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Commercial Enzymes for Hydrolysates from BSF, *Hermetia illucens* L.

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Abstract

Enzymes are proteinaceous bio-compounds serving for catalysis and accelerating rate of bio-chemical reactions. The enzyme involved hydrolysis is dealing with disintegration of the complex compounds through utilization of enzymes (or the source of enzymes) followed by the reaction with water. The present attempt is dealing with analysis of influence of chemical protein-extraction reaction, and hydrolysis through the use of enzymes (Alcalase, Papain and Pepsin) on the functional-properties, activity of antioxidation and composition of amino-acids of the protein contents of black soldier fly (BSF), *Hermetia illucens* (L). Hydrolysate derived through the alcalase was reported for the most significant degree of hydrolysis (DH) ($p < 0.05$). Hydrolysate derived through the pepsin was reported for the lowest oil holding capacity ($p < 0.05$). Lower Emulsifying-stability (ES) and foam-capacity (FC) were reported by hydrolysates in comparison with protein-concentrate ($p < 0.05$). The activity of antioxidation from protein hydrolysates derived from alcalase and from protein-concentrate was higher in comparison with that of hydrolysates derived from papain and pepsin ($p < 0.05$). The glutamic acid was reported as the presiding amino acid of the protein hydrolysates.

Keywords: Enzymatic hydrolysis, BSF, Hydrolysates, Alcalase, Papain, Pepsin

Introduction

According to the Food and Agriculture Organization (FAO), the population of the world is going to attain nine billion by the year: 2050. For the purpose to meet the demand of food at global level, it is a need to increase the production of the food by seventy percentages (FAO, 2020). However, the present practices of agriculture are not sufficient for sustainable supply of the food for such increasing global population. In addition, one out of eight number of people is facing the problem of food insecurity. One out of six children at global is not assured about next meal. The significance of security of the food has been underscored in the pandemic of COVID-19, during which many processors of food and the chain of supply of the food were closed and, thus created a shortage of food material and also increased insecurity of the food. Fifteen percentages of the total energy of diet of human being at global level correspond to the meat. Eighty percentages of land of agriculture are utilized for grazing the animals (or for the production of food or fodder for the livestock) (Herrero *et al*., 2015; Herrero *et al*., 2016). The meat is the most significant components for creating the insecurity of the food. Therefore, it is a need of modern age to reduce consumption of meat by human being (Kanianska, 2016). Kanianska (2016) recommend seventy percentages of reduction in consumption of meat. Reduction in consumption of meat by human being may solve the problem of insecurity of the food material. Loss of the food material appears to be one more challenge for the sustainability of the food material, economics and the status of nutrition of the food material. In United States, despite considerable progress of production of agricultural crops, practices of post-harvest and chain of management of supply, for each year, thirty to forty percentages (approximately) of the total production of the food are on the line of "loss" (Ziolkowska, 2017). Development of system of production of food of "Novel and Smart Qualities" is thus, prime concern for modern human society. The system of production of food of "Novel and Smart Qualities" is going to reduce wastage of food material and increase in the yield of production yield. The system of production of food of "Novel and Smart Qualities" is going to provide sustainable alternative-proteins. It may also exert minimum impacts on the environment.

On this line, entomophagy (eating the insects) is one of the sustainable systems of food and nutrition for human society. The human societies of Asian countries, African countries and of Latin America are presently following the system is entomophagy (or eating insects) as a part of their diet (Liceaga, 2019). According to Anaya *et al*. (2013), the insect groups are representing the significant and the largest sector of total global fauna. The insects are accounting for about ninety-five percentages of global biodiversity. The insects have historically been used for consumption at different stages (egg, larval instars, pupal stages and adults) of their life cycles. In the countries like Zambia and Nigeria, there is insufficient supply of the meat for human societies. Therefore, the life stages of the insects (especially, the

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larval stages) are the most significant source of proteins (Omotoso, 2006). The life stages of the insects are with sustainability. The life stages of the insects are with excellent value of nutrition. The life stages of the insects (especially, the larval stages) of are with protein (about fifty to seventy percentages), fats (about thirteen to thirty-three percentages) and fibers (about five to twelve percentages). The life stages of the insects are with low emissions of greenhouse gases (and their production too). They are with excellent ratio of conversion of feed. They need lowest quantities of water and non-expensive sources of feed. These qualities are responsible for accreditation of the life stages of the insects as "the most favourable source of food material for human being". The alternative proteins are developed by the life stages of insects. The insect derived proteins may be developed for food sources and the food products (Van Huis, 2013). The acceptance by the consumers appears to be the most significant hurdle in the system of utilization of insect life stages as food in the diet of human being. The fragmentation is going to increase the possibility of acceptance of life stages of the insects as a food material by human being. The life stages of the insects should be converted into powder rich in protein for easy acceptance and inclusion in a diet of human being (Borremans *et al*., 2020). There is possibility of recovery of the proteins, establishment of "Broad Food Spectrum" and the "Ingredients of the Feed" through the process of application of hydrolysis through enzymes. The concept of enzymatic hydrolysis is based on the principle of facilitation of breaking the bonds of complex compounds like proteins through the use of enzymes, with addition of water (elements of water) (Campbell & Reece, 2005). It may also help to establish improved and upgraded functional-properties of the protein contents of the life stages of insects (Ovissipour *et al*., 2013). On this line of attempts, there are few reports, which include: development of protein-hydrolysates through the use of insects like, cricket, *Gryllodes sigillatus* (L) (Hall *et al*., 2017); migratory locust, *Locusta migratoria* (L) (Purschke *et al*., 2018); mealworms, *Tenebrio molitor* (L) (Tang *et al*., 2018) and black soldier flies (BSF), *Hermetia illucens* (L) (Caligiani *et al*., 2018; Firmansyah & Abduh, 2019; Mintah *et al*., 2020; Zhu *et al*., 2020). There are no reports on enzymatic hydrolysis of the larval stages of black soldier fly (BSF) for the functional properties, antioxidant activities, nutritional values and the structure of the protein. On this much background, the present attempt has been planned.

