % which are agreed with the earlier findings of Suresh et al. [7] Paulino et al. [33]; Zhang et al. [6], Ni and Liang, 1999, Aruga [34] whom have reported that the dried silkworm pupae contain 2.5 to 4 % of chitin.

Chitosan yield (%): The same trend of chitin yield was observed in chitosan yield. However, significantly highest yield was found in cross breed male pupae (2.667 %), while, lowest was in bivoltine hybrid female pupae (2.210 %) (Table 3). No significant difference was observed in chitosan percentage based on chitin weight in pupae. Higher values were observed in bivoltine hybrid male pupae (78.333 %). The pupal chitosan yield of current findings ranged from 2.00 to 2.50 % which is agreed with the results of [Luo et al., 2019; Paulino et al., 2006] reported that *B. mori* pupae content 2.50-3.00 % chitosan. Similarly, Suresh et al. [7] who reported that chitosan content varied from 2.10 to 2.60 % in pure races of *B. mori* and which found to be higher (2.45 %) in male pupae than in female pupae (2.29 %).

In the present findings, male pupae were found to have more chitin than females. Despite females being larger with greater weight, fat, and protein, there were more male pupae by volume. When considering volume, male pupae have more cuticle material. These size and shape differences might explain variations in chitin content.

3.4 Physicochemical Properties of Chitosan Extracted from Silkworm Pupae

The results with respect to the physico-chemical properties of chitosan extracted from pupae of cross breed (PM × CSR2) and bivoltine hybrid are presented in Table 4.

The moisture content of pupal chitosan samples ranged between 7.3 to 7.4%. Sandford [35] emphasized that chitosan's moisture content should not exceed 10 % for it to be suitable for commercial applications. The present findings are in conformity with the results of Suresh et al. [7], Fini and Orienti [36]. Nitrogen content in pupal chitosan samples varies within 3.36 % which was lower than commercial chitosan (from crustacean waste). Similarly, Suresh et al. [7] reported that nitrogen content was 3.32 and 4.12 % in chitosan extracted from mulberry and eri silkworm pupae resp. The ash content of pupal chitosan samples found to be less than 1% which was supported by Nessa et al. [37] maintained that a premium-quality chitosan grade should boast an ash content of less than 1%.

The degree of deacetylation of pupal chitosan samples ranged between 95 to 97 % which was higher than commercial chitosan (92.88 %). These findings are in agreement with the observations of Suresh et al. [7] who reported the degree of deacetylation (DD) observed in chitosan samples extracted from silkworm pupae varied significantly, spanning a range from 46.5 to an impressive 97 %. The pupal chitosan has solubility (99%) supported by earlier study. Similarly, Luo et al. [38] found 99.3 % solubility in silkworm chrysalis chitosan. Among the pupal chitosan's pH ranged between 7.13 to 7.19, which was higher than that of commercial chitosan (6.72). Among the pupal chitosan, the male pupae of cross breed (DP_3) showed more viscosity (45.58 cP) and least was found in male pupae of bivoltine hybrid (DP_1) (44.67 cP). Chitosan with a lower viscosity offers distinct advantages compared to its high-viscosity counterpart when employed in the food and pharmaceutical industries. The viscosity range of chitosan, derived from the exoskeletons of

mature two-spotted field crickets (*Gryllus bimaculatus*), spanned from 21.6 to 62.4 cP [39-41].

4. CONCLUSION

In conclusion, silkworm pupae are a viable raw material for pupal oil and chitosan production which is an alternative raw material for food and biochemical industries. Further, chitosan production from various sources of silkworm may help to determine its suitability for various biomedical applications. From the present study it is evident that, chitosan extracted from silkworm pupae shows better physicochemical parameters like solubility, degree of deacetylation, ash content *etc.* which will be utilized as alternative source of chitosan.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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