

## The Potential Application of *Cupriavidus necator* as Polyhydroxyalkanoates Producer and Single Cell Protein: A Review on Scientific, Cultural and Religious Perspectives

Jiun Yee Chee<sup>1</sup>, Manoj Lakshmanan<sup>1,2</sup>, Iffa Farahin Jeeper<sup>1</sup>, Nabila Husna Mohamad Hairudin<sup>1</sup>, Kumar Sudesh<sup>1,2\*</sup>

1- School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.

2- USM-RIKEN Centre for Aging Science (URICAS), Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.

### Abstract

**Background and objective:** Polyhydroxyalkanoates are environmentally friendly bioplastic compounds produced via the microbial route that offer an alternative to synthetic plastics due to their comparable durability and thermal stability. However, the high production cost as a result of carbon feedstock for microorganisms and the downstream recovery process narrow the usage of polyhydroxyalkanoates in various fields. Conversion of by products from the food and agricultural industries such as waste cooking oil, glycerol, palm sludge oil, oil palm trunk sap and soya waste into polyhydroxyalkanoates is an attractive approach that can minimize and/or add value to waste.

**Results and conclusion:** Recently, there has been a lot of interest in exploring not just polyhydroxyalkanoates as valued-added products, but also PHA-producing bacteria as a nutritional food or feed source. It has been previously reported that the PHA-producing bacterium, *Cupriavidus necator*, can be utilized as a single cell protein (SCP) in animal feed owing to its high protein content. The mealworm beetle (*Tenebrio molitor*) has also been used as the model insect to evaluate the efficacy of *Cupriavidus necator* cells as a source of protein and to recover polyhydroxyalkanoate granules at the same time. The European Union has imposed strict regulations on the type of feedstock that can be used to ensure that the food chain is safe. In addition, there are religious and cultural concerns. This review will focus on the nutritional value of *Cupriavidus necator* as single cell protein and its safety as animal feed. The impact of using by-products from the agriculture and food industries as carbon feedstocks to produce single cell protein will be discussed, alongside societal acceptance of this practice.

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#### \*Corresponding author:

**Kumar Sudesh**,  
School of Biological  
Sciences and USM-RIKEN  
Centre for Aging Science  
(URICAS), Universiti Sains  
Malaysia, 11800 Minden,  
Penang, Malaysia.

Tel: +604-6534367

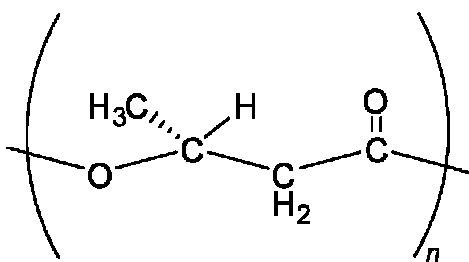
E-mail: [ksudesh@usm.my](mailto:ksudesh@usm.my)

## 1. Introduction

The use of synthetic plastics has become an integral part of our day-to-day activities. Synthetic plastics are designed in such a way to possess high performance and quality, which makes them have long lifespan. These properties in turn make them resistant to biodegradation, causing increased accumulation of plastics in the environment [1]. In 2016, it was estimated that approximately 335 million metric tonnes of plastics were produced globally. While a fraction of the plastics were taken up for recycling or contained in landfills, the rest still remain as litter across continents and oceans [2]. Synthetic plastics might be

harmful in some contexts-either they are able to absorb pollutants or the plastic itself is potentially toxic [3,4]. People are aware of the adverse effects of using these petrochemical plastics on the environment and human health. Most countries around the world have been carrying out various solid waste management programs, which include reducing the use of conventional petrochemical-based plastics by introducing bio-based plastics with or without biodegradable properties. These bio-based plastic materials should resemble the desired properties of conventional plastics and at the same time

should ideally be fully biodegradable by microbes, leaving behind no environmentally harmful by-products when discarded. Polyhydroxyalkanoates (PHAs) are considered a good choice of biodegradable polyester materials to replace some of the conventional plastics. PHAs consist of various hydroxyalkanoate monomers that are synthesized by microorganisms as storage compounds in stress conditions, in which there are excess carbon sources available while other nutrients are scarce [5]. Some of these polyesters have physical properties that are almost similar to polypropylene; besides having good biocompatibility and biodegradability, they are bio-synthesizable using renewable carbon sources. These superior attributes make PHAs one of the best candidates for next generation plastics for certain applications. When the bacteria are exposed to stress conditions that limit growth, the excess carbon sources available are assimilated and converted into 3-hydroxyalkanoic acid (3HA) monomer units. These monomers are in turn polymerized by the PHA synthase enzyme in the bacterial cells and stored as water-insoluble inclusion bodies, or granules, in the cell cytoplasm. PHA granules can be observed under phase contrast microscopes as discrete inclusions with a size range of 0.2-0.5  $\mu\text{m}$  due to their high refractivity [6]. The production of PHAs in bacteria can help achieve both biodegradable and biocompatible properties to guarantee complete stereo-specificity such as 3-hydroxybutyrate [all chiral carbon atoms that are located at the back bone are in the *R* (-) configuration] (Figure 1). The chemical composition of PHAs is determined by the type of bacteria and growth conditions that result in molecular weights that typically ranges from  $2 \times 10^5$  to  $3 \times 10^6$  Da [7].