Materials and Methods

The attempt concerned with the influence of food substrates on body weight and protein (soluble and Total) contents of black soldier fly (BSF), *Hermetia illucens* (L) was completed through the steps like: (A. Procurement of the larval-stages of black soldier fly (LBSF), *Hermetia illucens* (Linnaeus) (Order: Diptera, Family: Stratiomyidae); (B). Preparation of BSF Meal; (C). Protein Extraction from the Black Soldier Fly (BSF) Meal Through Method of One-Step Chemical Extraction; (D). Enzymatic Hydrolysis of the Black Soldier Fly (BSF) Meal and Measurement of Degree of Enzymatic Hydrolysis (DEH); (E). Properties of BSF-Protein-Concentrate and Hydrolysates; (F). Antioxidant Activity of BSF-Hydrolysates; (G). Quantification of the total protein in the hydrolysates; (H). Analysis of composition of Amino Acids and (I). Statistical Analysis of the Data.

Procurement of the Larval-Stages of Black Soldier Fly (LBSF), Hermetia Illucens (Linnaeus) (Order: Diptera, Family: Stratiomyidae)

For the present attempt, the larval-stages of black soldier fly (LBSF), *Hermetia illucens* (Linnaeus) (Order: Diptera, Family: Stratiomyidae) of twenty-one days old were procured from the Black Soldier Fly (BSF) Unit of ICAR-National Institute of Abiotic Stress Management (Malegaon-Karhavagaj Road, Khurd, Baramati, Taluka: Baramati, District: Pune Maharashtra State-413115 India). Here, at ICAR-National Institute of Abiotic Stress Management, in the well-established insectary, the laboratory staff initiated the rearing of the larval-stages of black soldier fly (LBSF), *Hermetia illucens* (Linnaeus) (Order: Diptera, Family: Stratiomyidae) through the use of commercial granular poultry feed. The weight of individual larval-stage of black soldier fly (LBSF), *Hermetia illucens* (Linnaeus) (Order: Diptera, Family: Stratiomyidae) was recorded. The larval-stages (twenty-one days old) of black soldier fly (LBSF), *Hermetia illucens* (Linnaeus) (Order: Diptera, Family: Stratiomyidae) were brought to Department of Zoology, Shardabai Pawar Mahila Mahavidyalaya, Shardanagar (Malegaon Colony) Tal. Baramati, Pune, Maharashtra State- 413115 India. Mahrashtra India.

Preparation of BSF Meal

The mature larval stages (pre-pupal stages) of the Black Soldier Fly (BSF), *Hermetiaillucens* L. (Diptera: Stratiomyidae) were selected randomly from the stock. They were kept in freezer at -35ºC for twenty-four hours. After twenty-four hours of freezing, they were subjected for thawing followed by washing thoroughly. The content was then processed for drying for forty-eight hours in oven (60 °C). Through the use of blender, the oven dried pre-pupal stages of the Black Soldier Fly (BSF), *Hermetiaillucens* L. (Diptera: Stratiomyidae) were subjected for grinding until smooth. The content thus obtained was titled as, "Black Soldier Fly Meal" (BSF Meal). The "Black Soldier Fly Meal" (BSF Meal) was stored in a refrigerator at -35ºC until use in experiments (Fukumoto, 1943).

Protein Extraction from the Black Soldier Fly (BSF) Meal Through Method of One-Step Chemical Extraction

The "Black Soldier Fly Meal" (BSF Meal) was processed for defatting. One part of the "Black Soldier Fly Meal" (BSF Meal) was mixed with two parts of ether (petroleum) (w/v). This mixture was processed for shaking through the use of shaking-incubator. Shaking was carried out for an hour for at room temperature. The petroleum ether (solvent) with lipid contents was separated. The whole process was repeated once again. This repetition was for the purpose to obtain the residues. The method of solvent evaporation (under a vacuum drier at 40 ◦C) was used for lipid-recovery. The defatted pellets were washed with deionized water. Washing the defatted pellets with deionised water is to remove solvent residue. For the perfection, washing the defatted pellets with deionised water was repeated three times. The BSF pellets (defatted) were weighed on electronic balance. The BSF pellets (defatted) was then treated with forty millilitres of sodium hydroxide (1 M NaOH). Treatment of BSF pellets (defatted) with sodium hydroxide (1 M NaOH) was carried out at 40 ◦C for an hour. The content was then

processed for centrifugation (at 4450× g for 30 minutes). The collected supernatant was precipitated with ten percentages of solution of trichloroacetic acid in acetone. The assay samples thus obtained were kept at minus twenty degree Celsius for overnight. The assay samples were then subjected for centrifugation (at 4450× g for 30 minutes). The residue (precipitation) was then washed with acetone. Washing the residue (precipitation) with acetone was carried for three times. To obtain concentrated protein (BSF Protein), the contents was dried in a freeze drier (Fukumoto, 1943; Taylor, 1957).

Enzymatic Hydrolysis of the Black Soldier Fly (BSF) Meal and Measurement of Degree of Enzymatic Hydrolysis (DEH)

Alcalase enzyme is also called as subtilisins. It belongs to the serine protease or to the group: subtilases. At the active site, the "Alcalase" enzyme initiate the nucleophilic reaction. It is acting on the bond of peptide (or amide) through the residue of serine-proteases. The "Alcalase Enzyme" is with molecular weight of about: 27kDa. Alcalase enzyme is derived from the soil bacteria, *Bacillus amyloliquefaciens* (L). This bacterium, *Bacillus amyloliquefaciens* (L) is considered as a "Growth Promoting Rhizobacterium". This bacterium, *Bacillus amyloliquefaciens* (L) was discovered by Fukumoto (Japanese scientist) in the year: 1943. Due to the nature of giving liquifying amylase, J. Fukumoto named the species as: amyloliquefaciens. The enzyme: alcalase, the protein digesting enzyme is commercially available. It was procured from Sigma-Aldrich through local dealer. Likewise, the crude powder (papain) ("Cysteine-Protease" derived from the latex of papaya, *Carica papaya* (L); the enzyme: pepsin (endoprotease enzyme derived from the gastric mucosa of porcine alimentary canal). Papain ("Cysteine-Protease" and pepsin were procured from Sigma-Aldrich through local dealer.

