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Extraction and characterization of silkworm *Bombyx mori* pupae protein

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Abstract
Entomophagy is a re-emerging terminology used to describe the practice of consuming insects as a source of nutrition by human beings. In present study 4-6 days old silk cocoons were procured, pupae were collected and subjected for drying at 70ºC for 48 hours, grounded and defatted (N hexane). Protein (crude) was extracted by acid- alkali pH (5.7) shift method. The results revealed that, dried pupae consist of 38.13% of protein, the true protein content of crude protein was 81.02%. proximate (AOAC), colour, water activity, protein solubility were analysed and characterized by analysing functional properties *viz.*, water absorption capacity (3.08± 0.02 g_{water}/g_{DM}), oil absorption capacity (4.05±0.03 g_{oil}/g_{DM}), emulsifying activity (1.93±0.09%), emulsifying stability (1.85±0.108%), foaming capacity (7.67±0.47%), foaming stability (5.83±0.23%) least gelation capacity (10.67±0.94w/v), bulk density (0.38±0.01g/ml) and tap density (0.46±0.008g/ml). Silkworm pupae protein proved to be cheap, edible and alternative protein source obtained as a by-product from sericulture industry.

Keywords: Entomophagy, pupae protein, functional properties, alternate protein source, by-product

Introduction
Proteins are employed as an integral source in food industry for the composition of food formulations. Food proteins and its concentrates and isolates have multiple applications such as it includes beverages, meat analogues, texturized vegetable protein, fat replacers, functional foods, cosmetics *etc.*, By 2050 it is expected that the desire for protein may increase to double, hence there is a requirement of innovatory and reasonable protein sources. Insects have been acknowledged as the novel, alternative and valid source to fulfil future protein demands.

Entomophagy describes the consuming of insects or bugs as food source by human. It is practiced in 3,000 ethnic groups around the globe, including Central and South America, Africa, Asia, Australia and New Zealand. There are around 1900 insect species were reported as edible and silkworm pupae (Bombyx mori) is one among the edible insects. Insects are consumed at different life stages including eggs, larvae, pupae and adults. The main edible insect species are beetles, caterpillars, ants, bees, wasps (Hymenoptera), grasshopper locusts (Orthoptera), aphids, leafhoppers, true bugs, termites, flies (Diptera), silkworm *etc.*, Silkworm (*Bombyx mori*) are edible insects. Life cycle of silkworm includes four stages *viz.*, egg, larvae, pupae and adult. After extraction of silk from cocoon. The pupae are discarded as a waste which is rich in many nutrients especially protein. Sericulture industry spent waste, silkworm pupae are highly degradable and are applied as a fertilizer sometimes discarded as waste in open environment. Utilization of silkworm pupae as feed and in sustainable protein production, helps to reduce the malnutrition and to enhance food security are the environmentally friendly methods. Silkworm pupae is a rich source of fat, proteins and essential amino acids. Due to its fascinating nutritional profile, it has wide range of applications as a food, medicine and as an animal feed in many Asian countries from long time (Dong *et al.*, 2017) [17].

Consumption of silkworm pupae is practiced in certain parts of world, they are consumed as a novel food source in China, it is a chief source of protein to the people living in mountain regions of Japan. In India, silkworm pupae consumption is practiced by tribal people from early days in north east region of the country.

Insects can be considered as an alternative protein source with less environmental impact (van Huis., 2013) [19].

Insects are ingested as a whole or can be processed into a less noticeable forms, which increases the consumer acceptability. Although the ingestion of insects has numerous advantages. Presence of allergens in some insects and Limited consumer acceptance in developed countries, they do not practice entomophagy remains a hurdle to its widespread adoption. Utilization of insect proteins in food composition depending on its functional properties may be the best solution to tackle the drawback. Based on this a study was conducted at IIFPT, Thanjavur using *Bombyx mori* pupae, crude protein was extracted, physicochemical and functional properties were analysed. Protein was purified and analysed. The following are the steps involved in extraction of pupae protein.

Materials and Methods

Materials

Silk cocoons of 4-6 days old were procured from Government cocoon market, Channapatna taluk, Ramanagara district, Karnataka.