**Figure 1.** The typical chemical structure of poly (3-hydroxybutyrate) [P (3HB)], the most common type of PHA which consists of 3-hydroxybutyrate repeating units. The number of this repeating unit in the polymer is represented by *n*.

PHAs can be divided into three major groups, which differ in terms of carbon number between each group. The three groups are: short chain length (SCL) PHAs that consist of less than 6 carbon atoms (C3-C5), medium chain length (MCL) PHAs with 6-14 carbon atoms (C6-C14) and copolymer, which is made up of the combination of SCL- and MCL-monomers [8]. There are some differences in the physical characteristics of SCL- and MCL-PHAs. MCL-

PHAs are elastic, amorphous and sticky, whereas SCL-PHAs are thermoplastic substances that have higher crystallinity than MCL-PHAs [6]. For SCL-PHAs, the oxidation of the monomer units can also be done at different positions other than the third carbon, while for MCL-PHAs, the monomer unit is usually oxidized at the third carbon. About 125 different monomers were reported to be the building blocks of PHAs 20 years ago and now the number has increased to more than 150 [9]. However, PHAs with certain monomer combinations were chosen to be produced in large quantities due to the ease of biosynthesis as well as the physicochemical properties of the polymers, which are useful for various industrial and biomedical applications.

## 2. Transformation of food waste into PHA

Food industries produce large quantities of liquid and solid wastes. In addition to that, significant amounts of leftover food are also of major concern. The need of using various kinds of wastes for beneficial bacterial fermentation is increasing especially when these wastes contain carbon which in return can be used for PHA production. However, the availability of these residual biomasses should also be considered. One of the factors that determine the availability of carbon sources is the geographical location of the production factory. This also means that to select a suitable carbon source, it should be available around where the products will be made to save transportation costs, manpower and other required facilities to meet the need of using selected waste streams. For example, in many European countries, enormous amount of whey is available mainly from the dairy industry. Approximately 180 to  $190 \times 10^6$  tons of whey is generated annually during the preparation of dairy products with an increase of around 1-2% each year [10]. As for the availability of molasses, lipids, methanol, starch and lingo-cellulosic materials are available for industrial scale production. Waste cooking oil, various plant oils and residual oil from palm oil production line could also contribute to the sources of waste lipids [11]. These waste lipid sources are available all year round and are not depending on any seasonal productions. Waste lipids are usually produced by the food processing industry, slaughterhouses, edible oil industry, dairy products industry and olive oil mills. It is known that food wastes only are produced around one third of the total 1.3 billion tons of food produced every year and with such a huge amount, waste lipid is considered enough for industrial scale applications [12]. The highest amount of potential residual biomass is contributed by lingo-cellulosic and cellulosic materials which are mainly provided by wood-processing, paper and agricultural industries [13]. The

world production of plant biomass which composed of 90% lignocellulose reaches  $200 \times 10^9$  tons per year [14]. Other agricultural wastes that also contain lingo-cellulosic and cellulosic materials such as wheat straw, rice straw, corn straw and sugarcane bagasse are produced in the range of 128 to 731 million tons as of 2012 [15].

The simultaneous production of rhamnolipids and PHAs is feasible and has been reported in the past for *Pseudomonas (P.) aeruginosa* [16-18]. It has been shown that *Burkholderia (B.) thailandensis* E264 was also able to produce PHAs besides rhamnolipids. In the study done by Kourmentza et al., they focused on the evaluation of the PHAs and rhamnolipids produced from used cooking oil derived from sunflower as the carbon source. According to the results, the strain *B. thailandensis* E264 was able to produce  $2.2 \text{ g l}^{-1}$  rhamnolipids and up to 60% P(3HB) of the cell dry weight. The residual biomass after 120 h was  $12.6 \pm 0.8 \text{ g l}^{-1}$  [19].