The "Black Soldier Fly Meal" (BSF Meal) and distilled water (in the ratio of 1:3 w/v) were mixed through the use of "Shaking-Incubator" at room temperature. Mixing the BSF Meal in distilled water was carried about two hours. Mixing the BSF Meal in distilled water was for hydration. After two hours, with constant stirring in shaker (mini), the temperature was increased to sixty degrees Celsius for about twenty minutes. Time duration and rate of this shaking was twenty minutes and 220 rpm respectively. There was addition of each enzyme (Alcalase, Cysteine-Protease and Pepsin) in the ratio of two percentages of "BSF-Meal". For the purpose of enhancement of efficacy of the process of "Enzymatic-Hydrolysis", one percentage of each of enzyme (Alcalase, Cysteine-Protease and Pepsin) was added for the initiation of the process of "Enzymatic-Hydrolysis" at the beginning. Remaining one percentage of enzyme (Alcalase, Cysteine-Protease and Pepsin) was added after an hour after the initiation of the process of "Enzymatic-Hydrolysis". In biochemistry, this process is called as, "Two Steps Hydrolysis". This process of "Enzymatic-Hydrolysis" was carried out for about two hours. The pH of medium for enzyme alcalase and papain were 6.85 and 3 respectively. For pepsin, the pH of medium of reaction was 3. The pH for pepsin was adjusted through the use of 0.1 M HCl. After two hours of process of "Enzymatic-Hydrolysis", the contents were subjected for heating for about ten minutes at ninety degrees Celsius in water bath. This heating was for the purpose to inactivate the reaction mixture at the end of process of "Enzymatic-Hydrolysis". After inactivation of the reaction mixture, the content (in the form of suspension) was subjected for centrifugation at 2500 rpm for about five minutes at room temperature. This centrifugation was resulted into appearance of three distinct phases, which include: phase of semisolid (at the bottom), intermediate supernatant phase of liquid and top supernatant phase of liquid. The bottom phase of liquid is containing proteins of insoluble nature and chitin. An intermediate supernatant liquid phase is containing hydrolysate of the proteins. A light liquid phase at the top is containing the fractions of the lipids. All the samples were kept in freeze at minus twenty degrees Celsius. An intermediate liquid phase of supernatant (the protein hydrolysates) was separated and processed for freeze-drying. After freeze drying, the protein hydrolysates were transferred into the polystyrene conical tubes and sealed them. The freeze-dried protein hydrolysates were stored at minus twenty degrees Celsius until further use. The weight of the oil, hydrolysates and solid layers were accounted for the calculation of the yield of fractions of hydrolysate on wet weight basis. All attempts of experimentation were performed in the sets of three replicates (N = 3). The formal titration as the proportion of alpha amino N terminal with respect to the total N in the sample was used for measurement of the degree of enzymatic-hydrolysis in triplicate set (Fukumoto, 1943; Taylor, 1957).

Properties of BSF-Protein-Concentrate and Hydrolysates

The methods explained by Shahidi *et al*. (1995) and Sathivel *et al*. (2005) were used for the analysis of the functional properties of "BSF-Protein-Concentrate" and "Hydrolysates". This analysis was carried in triplicate set. Five hundred milligrams of each sample were used for mixing in ten millilitres of oil of Canola. This mixture was used for the analysis of the "Fat Adsorption Capacities" of the "BSF-Protein-Concentrate" and "Hydrolysates". This mixture (Five hundred milligrams of each sample + ten millilitres of oil of Canola) was subjected for incubation for half an hour at room temperature. Thoroughly mixing the incubating mixture at the interval of ten minutes was followed. The contents were then subjected for centrifugation at 2500 rpm for about half an hour. This centrifugation allowed the oil content in reaction mixture to separate and free. The oil contents were removed. The resultant contents were used for evaluation of the "Oil Adsorption". Weight difference method was used for evaluation of the "Oil Adsorption". The results on the "Oil Adsorption" were expressed as "millilitres of oil adsorbed by one gram of the "BSF-Protein-Concentrate" and "Hydrolysates".

The stability of emulsification (or Emulsifying-Stability) (ES) of the content was determined through the method explained by Yasumatsu *et al.* (1972). Five hundred milligrams of each sample were taken in separate beaker (250 mL). Addition of fifty millilitres of 0.1 M sodium chloride (NaCl) (Yasumatsu, 1972). Addition of fifty millilitres of canola oil in each reaction mixture was made. Through the use of "Highspeed-Hand-Held-Homogenizer", the content was processed for homogenization for about two minutes. Aim of this process is to get maximum output and to create expected emulsion. From each emulsion, three portions (25 millilitres) were transferred into graduated cylinders.

The set was kept for about fifteen minutes at room temperature. The aqueous volume was measured. Likewise, total volume was also measured. The readings on aqueous volume and total volume were accounted for the calculation of stability of emulsification (or Emulsifying-Stability) (ES) of the content (Unit: Percentage). The mathematical formula used for the calculation of stability of emulsification (or Emulsifying-Stability) (ES) of the content (Unit: Percentage) is as below:

$$\text{The stability of emulsification (or Emulsifying-Stability) (ES)} = [(\text{Total volume} - \text{aqueous volume}) \div (\text{total volume})] \times 100 \quad (1)$$

The aeration method explained by Pacheco-Aguilar *et al.* (2008) was used for the determination of "Foam-Capacity and Foam-Stability" of the "BSF-Protein-Concentrate" and "Hydrolysates". In this method, twenty-five millilitres of deionized water were added in seven hundred and fifty milligrams of assay sample. The final pH= 6.5 (of the reaction mixture) was maintained. The content was mixed with the use of a stir bar. This mixing was carried out for about ten minutes at room temperature. The protein mixtures were subjected for aeration with the help of homogenizer. For the purpose to calculate the foam capacity, the volume of protein mixture before aeration was subtracted from the volume of protein mixture after aeration. The figure thus obtained was divided by volume of protein mixture before aeration. The quotient thus obtained was multiplied with hundred to obtain the percentage of foam capacity. The mathematical formula used for the calculation of foam capacity of protein mixture (Unit: Percentage) is as below:

$$\text{Foam capacity (\%)} = [(\text{volume after aeration} - \text{volume before aeration}) \div (\text{volume before aeration})] \times 100 \quad (2)$$

The readings on Foam Remaining After Ten minutes, thirty minutes and sixty minutes were -used for the determination of percentage of foam stability (FS).