Method

Sample Preparation

Procurement of 4-6 days old silk cocoons

Collection of pupae from healthy cocoons

Drying using hot air oven (70°C, 48 hours)

Collection of dried pupae and grounded

Defatting using N hexane

Protein extraction (Acid-Alkali pH Shift method)

Protein Extraction

Extraction of silkworm crude protein was done by using Acid-Alkali pH Shift method. For getting better yield pH was adjusted to 5.7 (Zhao *et al.*, 2016) [11]. Defatted silkworm pupae powder was taken and mixed with 0.25M sodium hydroxide solution (1:15 w/v) and subjected for stirring (Magnetic stirrer) at 40°C for 1 hour.

The alkali treated sample was loaded into the centrifuge tube and centrifugation done at 5000rpm for 20 minutes at 4°C, then supernatant was collected followed by discarding the pellet. To precipitate protein in the supernatant, the pH had been adjusted to 5.7 by using 2M hydrochloric acid. precipitated protein solution was centrifugation was done at 4500 rpm for 15 minutes at 4°C and here pellet was collected by discarding supernatant. Collected pellet was freeze dried and then the freeze dried powdered was collected and stored in refrigerated condition.

Extraction yield (%) and Extraction rate of protein (%) was calculated by using below formulas.

$$Extraction\ Yield(\%) = \frac{Extract}{Sample} X100$$

$$Extraction\ rate\ of\ protein\ (\%) = \frac{Protein\ content\ in\ extract}{Protein\ content\ in\ sample} XEY(\%)$$

Proximate Analysis

Every insect species will have their own nutritional profile and importance. The proximate composition of silkworm pupae protein powder (SPPP) was carried out as per AOAC

international methods and analysis, performed in triplicate. Moisture content was estimated by drying 3g sample at 105°C for 3 h, ash content was determined by incineration at 650°C for 2 h, crude protein content was determined by Kjeldhal method using a protein-to-nitrogen conversion factor of 6.25 and fat content was estimated using Soxhlet method.

Physicochemical Analysis
All physicochemical analysis of SPPP was performed in triplicates.

Water activity
Water activity was measured to determine the effect of different processing steps on moisture content of SPPP. It plays an essential role in determining the shelf life of the product. Water activity was measured by using water activity meter.

Colour
Colour was measured to know the effect of different processing steps on colour of protein. It was evaluated by using the Hunter Lab-system where differences in colour were recorded in L*a*b* scale in terms of lightness (L*), redness (a*) and yellowness (b*).

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^{2+} (\Delta b)^2}$$

Protein Solubility
It was determined according to the procedure of Haryati *et al.*, 2020 [10]. SPPP of 0.5 g of was weighed and placed in a centrifuge tube, distilled water was added to makeup to 10 ml and the mixture was homogenized for 5 min. The centrifuge tubes containing the samples were heated at 60 °C in a pre-heated water bath for 30 minutes and the tubes were cooled at room temperature and centrifuged at 3,000 rpm for 20 minutes. The supernatant of 5ml was poured into a petri dish and dried in an oven (105 ˚C) and the residue was weighed.

$$\text{Solubility}(\%) = \frac{\text{Weight of residue}}{\text{weight of Sample}} \text{X100}$$

Functional Properties
Water Absorption Capacity (WAC)
WAC of **SPPP** was estimated according to procedure of Zhao *et al.*, 2016 [11]. One gram of sample was mixed in 10ml distilled water, blended for 5 min and centrifugation was done at 2060 rpm for 10min. The final weight of sample in centrifuge tube after decanting water was recorded and result was expressed as

$$\text{WBC } [g_{water}/g_{DM}] = \frac{M1-M0}{M0,DM}$$

Where
M_1 - Final weight of sample after decanting the supernatant
M_0 - Initial weight of sample
$M_{0,\,DM}$ - Initial weight of sample

Oil Absorption Capacity (OAC)
OAC of SPPP was estimated according to procedure of Zhao *et al.*, 2016 [11]. An 0.3g of sample was weighed and mixed with 3ml of corn oil then centrifuged at 2060g for 30min Then final weight of sample in centrifuge tube after decanting the supernatant oil was recorded.

$$\text{OAC } [g_{oil}/g_{DM}] = \frac{M1-M0}{M0,DM}$$

Where
M_1 - Final weight of sample after decanting the oil.
M_0 - Initial weight of sample
$M_{0,\,DM}$ - Initial weight of sample

Emulsifying capacity
Emulsion capacity was determined according to the procedure of Ndiritu., *2018* [12]. One gram of sample, mixed with 100 ml of distilled water and homogenised for 10 min. At 5th minute of homogenisation, 100 ml of corn oil was added and continuously stirred. The emulsion was centrifuged at 3000 rpm for 10 min and the volume of the emulsified layer was recorded.