Waste that contains sugars or fatty acids is possibly the best candidate for PHA production. *Bacillus sp.* such as *Bacillus (B.) megaterium* and *Lactococcus (L.) lactis* were used to produce P(3HB) by using the following as substrates: glycerol reagent grade, Jatropha oil, castor oil, waste frying oil residual glycerol, by-product of biodiesel from palm oil and whey [20]. The study was conducted on different bacteria-substrate systems on a laboratory scale under various conditions of temperature, pH and substrate concentration. All three strains mentioned were cultivated with every substrate chosen to evaluate which bacteria-substrate system was able to produce significant amount of PHA. Jatropha oil has a higher proportion of oleic and linoleic acid followed by palmitic and stearic acids, while castor oil is rich in ricinoleic acid [21]. The frying oil was not characterized but is known to have free fatty acids (mono- and diacylglycerol), total polar material (oxidized monomeric, dimeric and oligomeric triglycerides) and compounds such as aldehydes and ketones, as well as polymerized triglycerides (dimeric and polymeric triglycerides with ring structure) [22]. The selection of the best bacteria-substrate system was made based on dry biomass and the production of PHAs. This experiment showed that the highest biomass produced was by *L. lactis*-whey combination which was at the 24<sup>th</sup> h with values of  $0.88 \pm 0.04 \text{ g l}^{-1}$ . This bacteria-substrate combination was the highest among all the strains. However, the highest concentration of PHA produced was observed in the *B. megaterium*-whey combination. This combination also produced much lower total biomass ( $3.1 \text{ g l}^{-1}$ , 36 h) compared to when the strain was cultivated in a broth enriched with glucose under the same cultivation conditions. The best response was shown in the combination of *B. megaterium* with glycerol as the carbon source, followed by the same strain with castor oil as the

carbon source: *Bacillus sp.*-waste frying oil and *Bacillus sp.*-castor oil [20].

Another study that was reported recently utilized sugarcane bagasse as carbon source by using an isolated strain designated as ART\_MKT2E [23]. The sugarcane bagasse contained residual sugars and was used as carbon source for PHA production. Prior to utilization, the sugarcane bagasse was first boiled in water for 5-7 min and filtered. The optimum concentration of bagasse filtrate was 60% with the addition of yeast extract and salts. The maximum amount of PHA produced was 55% wt in the  $0.160 \text{ g l}^{-1}$  cell dry weight (CDW). Acosta-Cardenas and colleagues found that sugarcane molasses and vinasse can also be used as one of the substrates for PHA production [24]. In this experiment, *Ralstonia eutropha* ATCC 17699 also known as *C. necator* H16 was used and the substrates were first treated before being used to produce P(3HB). The vinasse was centrifuged to eliminate particulate matter while the sugarcane molasses was diluted due to high sugar content until the ratio and concentration of sugar required was obtained. Results showed that  $2.71 \text{ g l}^{-1}$  of P(3HB) was obtained from molasses/vinasse medium at the ratio of 25:75 with the biomass concentration of  $3.90 \pm 0.32 \text{ g l}^{-1}$ . The P(3HB) accumulation was 97.8% with respect to the biomass produced.

*Acinetobacter junii* BP 25 was evaluated to have the ability to produce P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV) from parboiled rice mill effluent as substrate [25]. The parboiled rice mill effluent was autoclaved and filtered first before being used. The experiment was conducted using shake flask under batch mode in a two-stage cultivation process. The culture was first incubated in nutrient rich medium and then transferred into nitrogen-limiting medium with the supply of autoclaved rice mill effluent. The process optimization was done by one factor at a time resulting in P(3HB) production of  $2.64 \pm 0.18 \text{ g l}^{-1}$ . As for the copolymer production, valeric acid was added as the precursor at a concentration of 20 mM and *A. junii* BP 25 produced  $2.56 \pm 0.12 \text{ g l}^{-1}$  of biomass and  $2.20 \pm 0.15 \text{ g l}^{-1}$  of P(3HB-co-3HV).

Cheese whey is also one of the possible candidates for substrate source in producing PHA. Das et al. evaluated whole and ultrafiltered cheese whey for P(3HB) production by *B. megaterium* NCIM 5472 [26]. By optimization, the bacterium was able to produce about 75% wt P(3HB) of the CDW at a yield of  $8.29 \text{ g l}^{-1}$ . *B. megaterium* NCIM 5472 was able to produce 75% of P(3HB) of the cell dry mass with the P(3HB) concentration of  $8.29 \text{ g l}^{-1}$ .

Animal fats are potential substrate for PHA production. They are cheap, abundant and available in sustainable manner because of the meat industry. Several studies have evaluated various grades of animal fats for PHA production. Titz et al. [27] used low quality fats from the

waste stream of cattle slaughtering for PHA production. Koller et al. [28] reviewed on the usage of waste streams of the animal-processing industry as feedstocks for PHA production. Riedel et al. [29] used low quality waste animal fats as substrate in the production of PHA by wild type and recombinant *C. necator* Re2058/pCB113. An emulsification strategy was used without the need for mechanical and chemical pre-treatment to produce both P(3HB) and poly(3-hydroxybutyrate-co-3hydroxyhexanoate), P(3HB-co-3HHx) from animal fats. Comparisons were made between various types of fats such as waste plant oil, waste animal fats, tallow and by-products (fats of different qualities) from the protein hydrolysates production. The waste animal fats were obtained from ANiMOX GmbH (Ani-FATs) and suet was obtained from the local butcher. Tallow was produced during the study from the suet after undergoing several processes of impurities elimination. Different qualities of fats as by-products from protein hydrolysates production was also obtained from ANiMOX GmbH. The wild type *C. necator* H16 was able to produce 79-82% (w w<sup>-1</sup>) of P(3HB) per CDW when grown with various fats and a total of 24 g l<sup>-1</sup> P(3HB) production was achieved when cultivated with tallow. The CDW of *C. necator* H16 ranged from 3.1-4.5 g l<sup>-1</sup> when various fats and plant waste frying oil were used while 2.5 g l<sup>-1</sup> with tallow as carbon source. As for the recombinant *C. necator* Re2058/pCB113 strain, 49-72% (w w<sup>-1</sup>) of PHA produced with the copolymer 3HHx content of 16-27% mol in shake flask scale using various fats as carbon source. The CDW of the Re2058/pCB113 in shake flask scale ranged between 1.5-4.6 g l<sup>-1</sup> for all carbon sources used. The recombinant strain was then subjected to cultivation with waste animal fats of the lowest quality in lab fermenter scale which resulted in 45 g l<sup>-1</sup> CDW with 60% (w w<sup>-1</sup>) PHA content. The PHA copolymer obtained composed of 19% mol of 3HHx.