Antioxidant Activity of BSF-Hydrolysates

The DPPH (Di-Phenyl-Picryl-Hydrazyl) free radical scavenging assay method explained by Valco *et al*. (2007) was utilized for determination of antioxidant activity of **BSF-Hydrolysates**. The solution of DPPH (Di-Phenyl-Picryl-Hydrazyl) with the strength of 0.2 mM was prepared in DMSO (Dimethyl Sulfoxide). The DPPH (Di-Phenyl-Picryl-Hydrazyl) was dissolved in seventy-five percentage of DMSO (Dimethyl Sulfoxide). One millilitre of **BSF-Hydrolysates** was mixed with one millilitre of fresh DPPH (Di-Phenyl-Picryl-Hydrazyl). This mixture was processed for incubation in dark for about an hour. Thereafter, the optical density of the content was measured at 515 nm. The spectrophotometer used was "Evolution 60 S spectrophotometer. The optical density readings were against seventy-five percentage of DMSO (Dimethyl Sulfoxide) as a blank. The readings on optical densities (OD) of sample and optical densities (OD) of the blank were accounted for the calculation of DPPH Free Radical Scavenging Activity. The reading on optical density (OD) of the assay sample was subtracted from the reading on optical density (OD) of the blank. The figure obtained was divided by the reading on optical density (OD) of the blank. The quotient thus obtained was multiplied by hundred. The mathematical formula is as below:

$$\text{Percentage of DPPH-Radical-Scavenging-Activity} = [(1) - (\text{As} \div \text{Ac})] \times 100 \quad (3)$$

Protein Bioassay

Bioassay of proteins total proteins and soluble proteins from hydrolysates was carried out through the method of Lowry *et al*. (1951) explained by Khyade (2021). Protein concentration in assay sample was quantified measured with a Nano-Drop 2000 spectrophotometer with reference to the absorbance (optical density readings) obtained for a series of standard proteins (Bovine Serum Albumen). The unit for expressing the results was microgram (µg) proteins per mg hydrolysate obtained through enzymatic hydrolysis of the larval-stages of black soldier fly (LBSF), *Hermetia illucens* (Linnaeus) (Order: Diptera, Family: Stratiomyidae) (Khyade, 2021).

Amino Acid Composition

The assay samples were subjected for hydrolysis at 130ºC, for about sixteen hours in hydrochloric Acid (HCl) (vapor phase). It was followed by the process of derivatization with "Waters-AccQ-Tag-Derivatization Reagents". The quantification of amino acids of derivatized category was carried out with "Reverse Phase Ultra Performance Liquid Chromatography (RP-UPLC) with a C18 analytical column (1.7 µm, 2.1 × 100 mm) and acetonitrile/water as buffers.

Statistical Analysis of the Data

Each attempt in the experimentation was repeated for three times (n=3). This repetition is to ensure reproducibility and to achieve consistency in results. The statistical mean (of three replicates) and standard deviation were used to express the results. The statistical "Student's-t-test" was used for comparison of the mean values amomg the two groups (Altman, 1990; McDonald, 2009). "One way analysis of variance" was used for the determination of the significance of difference in the treatments and was considered as significant at $p < 0.05$.

Results and Discussion

The results on present attempt are summarized in **Tables 1-4** and presented in **Figures 1-3**. The degree (percentages) of enzymatic hydrolysis (DEH) and the yield (Wet Weight Basis Percentage) of Black Soldier Fly (BSF) meal through the use of enzymes (Alcalase, Papain and Pepsin) in the process of two-steps-enzymatic-hydrolysis, in two hours of duration are summarised in **Table 1** and presented in **Figure 1**. The results are discussed according to the parameters considered in the attempt, which include: Degree of Enzymatic Hydrolysis (DEH); Yield; Oil Holding Capacity; Emulsifying Stability; Protein (SP and TP) contents; Amino Acid Composition and Activity of Antioxidation. The highest degree of enzymatic hydrolysis (DEH) was reported in the use of Alcalase (Serine Protease). The degree of enzymatic

hydrolysis (DEH) of black soldier fly (BSF) meal through the use of Alcalase (Serine Protease) was 18.860 (±1.531) percentage and it was the most significant. The degree of enzymatic hydrolysis (DEH) of black soldier fly (BSF) meal through the use of Papain (Cysteine-Protease) was 15.786 (±1.132) percentage. The degree of enzymatic hydrolysis (DEH) of black soldier fly (BSF) meal through the use of Pepsin (Endoprotease) was 10.112^{*} (±2.473) percentage**.** The present attempt is suggesting that, alcalase (Serine Protease) is the most favourable enzyme to be utilized for the enzymatic hydrolysis and maximum yield (Wet Weight Basis Percentage) from Black Soldier Fly (BSF) meal. If alcalase (Serine Protease) is not available, the Papain (Cysteine-Protease) and (or) Pepsin (Endoprotease) should be utilized for the process of the enzymatic hydrolysis of Black Soldier Fly (BSF) meal. The results of present attempt are similar as reported by Purschke *et al*. (2018); Hall *et al*. (2017); Zhu *et al*. (2020) and Leni *et al*. (2020) in enzymatic hydrolysis of larval stages of the migratory locust, *Locusta migratoria* (L); the tropical-banded cricket, *Gryllodes sigillatus* (L); the black soldier fly, *Hermetia illucens* (L) and the lesser-mealworm (LM), *Alphitobius diaperinus* (L) respectively.