$$\text{Emulsion capacity (EC) \%} = \frac{\text{Volume of emulsifies layer}}{\text{Volume of the suspension}} \text{X100}$$

Emulsifying stability
Emulsion capacity was determined as per the method given by Ndiritu., 2018 [12]. one gram of sample was mixed with 100 ml of distilled water and homogenised for 10 min. At 5th minute of homogenisation corn oil was added and stirred continuously and the emulsion was heated (85˚C, 30 min) and cooled back to room temperature. The emulsion was centrifuged at 3000 rpm for 10 min and the volume of the final emulsified layer was recorded.

$$\text{Emulsion Stability (ES) \%} = \frac{\text{Volume of emulsifies layer}}{\text{Volume of the suspension}} \text{X100}$$

Foaming capacity (FC)
The foaming capacity and foaming stability were determined according to the procedure of Coffmann and Garciaj 2005 [13]. SPPP of 2 grams was weighed and suspended in 100 mL of distilled water. The suspension was stirred for 5 min at a medium speed. Then the mixture was transferred to a 250 ml graduated cylinder and the increase in volume was recorded as the capacity to produce foam.

$$\text{FC \%} = \frac{V2-V1}{V1} X100$$

Where
V_1 - Initial volume of the solution
V_2 - Final volume after mixing

Foaming Stability (FS)
Foaming stability was determined by measuring the foam's volume that persisted after 30 min of measuring foaming stability

$$\text{FS\%} = \frac{V3-V1}{V1} X100$$

Where
V_1 - Initial volume of the solution
V_3 - Final volume of foam that persisted after 30 min

Least gelation concentration (LGC)
The LGC was determined by the procedure of Kaur and Singh *2007* [17]. Test tubes containing suspensions of samples 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, and 20% (w/v) in 5 ml distilled water, heated in a boiling water for 1 hour, then

followed by rapid cooling and further cooled at 4°C for 2 hours. Then test tubes were inverted. The concentration of above which the sample did not fall down or slip is the LGC of that sample.

Bulk density

Bulk density was analysed according to the procedure of Haryati *et al.*, 2020 [10]. Fifty grams of sample was weighed and filled into measuring cylinder to measure the volume. Bulk density was calculated by comparing the sample weight with the its volume inside the container used (g/mL).

$$\text{Bulk density (g sample/mL)} = \frac{Sample\ weight}{Volume\ of\ measuring\ cup}$$

Tap density

50g of SPPP was placed into a 100 mL measuring cylinder and the measuring cup was taped no more than 30 times. The final volume of the sample was recorded.

$$\text{Tap density (g sample/mL)} = \frac{Sample\ weight}{Final\ tapped\ volume}$$

Result and Discussion

Characteristics of silkworm pupae (*Bombyx mori*) protein

The raw material used for this research was 4-6 days old silk cocoon (Mulberry silkworm).

This study was intended for extraction of protein from silk pupae and to characterize the SPPP by analyse its physicochemical and functional properties. After procuring silk cocoons, pupae were collected by cut opening the cocoon. The average yield of pupae for 1kg silk cocoons was approximately 800 grams (80%).

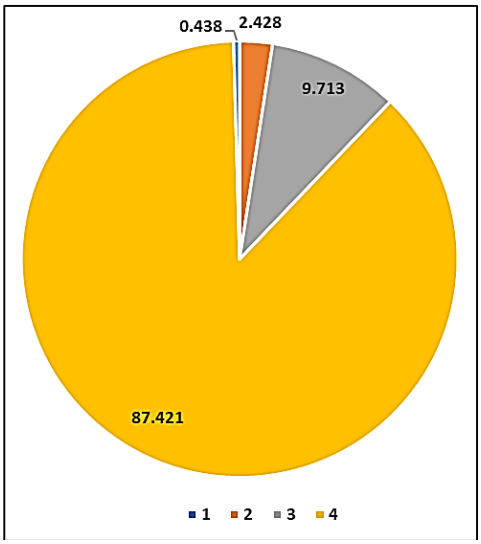

Fig 1: The average percentage of Silk cocoon components

[1- Exuvia, 2- Exterior Silk fibre, 3 – cocoon, 4 – Pupae]

The percentage of components of cocoons in this study is showed in Fig-1. The proportion of weight of cocoon to pupae was 1:9. Among all the components, the pupae constitutes the heaviest weight followed by cocoon shell, silk fibre and exuvia.