Another study was reported to have utilized vegetable-oil-degrading bacteria, that was first isolated from a rice field using enrichment cultivation as PHA producers [30]. Vegetable-oil-degrading bacteria can utilize waste vegetable oil to produce PHAs, thus lowering production costs. In this research, *Pseudomonas* sp. strain DR2 was isolated and PHA granules were detected by clear orange or red spots when stained with Nile blue A. The strain was grown on nitrogen and phosphate limiting media, containing waste vegetable oil as the sole carbon source. The strain was able to produce up to 37.3% (w w<sup>-1</sup>) of PHA from corn oil with CDW concentration of 0.96 g l<sup>-1</sup> and 23.5% (w w<sup>-1</sup>) of PHA<sub>MCL</sub> from waste vegetable oil.

Fernandez et al. (2005) used *P. aeruginosa* 42A2 to produce PHA from agro-industrial oil wastes such as technical oleic acid, used cooking oil and waste-free fatty acids from soybean oil [31]. The authors also described the taxonomic classification of the strain, the characterization

of the PHA produced by the isolated strain and the influence of cultivation parameters. Approximately 54.6% PHA accumulation per CDW was obtained with the use of technical oleic acid as carbon source. The strain was able to accumulate 66.1% PHA per CDW when waste-free fatty acids from soybean oil was used and 29.4% from waste frying oil. The utilization of used cooking oil as the sole carbon source for PHA production was described by Kamilah et al. as well. *C. necator* H16 and transformant *C. necator* PHB<sup>-</sup>4 harbouring *A. caviae* PHA synthase gene (PHB-4/pBBREE32d13) were used to synthesize P(3HB) and P(3HB-co-3HHx) [32]. *C. necator* H16 yielded 25.4 g l<sup>-1</sup> CDW with 71% wt P(3HB) content while transformant *C. necator* PHB<sup>-</sup>4 produced 85% wt P(3HB-co-3HHx) with 22.3 g l<sup>-1</sup> CDW [32].

Apart from using waste lipids as one of the carbon sources, underutilized plant oils can be also considered as waste owing to their low significance in food and feed applications. Thus, these oils have higher potential to be utilized as carbon feedstocks for PHA production. In a study conducted by Zainab-L et al., desert date oil, bitter apple oil, African elemi oil and *Amygdalus pedunculata* oil were used as novel carbon sources for PHA production [33]. Biosynthesis was carried out by one-stage batch cultivation in shake flasks using *C. necator* H16 and *C. necator* Re2058/pCB113 bacterial strains. It was found that these bacterial strains were able to efficiently utilize the oils to produce P(3HB) and P(3HB-co-3HHx), respectively. Maximum CDW of 8-9 g l<sup>-1</sup> was achieved by *C. necator* H16 from those various oils with P(3HB) content at a range of 36-71% wt. On the other hand, *C. necator* Re2058/pCB113 produced a maximum of 6-8 g l<sup>-1</sup> CDW and P(3HB-co-3HHx) content in the range of 50-70% wt with 3HHx monomer content as high as 31% mol [33]. Date seed oil and date molasses were also used as alternative renewable carbon source for P(3HB-co-3HHx) production by Purama et al. In this study, PHA biosynthesis was conducted via one-stage shake flask cultivation system. *C. necator* H16 Re2058/pCB113 was found to effectively utilize date molasses, giving a yield of 28% wt P(3HB) in the lyophilized cells [34]. On the other hand, a maximum yield of 80% wt P(3HB-co-3HHx) with 28% mol 3HHx were successfully produced from date seed oil combined with date molasses as carbon sources. Biosynthesis using date seed oil was reportedly to yield between 0.38-0.62 g of PHA per gram of oil fed to the cultures [34].