The results on degree of enzymatic hydrolysis (DEH) of BSF meal in present attempt are illustrating the yields of highest-hydrolysates and the oil-fraction are possible through the use of enzyme: Alcalase, with the lowest-solid-fraction. In contrast, the degree of enzymatic hydrolysis (DEH) with papain (Cysteine-Protease) resulted in the lowest-hydrolysate and oil-fraction-yield, with the highest level of solid-layer. Higher oil-recovery and hydrolysate-recovery with the enzyme: Alcalase compared to Protamex (protease) and Flavourzyme peptidase-preparation belongs to Asperigillus oryzae (L) during the attempt of hydrolysis of fish sardine, *Sardina pilchardus* (L) (Kechaou *et al*., 2009), and the enzyme: Alcalase compared to many other commercial enzymes during the attempt of hydrolysis of oily fish of family: Engraulidae (anchovies, *Clupeonella engrauliformis* L.) have been reported by Ovissipour *et al*. (2009). The degree of enzymatic hydrolysis (DEH) of the hydrolysates of black soldier fly (BSF) with enzyme Alcalase, papain, pepsin and pancreatin has been reported as six percent, twenty-five percent, seventeen percent and twenty-five percent by Caligiani *et al*. (2018) in one of the attempts. That is to say, the lowest degree (six percent only) of enzymatic hydrolysis (DEH) of the hydrolysates of black soldier fly (BSF) with enzyme Alcalase has been reported by Caligiani *et al*. (2018). The difference in ratio of enzyme to substrate and reduction in the rate of enzyme catalysed reaction (possibly, due to limitation of the activity of enzyme) are the two possible reasons may be cited here to explain the contrast (or difference) in the two results. Yield of lowest degree of enzymatic hydrolysis (DEH) (six percent only) of the hydrolysates of black soldier fly (BSF) with enzyme Alcalase in the attempt by Caligiani *et al*. (2018) may be due to the formation of the products inhibiting the enzyme activity and deactivation of the enzyme (Ovissipour *et al*., 2009; Valencia *et al*., 2014). The ratio of one percent enzyme to substrate was used by Caligiani *et al*. (2018) in one of the attempts, Caligiani *et al*. (2018) for hydrolysis of BSF-meal for twenty-four hours. Present attempt however, is dealing with the ratio of two percentages to the substrate in a "Two-Steps- Enzymatic-Hydrolysis". This change in the ratio of the enzyme to the substrate exerted for improved enzymatic reaction and increased enzymatic-degree of hydrolysis (DEH) through addition of one percent of enzyme to the substrate at the initial step of reaction and addition of remaining one percent of the enzyme to the substrate after an hour of enzymatic hydrolysis. Moreover, there is a strong relationship among the "ratio of enzyme to substrate" and "degree of enzymatic hydrolysis (DEH)" during the process of enzymatic-hydrolysis of tropical-banded cricket, *Gryllodes sigillatus* (L) (Hall *et al*., 2017) and during the process of enzymatic-hydrolysis of black soldier fly, *Hermatia illucens* (L) BSF (Firmansyah & Abduh, 2019).

Table 1. Degree of Hydrolysis and the Yield (Wet Weight Basis Percentage) of Black Soldier Fly (BSF) Meal Through the Enzymes (Alcalase, Papain and Pepsin)

Protease Enzyme	pH	Temperature	Degree of Hydrolysis (DH) (Percentage)	Percentage of Yield (Wet Weight Basis) for Hydrolysates	Percentage of Yield (Wet Weight Basis) for Oil	Percentage of Yield (Wet Weight Basis) for Solid Layer
Alcalase (Serine Protease)	6.86	60ºC	18.860^{***} (±1.531)	52.685^{***}(±3.748)	7.319^{***} (±1.649)	42.384^{***} (±3.786)
Papain (Cysteine-Protease)	6.86	60ºC	15.786^{***}(±1.132)	38.934^{**} (±3.398)	4.741^{**} (±0.618)	58.387^{***} (±3.351)
Pepsin (Endoprotease)	3.10	37ºC	10.112^{*} (±2.473)	45.357^{*} (±4.796)	3.378^{*} (±0.633)	53.614^{**} (±7.756)

- Each figure is the mean of the three replications.

-Figure with ± sign in the bracket is standard deviation.

-Figure below the standard deviation is the increase for calculated parameter and percent increase for the others over the control. *: $P < 0.05$; **: $P < 0.005$; ***: $P < 0.01$

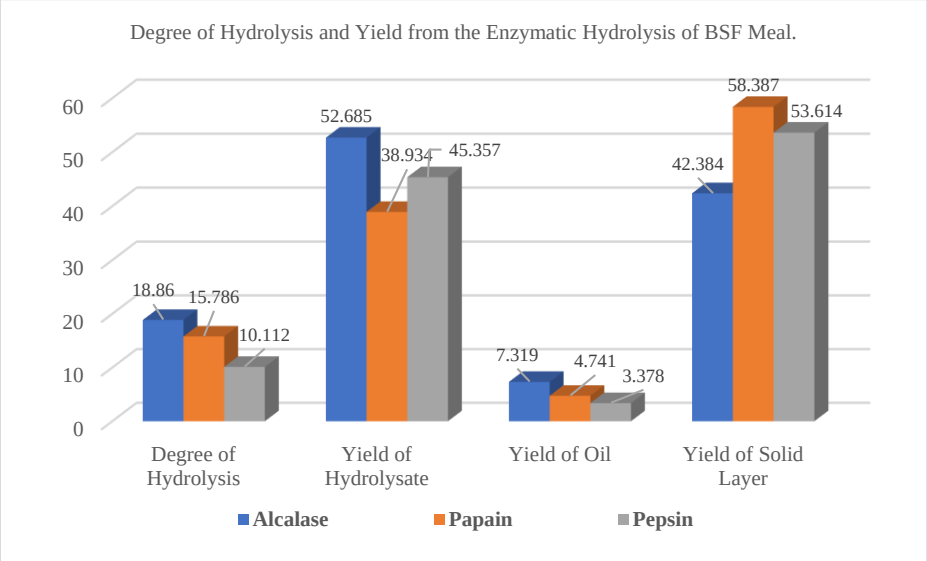

Figure 1. Degree of Hydrolysis (DH) and the Yield (Wet Weight Basis Percentage) of Black Soldier Fly (BSF) Meal Through the Enzymes (Alcalase, Papain and Pepsin)

Table 2. Oil Holding Capacity (OHC); Emulsifying Stability (ES) and Foam Capacity (FC) of the Protein Concentrate and Hydrolysates Derived from Black Soldier Fly (BSF) Meal Through Different Enzymes (Alcalase, Papain and Pepsin)