Sample preparation for protein extraction

After collection of pupae, the pupae collected were subjected for drying at 70°C for 48 hours. Dried samples were collected and grounded into powder and defatted using N hexane. For every 100 grams initial weight of live pupae, the weight of dried pupae was found to be 19 grams and after defatting the final weight of was found to be 10.41 grams. Proximate analysis of dried silk pupae powder (DSPP) was carried out using AOAC protocols Fig-2.

Protein content of dried silkworm pupae powder was 38.13± 1.05%. This value indicates that the silkworm pupae have a good nutrition potential, especially a good source of protein, takes part in various biochemical functions in the body like antibodies, enzymes, repairing of damaged tissues, formation of new tissues in the body *etc.*, In addition, it is also used as an energy source that gives equal calories as carbohydrates. Fat was the second most abundant component found (27.19±0.93%), which is used as an energy source. Ash was the third most abundant chemical component (9.47±0.143%) found in DSPP.

The ash content in DSPP is an inorganic component in the form of minerals. The ash content of DSPP was little high because silkworm pupae is rich in different minerals, which is constituted of both major and minor minerals such as Zinc, sodium, Calcium, Magnesium, Phosphorous, Copper, Lead, Arsenic, Manganese and potassium. The moisture content of dried silkworm pupae powder was found to be less (1.88±0.07%).

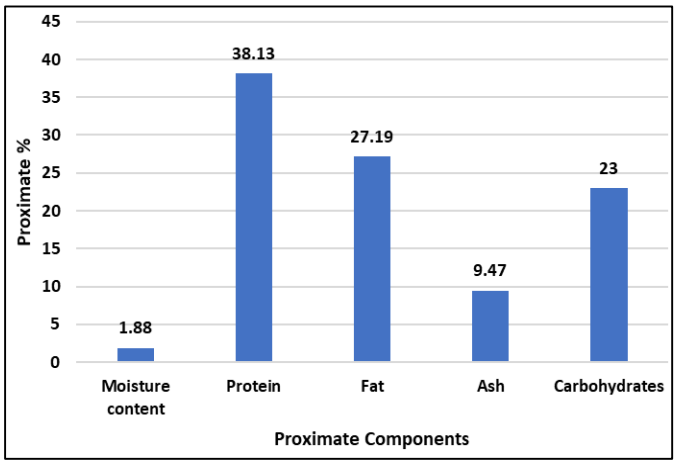

Fig 2: Proximate composition of DSPP (%)

Protein extraction

The silkworm pupae, used as raw material for protein extraction.

The selection of silkworm pupae is important form the proximate estimation it was showed to be high in protein and fat content among other chemical constituents. Protein was extracted by using Acid-Alkali pH (5.7) Shift method. It involves two main stages, namely the process of protein solubility in alkaline condition and the precipitation process by adjusting pH value to 5.7.

Extraction yield and Extraction rate of silkworm protein was calculated and are depicted in Table 1. The yield percentage of protein is an important parameter to determine the effectiveness and economic value of a product. The average yield of crude protein from DSPP was found to be 35%.

Table 1: Protein extraction, extraction yield and protein extraction rate

Sl.No	Observations	Values (%)
1	Weight of defatted pupae powder	100
2	Protein powder after freeze drying	35
3	Extraction yield	35
4	Extraction Rate of Protein	74.4

Proximate analysis of SPPP was done by following AOAC method. The results of the proximate analysis of SPPP is shown in Fig-3.

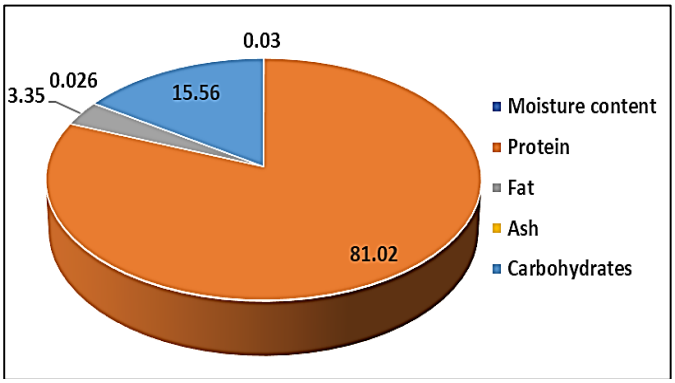

Fig 3: Proximate composition of SPPP (%)