Furthermore, fruit residues such as fruit skins, pulp, stalks and seeds are considered as food wastes too. Fruit pomace can be another good option for carbon feedstock for fermentation due to its high polysaccharides content. In the work done by Follonier et al., pomaces from apricots, cherries and grapes as potentially cheap and sustainable

carbon substrates for the production of MCL-PHA using *Pseudomonas* strain was explored for the first time [35]. A two-stage cultivation system with *P. resinovorans* was employed using hydrolysed pomace from apricots or grapes as carbon feedstocks and waste frying oil as precursor [35]. Approximately 47, 49 and 106 g l<sup>-1</sup> glucose were recovered from pomaces of apricots, cherries and Solaris grapes respectively. With the highest sugar content, Solaris grapes was undoubtedly proven to be a favorable growth substrate for PHA production with a total yield of 21.3 g PHA (L pomace)<sup>-1</sup> compared to only 1.4 g PHA (L pomace)<sup>-1</sup> using apricots [35]. With this work, it was demonstrated that although process optimization is further required, the feasibility of pomace as carbon substrate for PHA production could still be successfully established.

### 3. Controlling PHA biosynthesis in terms of availability of carbon feedstocks

As explained in the sub-sections above, many studies have used various kinds of agricultural and food waste in the production of PHAs. However, looking at the bigger picture, the continuous and sustainable production of PHAs on an industrial scale requires consistent supply and effective conversion of this waste by bacteria to produce PHAs with negligible variances in their monomer composition and physicochemical properties. As such, many factors play a concerted role in enabling this production process to work well in the long run. At the most basic level, although factors such as strain compatibility, development of new strains for optimized PHA production via genetic engineering, fermenters and fermentation conditions directly affect the productivity of PHAs, this review emphasizes the feed-stocks that act as the carbon source in PHA biosynthesis by microbes. Feedstocks play a major role in the fermentation process, because only by assessing the quality and availability of feedstocks can the process design for fermentation be outlined. Traditionally, PHA biosynthesis has been carried out by using food quality sugars, edible oils and expensive fatty acids. One of the major contributing factors to the bottleneck in the large-scale production of PHAs is the cost. The use of these expensive carbon sources not only increases production costs, but also competes with the food and feed applications for humans. A review by Koller and Brauneegg [36] has outlined what are referred to as the 'eight pillars of cost-effective and sustainable PHA manufacturing'. One of the eight pillars mentioned in this review is raw materials or feedstocks for PHA biosynthesis. In order to slash production costs, for which 50% of the total cost is accounted for by raw materials, the use of agricultural and food waste has been widely explored. However, it must be noted that the choice of raw

materials can directly affect the production and quality of PHAs in terms of molecular weight, monomer composition, odour and pigmentation [36].

Therefore, for these kinds of waste to be effectively used in the PHA production line, Koller and Brauneegg devised criteria that must be fulfilled before the waste can be converted as feedstocks for PHA production. The criteria are: the consistent availability of feedstocks, constant feedstock quality with minimal batch-to-batch variations in their compositions, easy logistics for the transportation of these feedstocks, the stability of feedstocks, especially for long term storage, and no competition with food and feed applications [36]. It was previously reported that the use of inexpensive feedstocks, such as agricultural and food waste, can lead to lower PHA productivity compared to the process that uses purified feedstock. This is due to the low adaptation of the microbe to the feedstocks, the low concentration of the carbon source in the raw feedstocks and the presence of substances in the feedstocks that inhibit efficient PHA biosynthesis processes [37]. Therefore, proper strategies are needed to optimize and convert these feedstocks into carbon rich substrates while reducing the inhibiting substances for maximized PHA production. Many studies have in fact demonstrated and successfully implemented strategies to maximize the availability of carbon sources in the waste feedstock that was used for PHA production, while reducing the inhibiting substances.

One of the successful uses of waste was reported on whey. The availability of carbon sources from sugars in whey was maximized up to 50% from the initial availability of 4-5% in sweet whey by subjecting them to ultrafiltration to obtain the sugar rich whey permeate. This was further hydrolysed enzymatically to enhance the availability of sugars up to 50% [38]. In another report, the inhibiting by-products resulting from the hydrolysis of the lingo-cellulosic-like substance furfural were successfully removed using charcoal or lignite, making the sugars that were produced as a result of lignocellulose hydrolysis fully accessible to the bacterial strains that were used in PHA biosynthesis [23]. A similar technique was used by Silva et al., who converted the toxic bagasse hydrolysate into a substrate that is suitable for PHA production [39]. Crude glycerol phase (CGP) is the major by-product generated during biodiesel production. Despite having 65% of glycerol substrate, CGP still needs to be pre-treated to remove methanol, which exists as one of the secondary products in CGP. Proper demethanolization ensures that CGP can be effectively used as a fermentation substrate to produce PHAs, as methanol acts as a major inhibitor of microbial growth [40]. Demethanolization can be done using thermal- or vacuum-assisted evaporation methods [41]. The availability of glycerol in CGP can also be

increased by the removal of water using vacuum dehydration, distillation or more advanced phase separation methods. Apart from that, a lot of efforts have been also made to genetically modify the PHA producers to better utilize the wastes as carbon feedstocks whereby the substrates are re-directed to PHA biosynthesis pathway while deleting the unrelated pathways. These works have been comprehensively reviewed by Chen and Jiang and Nielsen and co-workers in their respective review articles [42,43].