Serial No.	Parameter	Protein Concentrate	Hydrolysates from Enzyme: Alcalase	Hydrolysates from Enzyme: Papain	Hydrolysates from Enzyme: Pepsin
1.	Oil Holding Capacity (OHC)	04.449* (±0.088)	04.927* (±0.093)	04.617* (±0.087)	04.218* (±0.071)
2.	Emulsifying Stability (ES)	100.00* (±17.913)	54.681* (±05.942)	52.729* (±08.786)	42.584* (±03.293)
3.	Foam Capacity (FC)	20.913* (±00.568)	07.573* (±00.789)	04.786* (±00.847)	00.000* (±00.000)

- Each figure is the mean of the three replications.
-Figure with ± sign in the bracket is standard deviation.
-Figure below the standard deviation is the increase for calculated parameter and percent increase for the others over the control. *: P < 0.05; **: P < 0.005; ***: P < 0.01

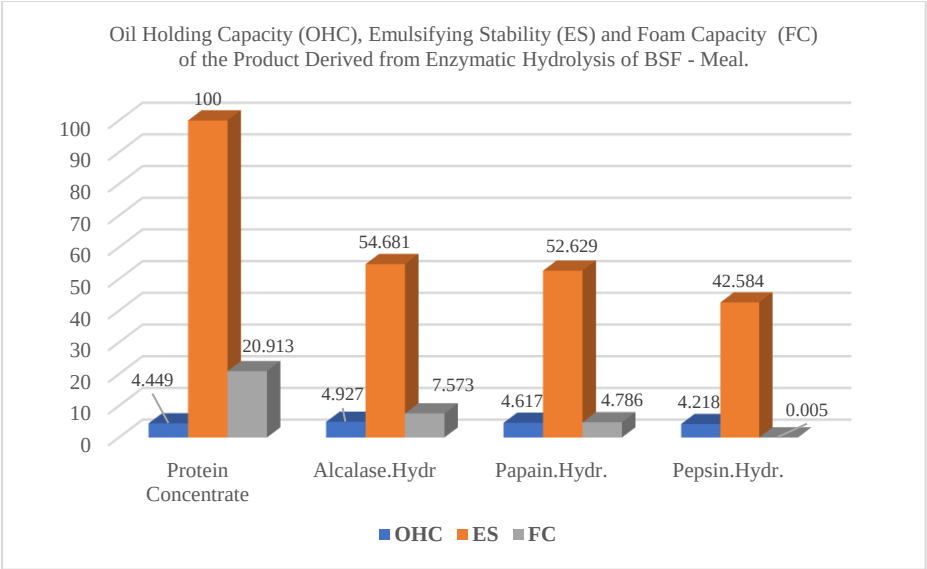

Figure 2. Oil Holding Capacity (OHC); Emulsifying Stability (ES) and Foam Capacity (FC) of the Protein Concentrate and Hydrolysates Derived from Black Soldier Fly (BSF) Meal Through Different Enzymes (Alcalase, Papain and Pepsin)

Table 3. Protein Contents (Soluble and Total) and Antioxidant Activity (Micromoles DPPH per milligram protein) of the Protein Concentrate and Hydrolysates Derived from Black Soldier Fly (BSF) Meal Through Different Enzymes (Alcalase, Papain and Pepsin)

Parameter	Protein Concentrate	Hydrolysates Through Enzymes: Alcalase	Hydrolysates Through Enzymes: Pepsin	Hydrolysates Through Enzymes: Papain
Soluble Protein	197.19 (±36.786)	474.26 (±123.73)	293.68 (±91.574)	235.89 (±72.751)
Total Protein	213.93 (±42.662)	415.45 (±117.89)	317.76 (±93.071)	254.82 (±69.824)
Antioxidant Activity (µmoles DPPH per mg protein)	46.742* (±7.733)	62.496*(±11.249)	37.082* (±4.586)	29.886* (±4.234)

- Each figure is the mean of the three replications.
-Figure with ± sign in the bracket is standard deviation.
-Figure below the standard deviation is the increase for calculated parameter and percent increase for the others over the control. *: P < 0.05; **: P < 0.005; ***: P < 0.01

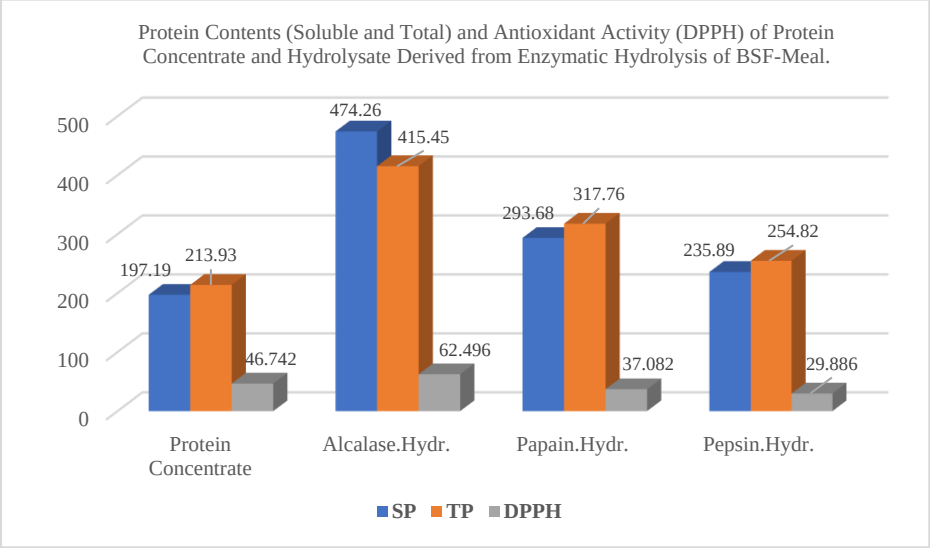

Figure 3. Protein Contents (Soluble and Total) and Antioxidant Activity (Micromoles DPPH per milligram protein) of the Protein Concentrate and Hydrolysates Derived from Black Soldier Fly (BSF) Meal Through Different Enzymes (Alcalase, Papain and Pepsin)

Table 4. The Composition of Amino-acids Intact Protein, Protein Concentrate and Hydrolysate composition in BSF intact Protein, protein, protein isolate and hydrolysates Derived from Black Soldier Fly (BSF) Meal Through Different Enzymes (Alcalase, Papain and Pepsin)