The Protein contents of a product varies from sample to sample and it is affected by several factors such as the type of raw material, type of extraction, solvent, extraction time, centrifugation conditions drying method *etc.*,

Physicochemical Analysis

Water activity (aw)

It refers to the availability of free moisture content in the product. It indicates the safety and stability of food with respect to microbial growth rate of deteriorative reactions as well as physical and chemical properties or reactions. Water activity measurement is an essential parameter in the quality control of hygroscopic or moisture sensitive products or materials. If water activity is high, then there is a risk of microbial growth and water migration. The water activity of SPPP was found to be 0.34±0.005, which is insufficient for growth of food spoilage or pathogenic microbes like bacteria and fungi. Water activity is used in food preservation, food supply stabilization, and to develop different types of shelf-stable foods. Freeze drying is a method will reduce the water activity of foods. Dried or low- moisture foods do not contain more than 25% moisture (Erkmen & Bozoglu., 2016) [15].

Colour Measurement

Colour, has an important attribute which implies on the minds of people about food is concerned. Colour is a major attribute that influences the acceptance or rejection of edible insects (Tan *et al.*, 2015). Food colours influence appetite and choice of food. Consumers expect foods to have their own characteristic appearance. The colour value of SPPP was found to be 17.33±0.002.

Though the colour of dried silkworm pupae powder was dark it is similar to some of the commercially available health mixes. In comparison with the defatted sample, the results of the colour analysis showed that the lightness (L*) value of SPPP was decreased by almost 28%, redness (a*) value was increased by almost 13%, and yellowness (b*) value was decreased by almost 9%. Evidently the sample preparation method and protein extraction methods will significantly affect the yield and colour characteristics of the protein.

Protein Solubility

Protein Solubility is one of the most important physicochemical as well as functional properties of protein that depends on hydration and the degree of hydrophobicity of protein molecules. The protein solubility of the SPPP was found to be 9.05%. Protein solubility is most important for formation of emulsions, foams, and gels in designed food products**.** Water solubility of proteins is mainly governed by the net result of electrostatic repulsion and hydrophobic interaction but as hydrophobicity increases, solubility decreases.

Table 2: Physicochemical Parameters of SPPP

SL.NO	Physicochemical Parameters	Values
1.	Colour	17.33±0.002
2.	Water activity	0.34±0.005
3.	Protein Solubility	9.05±0.019

Functional properties

Water absorption capacity (WAC)

Water absorption capacity is the most important characteristic of protein**.** It refers to the ability of the protein to retain water during food processing against gravity which includes bound water, hydrodynamic water, capillary water and physically entrapped water. Amino acid profile, charge characteristic, hydrophobicity, pH, temperature, ionic strength and protein concentration are the factors affecting WAC of proteins. WAC depends on the protein content present in the food. The important properties such as hydration, solubility, viscosity, gelation in product development depends on protein-water interaction. The results showed that water absorption capacity of SPPP was 3.08± 0.02 (g_{water}/g_{DM}). This SPPP has a quite highwater absorption capacity. The high-water absorption capacity of the SPPP indicates the presence of high porosity, so that water get trapped inside the spaces among particles.

Oil absorption capacity (OAC)

The OAC of SPPP was found to be 4.05±0.03 (g_{oil}/g_{DM}). Oil absorption capacity, refers to the physical entrapment of oil to the protein and also to the number of non-polar side-chains of proteins that bind the fatty acids in the oil. The SPPP in this study has the ability to absorb amount of fats. The OAC is an important property, especially for food ingredient usually used in making dough, making cakes, sausages, salad sauces, and mayonnaise (Haryati *et al.*, 2020) [10]. OAC is greatly affected by Protein-oil interactions.

Emulsion capacity (EC) and Emulsion stability (ES)

The balance between hydrophilic and lipophilic bonds in food matrix effects the protein emulsion capacity. The balance in food between absorption of water and oil will affect the ability to form food emulsions. The emulsion capacity of SPPP was found to be 1.93±0.09%. The hydrophobic group in protein will tend to have strong affinity for lipid-soluble molecule, while the hydrophilic group had an affinity for water. In the formation of emulsion properties, there is an interaction of hydrophobic amino acids that bind to fat, and hydrophilic amino acids which forms a matrix network of protein molecules trapping water, thus forming surface molecules with low tension.