All the strategies outlined above have one objective in common, which is to maximize PHA production. Despite increased efforts to optimize the production of PHAs, there are still concerns about the fate of the huge cell biomass that would be left after PHA extraction in a large-scale fermentation plant. Discarding the cell biomass would also incur additional costs, especially if the downstream PHA recovery process uses harsh chemicals. Some PHA-producing bacteria, such as *C. necator* have been explored for their potential to be a source of single cell protein (SCP) for humans and animals. Although many studies still need to be done to assess the suitability of these bacterial cells for human consumption, several studies have already reported the successful use of these bacterial cells as a protein source for animals and insects [44-46]. Keeping this in mind, the fermentation process for PHA production needs to be controlled by applying strategies to make maximum carbon sources available from the waste feedstocks, while at the same time optimizing the feeding strategy to produce high cell density cultures. This will ensure that cell biomass as well as PHA productivity is well balanced. An ideal PHA production process would not only produce high yield PHAs, but also high cell biomass, which could be used as a nutritional protein feed for animals and insects. Animals or insects that would feed on this high protein diet would naturally incur high protein content in their bodies. These organisms could in turn be converted into protein-enriched food for livestock. When fed to animals and insects, the lyophilized cells containing PHAs would result in the assimilation of the bacterial cells by the host animals, while the undigested PHAs would be excreted in the faeces, resulting in up to 90% recovery of PHAs from the cells. The faecal pellets would be subjected to simple green-purification procedures to remove the impurities to yield PHAs that have similar physic-chemical properties with the solvent-extracted ones. This new biological recovery approach has previously been proven to be highly successful by our team [44-47]. A recent review on this biological recovery approach has also covered the feasibility and challenges of this method in the long run [48]. The use of bacterial cells as SCP will be reviewed in more detail in the subsequent sections.

#### 4. The potential of *Cupriavidus necator* as a single cell protein

The challenge of supplying food to fulfil global demand poses difficulties when it comes to the issue of protein deficiency, which plays a role as the main component of all cellular processes [49]. SCP is microbial biomass in dried and non-alive forms such as yeast, bacteria, fungi and algae grown on various media [50,51]. Tracing the history of SCP, the name 'single cell protein' refers to protein sourced from microorganisms and was coined for the first time by Professor Carol Wilson from Massachusetts Institute of Technology [50-52]. In terms of energy input per gram of protein produced, SCP production consumes higher energy than vegetable proteins, but lower energy than livestock proteins. In general, the estimated average composition of most bacterial cells is 50% protein, 15% nucleic acids and 20% cell wall substances (Table 1) [53]. In the 1970s, Pruteen was the first commercial SCP, which used dried *Methylophilus methylotrophus* as an animal feed additive. However, the demand for SCP was not overwhelming due to competition from soy-based products. The perception of consumer acceptance towards SCP changed with rising soy prices [54]. Another advantage that places SCP in a better position is its shorter doubling time compared to other traditional protein producers (Table 1), e.g., plants and poultry [52], which causes less pollution and independent of land usage.

The first study on *C. necator* as SCP was published in 1964 by Foster and Litchfield [55]. One of the interesting aspects was the biomass's high protein content, reaching up to 74% of the total cellular content compared to yeast, fungi and algae which was around 50% (Table 1). The compositional features of amino acids found in this bacterium showed that it was nearly equal to casein protein with limitation in sulphur-containing amino acids [56]. Besides that, other nutritional values were studied by Waslien and Colloway which showed that log-phase *C. necator* contained 12-14% protein, 9% lipid and some minerals (Table 1) [57]. In the 1970s, *C. necator* H16 was developed as an SCP for human food consumption and also as animal feed [51]. Imperial Chemical Industries Ltd. was the first company to introduce *C. necator* into an SCP programme before its potential as a PHA producer was realized. The crucial factor considered in the application of SCP as food supplement is safety from harmful and carcinogenic substances, either as raw materials fed to microorganisms, by-products synthesized by microorganisms or substances formed during processing [58]. A sub-chronic toxicity study of rats completed by Molek et al. [59] to address the safety aspects of bacterial nucleic acid-reduced SCP, aside from its nutritive value, revealed the induction of immune response and proliferation of phagocytic cell lines towards the bio-based