Serial No.	Amino Acid	Quantity (mg/Gram) In Intact Protein	Quantity (mg/Gram) In Protein Concentrate	Quantity (mg/Gram) In Alcalase Hydrolysate	Quantity (mg/Gram) In Papain Hydrolysate	Quantity (mg/Gram) In Pepsin Hydrolysate	Reference Protein (FAO/WHO, 1985)
1.	Alanine (Ala)	10.953	38.941	41.901	11.953	34.716	-
2.	Arginine (Arg)	06.345	31.812	28.866	09.467	22.869	-
3.	Asparagine (Asn)	13.972	82.421	58.517	18.711	46.291	-
4.	Glutamic acid (Glu)	16.753	95.483	74.866	35.198	68.312	-
5.	Glycine (Gly)	07.821	31.673	33.152	11.611	32.162	-
6.	Histidine (His)	05.093	19.351	20.712	10.394	18.693	15
7.	Isoleucine (Ile)	06.915	35.972	25.441	05.173	16.945	30
8.	Leucine (Leu)	09.863	50.631	37.437	05.542	24.973	59
9.	Lysine (Lys)	08.641	54.083	36.122	07.523	27.041	45
10.	Phenylalanine (Phe)	06.063	34.431	20.213	03.786	12.312	38

11.	Proline (Pro)	09.151	31.111	38.474	14.512	34.301	-
12.	Serine (Ser)	03.843	13.742	14.423	04.111	11.991	-
13.	Threonine (Thr)	04.421	16.633	18.142	04.451	14.43	23
14.	Tyrosine (Tyr)	07.102	27.621	30.511	07.012	23.853	-
15.	Valine (Val)	0.791	43.402	38.602	7.753	30.311	39
16.	Hydrophobic Amino Acids (HAA)	59.832	262.11	232.561	55.702	177.372	-
17.	Positively Charged Amino Acids (PCAA)	20.061	105.23	85.682	27.371	68.593	-
18.	Negatively Charged Amino Acids (NCAA)	30.722	177.90	133.37	53.911	114.61	-
19.	Total Essential Amino Acids (TEAA)	50.781	254.49	196.65	44.601	144.39	-
20.	Essential Amino Acid Index (EAAI)	00.322	01.101	00.851	00.272	00.701	-
21.	Aromatic Amino Acids (AAA)	19.311	79.381	77.452	22.591	63.142	-

The results on oil holding capacity (OHC) of protein concentrate and protein hydrolysate in present attempt indicated that, the hydrolysate derived through pepsin hydrolysis appear significantly lower in comparison with the hydrolysate derived through alcalase and papain hydrolysis **(Table 2 and Figure 2)**. No significant difference was observed for oil holding capacity (OHC) of the protein concentrate and hydrolysates derived through alcalase and papain hydrolysis. The enzyme pepsin is not concerned with enhancement of functional properties of the peptides. The enzyme pepsin is not concerned with development of the peptides with residue of proper hydrophobic nature. The enzyme pepsin had significantly lowest amino acids of aromatic nature. The amino acids of enzyme pepsin include: phenylalanine, histidine, tyrosine (Vioque *et al*., 2000).

The emulsifying stability (ES) of the content is significant with reference to storage. Instability of the content is going to exert the floating the droplets to the uppermost surface. Instability may also lead to achieve the ability to remain (cohesion) or fit together well. Final product of instability of the content is the separation in the form of cream. The emulsifying stability (ES) of the protein concentrate, Hydrolysates from Enzyme: Alcalase, Hydrolysates from Enzyme: papain and Hydrolysates from Enzyme: pepsin in present attempt were reported as: 100.00* (±17.913); 54.681* (±05.942); 52.729* (±08.786) and 42.584* (±03.293) respectively **(Table 2 and Figure 2)**. The emulsions obtained from the protein concentrate was found creaming within a week and the most stable.

The foam capacity (FC) of the protein concentrate, Hydrolysates from Enzyme: Alcalase, Hydrolysates from Enzyme: papain and Hydrolysates from Enzyme: pepsin in present attempt were reported as: 20.913* (±00.568); 07.573* (±00.789); 04.786* (±00.847) and 00.000* (±00.000) **(Table 2 and Figure 2)**. The highest and the most significant foam capacity was reported by the fraction of protein concentrate in the attempt. Generally, all the proteins exhibit unstable nature with reference to their foam capacity. The foam capacity of most of the insects is ranging from poor to zero foaming capacity. The foaming capacity of pallid emperor moth, *Cirina forda* (L) has been reported as six percent. This insect has the lowest foaming capacity and stability (Omotoso, 2006). Hall *et al*. (2017); Zielinska *et al*. (2018) and Leni *et al*. (2020) reported improvement in both the foam-capacity and foam-stability through moderate enzymatic hydrolysis. Thorough hydrolysis through the enzymes of the proteins exerts higher degree of hydrolysis. Smaller peptides are generally with poor grade of foam capacity. Thorough hydrolysis through the enzymes of the proteins belong to lesser mealworm, *Alphitobius diaperinus* (L) reported decreased nature of foam capacity. Five to ten percent degree of hydrolysis show five to seventy-three foam capacity (Leni *et al*., 2020). The present attempt used the enzymatic-hydrolysis of "Two Step Type", which effected into protein hydrolysate with higher degree of hydrolysis (DH) and with lower foam capacity. Moreover, the earlier researchers compared the foam capacity of protein hydrolysate with the intact protein (protein powder). The extracted and isolated proteins belong to edible insects, like mealworm, *Tenebrio molitor* (L), tropical house *cricket, Gryllodes sigillatus* (L) and desert locust, *Schistocerca gregaria* (L), has reported strong foam-capacity and foam-stability in comparison with that of the insect protein of intact nature (Zielińska *et al*., 2018). The highest foam-capacity has been reported through the extraction of the protein from black soldier fly (BSF), *Hermetia illucens* (L) through the use of three methods was reported that, the method of "One Step Chemical Extraction of Protein" (Caligiani *et al*., 2018).