Emulsion stability is to test the effect of heating on formed emulsion. The emulsion stability of SPPP was 1.85±0.10%. This shows that emulsion capacity slightly decreases upon heating.

Table 3: Functional properties of SPPP

SL. No	Functional Parameters	Values
1.	Water Absorption Capacity (g_{water}/g_{DM})	3.08± 0.02
2.	Oil Absorption Capacity (g_{oil}/g_{DM})	4.05±0.03
3.	Emulsion Capacity (%)	1.93±0.09
4.	Emulsion Stability (%)	1.85±0.108012
5.	Foaming Capacity (%)	7.67±0.47
6.	Foaming Stability (%)	5.83±0.235
7.	Least Gelation Capacity (W/V)	10.67±0.94
8.	Bulk Density (g/ml)	0.38±0.01
9.	Tap Density (g/ml)	0.46±0.008

2Foaming capacity (FC) and Foaming stability (FS)

Dispersion structure containing colloidal fluid is called foam, it consists of two constituents namely, dispersing medium (protein solution) and dispersed phase (gas or air). The factors such as viscosity, surface tension, and the nature of the film formed on the surface of the liquid are factors that influences the foam formation. The ability of proteins in trapping gas is the main factor determining the characteristics of protein foam. The foaming capacity of SPPP was 7.67±0.47. The foam formed by SPPP was stable up to 17 minutes, then it decreased with the length of time of observation. The foaming capacity of this study was found to be lower; the foaming capacity of the sample was higher than the stability of the formed foam. At high viscosity and low surface tension foam will be relatively stable. Foaming capacity depends on the flexibility of the molecule and the physicochemical properties of the protein [Chamalaiah *et al*., 2011] [16].

Least gelation concentration (LGC)

SPPP showed its ability to form gels at concentrations of 10.67±0.94 (w/v). The interaction between protein and protein or a protein and water through disulfide bonds and hydrogen bond against hydrophilic amino acid groups are responsible for the formation of gel strength. Disulfide bonds have a big contribution to gel formation. Gel formation takes place due to the hydrophobic interaction in the protein network. Sulfhydryl bonds formation can cause the water around it to be trapped.

The more water trapped inside the gel, the bigger the gel will be. The functional characteristics of SPPP in this study shows its ability to use it as an ingredient, emulsifiers, substitutes, binders and gelling agents in various applications of high protein-based food products.

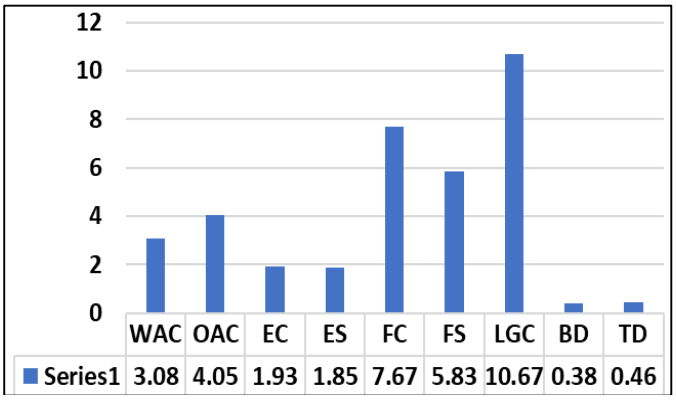

Fig 4: Functional Properties of SPPP

Bulk density

Bulk density is the ratio of the mass of an untapped powder sample and its volume including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed and it is expressed in units of g/ml. The smaller the density of the sample, the bulkier the material is. It is an important characteristic in designing packaging material. The value of the bulk density of SPPP was 0.38±0.01 g/ml. The bulking properties of a powder are dependent upon the preparation methods, treatment and storage condition of the sample.

Tap density

The tapped density refers to the increased bulk density attained after mechanically tapping a container containing the powder sample. Tap density can be used to index the ability of powder to flow. The value of the tap density of SPPP was 0.46±0.008 g/ml.

Conclusion

The Silkworm pupae contains 81.02 ± 0.24 true protein, and it is also a good source of fat as well as minerals. The physicochemical properties and functional properties of SPPP were found to be suitable for new product development or in developing protein rich food product development. Although silkworm pupae were consumed in various parts of the world, there are reports available on the allergens present in silkworm pupae. Hence there is a need to study the safety aspects of silkworm pupae.

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