protein. The induced immunoglobulin was reported to be IgA, existing both in blood and saliva [60]. As a result of *C. necator*'s role as an SCP, in 2013 Kunasundari et al. established an eco-friendly method to recover PHAs from bacterial cells using a biological process [44]. This patented process involved feeding rats with freeze-dried *C. necator* H16 cells bearing a moderate PHA content. They found that the cellular material, for instance cytoplasm, was digested by the rats' digestive system, which in turn resulted in the release of PHA granules during the excretion process [44]. The PHA granules were not digested presumably because of the absence of PHA depolymerases or related enzymes in the rat's digestive system. After three years, the same research group scaled up the process using insect larvae as the recovery agent instead of rats. The freeze-dried biomass of *C. necator* that was given to the mealworm (*Tenebrio molitor*) contained approximately 54% wt PHA of the CDW [45]. Based on the preliminary results reported, higher protein content was determined in mealworm beetles that were fed *C. necator* cells compared to mealworm beetles that were fed with oats [45]. Nevertheless, the PHA granules recovered from the mealworms' excretions still contained a significant amount of protein, both from bacteria and the mealworms themselves, which made the washing process difficult. Kunasundari et al. [44] revealed that the outer layer of the yellowish-white faecal pellets secreted by Sprague Dawley rats after consuming *C. necator* H16 could be partially removed by soaking them for a day in distilled water, resulting in a purity of P(3HB) of up to 94% wt. These biologically recovered PHA granules retrieved from faecal pellets contained minor quantities of proteins and other impurities attached on the surface of the granules. The detected proteins were identified as enzymes involved in PHA biosynthesis of *C. necator*, e.g., acetyl-CoA acetyltransferase, 3-ketothiolase, acetoacetyl-CoA reductase and phasins, along with other proteins such as amylase and larval cuticle protein that belongs to mealworms [45].

Proteins from unicellular microorganisms are deficient in sulphur-based amino acids, such as cysteine and methionine, and thus require additional supplementation even though they exhibit a higher composition of lysine. Moreover, high nucleic acid content inside mono-cellular protein is always the main problem, impairing the usefulness of SCP, especially in fast-growing organisms [34]. Nucleic acid is naturally metabolized into uric acid in human and animal bodies. Consumption of high amounts of nucleic acid will lead to an increased level of uric acid in humans', birds' and reptiles' blood streams. It has been postulated that an increase of uric acid in the body may lead to the formation of hyperuricemia or gout [44]. A study carried out by Kunasundari et al. [44] on the feeding of *C. necator* H16 cells to rats showed a significant increase of urea in blood serum after 14 days compared to the respective control groups, while the serum triglyceride and glucose levels were statistically lower. Interestingly, neither diarrhea nor liver malfunction was recorded during the study. The consumption of a high-protein diet initiated a depression in energy intake, causing a reduction in body fat and achieving a higher ratio of lean to fat mass [66]. In addition, elevated levels of hemoglobin were found in rats that were fed with the high-protein diet.

A decrement in the albumin to globulin ratio was identified in blood samples of broilers aged between 30-62 days when fed with mealworm diet (0.30) compared to soybean meal (0.44) [67]. At the same time, it has also resulted to a better disease resistance and immune response to broilers, perhaps due to the prebiotic effects resulting from chitin from mealworms [68,69]. Chitin is believed not to pose a health risk to humans, but it is poorly digested and absorbed by the small intestine. Being the second most abundant natural polysaccharide, it can be mediated by the microbiota in the large intestine, where chitin also acts as a prebiotic for wellbeing [67].

**Table 1:** Efficiency of protein production and composition between *Cupriavidus necator* and other SCP producers

	<i>C. necator</i>	Bacteria	Yeast	Fungi	Algae	References
Doubling time	NA	10-120 min	10-120 min	2-6 week	2-6 h	[52,61]
Efficiency of protein production in 24 h	NA	100 × 10,000,000 tonne	100 tonne			[52]
Protein percent (% dry weight)	74	50-65	45-55	30-45	40-60;	[52,55]
Nitrogen (%)	12-14	11.5-12.5	7.5-8.5	5-8	7.5-10	[57,62]
Fat content (g 100 <sup>-1</sup> g of dry weight)	9	8-10	1-8.1	1.3-4.4	3-16	[57,63]
Nucleic acid (% dry weight)	78	8-12	6-12	7-10	3-8	[52,64]
Moisture (% dry weight)	74.55					[55]
Ash (% dry weight)	1.73	3-7	5-9.5	9-14	8-10	[50,65]
Other minerals (mg 100 <sup>-1</sup> g of dry cells)	Ca 66; Mg 98 Na 280; K 590	NA	NA	NA	NA	[57]

**Abbreviation:** NA: not available

Additionally, van Huis and co-workers [70] have observed that the use of antibiotics may be reduced by feeding insects to broilers in the poultry industry. Furthermore, chitin exhibits antimicrobial effects on Gram-negative bacteria such as *Escherichia coli*, *Vibrio cholerae*, *Shigella dysenteriae* and *Bacteriodes fragile* [71]. The main drawback of using mealworms as animal feed is the high fat content, which is more than 30% on dry weight basis when the mealworms were raised using oats as the primary diet. Apart from that, the higher cost of mealworm compared to fishmeal is another bottleneck in utilizing them as an animal feed. By adopting the concept of industrial symbiosis for the production of PHA and mealworm, it may be possible to reduce both the cost and fat content of mealworms by feeding the mealworms with bacterial cells that contain PHA. This process will not only reduce the cost of recovering PHA from bacterial cells but at the same time will be more eco-friendly because the residual cell material will be used as protein rich feed for the mealworms.