The part and partial of all the cells and tissues are the proteins. The analysis of the total proteins at individual level is possible. Expeditious and cheap analysis is possible for the total proteins. The proteins of soluble category are concerned with translocation of themselves across the endoplasmic reticulum (ER) and then into the lumen. The proteins of soluble category are going to remain either in the endoplasmic reticulum (ER) or going to be secreted from their mother cells. The soluble proteins of protein concentrate, Hydrolysates from Enzyme: Alcalase, Hydrolysates from Enzyme: papain and Hydrolysates from Enzyme: pepsin in present attempt were reported as: 197.19 (±36.786); 474.26 (±123.73); 293.68 (±91.574) and 235.89 (±72.751) respectively **(Table 3 and Figure 3)**. The soluble proteins of all the four fractions in present attempt appeared as significant. The highest soluble protein contents were measured in the Hydrolysates from Enzyme: Alcalase.

The total proteins of protein concentrate, Hydrolysates from Enzyme: Alcalase, Hydrolysates from Enzyme: papain and Hydrolysates from Enzyme: pepsin in present attempt were reported as: 213.93 (±42.662); 415.45 (±117.89); 317.76 (±93.071) and 254.82 (±69.824) respectively **(Table 3 and Figure 3)**. The total proteins of all the four fractions in present attempt appeared

as significant. The highest total protein contents were measured in the Hydrolysates from Enzyme: Alcalase.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a dark in colour, crystalline in nature and with a molecule of stable free radical. It is used to monitor the chemical reaction with radicals. It is special for assaying the antioxidant activity (Sharma & Bhat, 2009). The antioxidant activity (µmoles DPPH per mg protein) of protein concentrate, Hydrolysates from Enzyme: Alcalase, Hydrolysates from Enzyme: papain and Hydrolysates from Enzyme: pepsin in present attempt were reported as: 46.742* (±7.733); 62.496*(±11.249); 37.082* (±4.586) and 29.886* (±4.234) respectively **(Table 3 and Figure 3)**. The antioxidant activity (µmoles DPPH per mg protein) of all the four fractions in present attempt appeared as significant. The highest antioxidant activity (µmoles DPPH per mg protein) was measured in the Hydrolysates from Enzyme: Alcalase.

Table 4 is dealing with amino acid contents of black soldier fly (BSF) meal, BSF protein concentrate and the hydrolysates. The contents of hydrophobic amino acids of the fractions of enzymatic hydrolysis, in present attempt were illustrated that, they are significantly lower in the pepsin hydrolysate. The glutamic acid was appeared as dominant amino acid. These results are parallel with the results on enzymatic hydrolysis of black soldier fly (BSF) meal by earlier attempts on black soldier fly (BSF) meal (Janssen *et al*., 2017; Caligiani *et al*., 2018; Firmansyah & Abduh, 2019; Zhu *et al*., 2020). The protein hydrolysate derived from the tropical banded cricket, *Gryllodes sigillatus* (L) and the larvae of housefly, *Musca domestica* (L) has reported the glutamic acid as the most dominant amino acid (Wang *et al*., 2013; Hall *et al*., 2017). Hydrophobic Amino Acids (HAA) like alanine (Ala), isoleucine (Ile), phenyl alanine (Phe), proline (Pro), Tyrosine (Tyr), and valine (Val) are associated with peptides with bioactive functions (ability of antioxidation) (Saadi *et al*., 2015). Such hydrophobic Amino Acids (HAA) were significantly higher in the protein concentrate in the present attempt followed by the hydrolysate derived through Alcalase and papain. Such hydrophobic Amino Acids (HAA) were lowest in the associated hydrolysate of pepsin. Similar types of the results were reported by the present attempt with reference to the Essential-Amino-Acids (EAA); the Positively charged Amino acids (PCAA), Negatively-Charged-Amino-Acids (NCAA) and the Aromatic Amino Acids (AAA). The composition of amino acids of hydrophobic nature, that is "Hydrophobic Amino Acid" (HAA) content was significantly lower in pepsin hydrolysates than Alcalase and papain hydrolysates and protein concentrate in present attempt. The results of present attempt were illustrating the increasing the qualities and properties of functional nature for the proteins from black soldier fly (BSF) meal.

The animals use to obtain amino acids through the process of consumption of food material with proteins. Through the digestive system, the ingested proteins are converted into amino acids. There is denaturation of the proteins through the digestive enzyme: proteases. In the animal body, some of the amino acids are utilized for biosynthesis of the proteins. Others amino acids are processed for gluconeogenesis for conversion into the glucose. Amino acids may also be used to enter into the tricarboxylic acid cycle (Kreb cycle or citric acid cycle). In starvation condition, the proteins are used as a fuel as they allow to support life (Brosnan, 2003).

The practical fact is that, the properties of functional nature and bioactivity of the proteins may be affected in negative manner depending on process of enzymatic-hydrolysis and the type of enzyme used for hydrolysis. Most of the earlier researchers have recommended the alcalase enzyme for increased qualities and composition of amino acids of protein-hydrolysates from insects (Hall *et al*., 2017; Firmansyah & Abduh, 2019; Zhu *et al*., 2020). Through the comparison between the effects of several enzymes of commercial nature on the protein hydrolysates of black soldier fly (BSF), Leni *et al*. (2020) reported the lowest free amino acid contents through the use of enzyme: pepsin and the highest free amino acid contents through the use of papain.

Conclusion

The enzyme involved hydrolysis is dealing with disintegration of the complex compounds through utilization of enzymes (or the source of enzymes) followed by the reaction with water. Through the hydrolysis of "Two Step" type hydrolysis, the enzyme: Alcalase was found yielding the hydrolysates of protein with the highest degree of hydrolysis, improved functional-properties, greater levels of activity of antioxidation and composition of the amino acids with higher levels of hydrophobic Amino Acids (HAA). In Comparison with the common extracted proteins (protein-fractions), the method of enzymatic hydrolysis is appearing to reduce the functional-properties of hydrolysates of the black soldier fly (BSF). The enzyme: pepsin reported the lowest measurements of parameters, which may be associated with the poor composition of amino acids. The proteins derived through the enzymatic hydrolysis of black soldier fly (BSF) with Alcalase and papain are offering sustainability for the method. This may be reason for higher contents of amino acids of hydrolysates. The results of the present attempt are forming baseline on the way of development of sustainable alternative food material from insects.

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