## 5. Challenges in the use of insects as a food source

One of the challenges in using insects as food may include anti-nutrient properties. Chitin, which makes up most of the insect's exoskeleton, has the potential to contribute negative effects on protein digestibility [72]. On the other hand, chitin is a good source of fibre and most relevant authorities have approved the use of its extract from shellfish. In Japan, chitin is used in the production of cereals [73].

Another potential challenge is microbial risk. There are reports on spore-forming bacteria within the guts of mealworms and crickets, especially those that have been crushed. This may have been due to the release of microorganisms from the gut [74]. However, the risk can be greatly reduced by simply adding a blanching step during the processing phase [75].

Allergic reactions can also be a challenge. Most animals within the group of arthropods, which includes insects, can cause allergic reactions. For example, a positive cross-reaction has been reported between mealworm proteins and a group of people with known dust mites and crustacean allergies [76,77].

Challenges also remain in terms of government policies and regulations. The acceptance of insects as food could also be dependent on the permission of a particular country's rules and regulations. To date, there have been no regulations imposed on the eating of insects in countries that have been practicing this as a tradition. However, in most Western countries, rules are in place that creates a barrier to the use of insects as both food and feed. A statement by the European Food Safety Authority (EFSA) has revealed that each product that includes insects as an

ingredient for human consumption will be categorized as 'Novel Food' and must be approved before it is allowed to stay on the market [78].

Furthermore, parasitical hazard also presents another potential challenge in relation to insect consumption. It is known that the tradition of insect consumption is well accepted in the Asian countries thus the work linked the acceptance of consuming insects to the geographical area investigated [79]. There were about six fluke species isolated from insect gut and one of them was *Phanerosolus bonnei* (Lecithodendriid) which was first found from a human autopsy in Jakarta, Indonesia. Other infamous potential foodborne and waterborne pathogens were Protozoa, such as *Entamoeba histolytica* and *Giardia lamblia* which were found within cockroaches [80]. These parasites could be also found in edible insects and thus, should be carefully considered for human consumption.

The risk of consuming chemicals also adds on to the existing list of challenges. The consideration for accidentally ingesting chemicals during the consumption of insects as dietary supplements arose when the products were obtained by wild harvesting rather than controlled farming. This statement is supported by a case that occurred in Thailand where a major disinfestation has taken place. Dead insects which were placed on the market have been reported to cause health problems for the consumers [81]. Heavy metals are also not an exception where there was a case involving a high lead content in chapulines (dried grasshopper) which was related to elevated blood lead levels in Californian children and pregnant women [82].

## 6. Challenges and societal acceptance of mealworms in food security

In addition to the challenges reported above, there is also the issue of public acceptance of mealworms consumption by humans. The acceptability of mealworms as a human food does not only rely on its safety and nutritional value, but also on the social and ethical concerns that includes physiological, social and religious implications. The growing world population has increased food insecurity. Thus, there is a need for new human food sources and animal feed. Insects are now considered as one of the potential food sources owing to their comparable protein levels with other available food sources and relatively high content of nutrients and unsaturated fats [70,72,83]. Other advantages are that insects can also offer lower land requirements for production and have a lower environmental impact, in the sense that the emission of greenhouse gases is significantly reduced [83,84].

### 6.1 Consumer acceptability

In some parts of the world, mainly in Asian and African countries, insects are considered to be a valuable protein



source for human consumption. The suitability of insects as an alternative protein source is thought to be a local wisdom that has been passed down from generation to generation. In contrast, Westerners view entomophagy as somewhat unappetizing. A study conducted on Western views showed that only 12.8% of males and 6.3% of females were likely to consume insects as a meat replacement [85]. There were studies conducted on Westerners' willingness to consume insects which showed low acceptance [86-88]. One of the major reasons is their view on entomophagy as something disgusting and they falsely see insects as a pathogen risk which leads to the thinking of insects as food contaminants [89]. The Westerners' views on entomophagy is also considered as a product of cultural transmission in which they might think of it as taboo [90]. Studies have suggested that insect-disgust showed by Westerners does reflect the actual fear of food contamination [91,92]. It was found that there were differences in individual's trait-level of disgust sensitivity among the Swedish. The acceptance towards entomophagy is also related to one's own susceptibility to infectious diseases in relation to insect-disgust. It has been found that perceived vulnerability towards diseases is correlated to the fear of consuming contaminated food [93]. As for the Westerners who think of insects as a source of food contamination, this might be the contributing factor to the low acceptance of entomophagy.

Another factor contributing to the low acceptance of the Westerners is due to the product availability which is also considered as a main challenge of entomophagy especially in the West [94]. This is strongly related to social food norms which bring the debate into the lack of availability of insects as food products and familiarity of the food itself depending on social preferences. A person would prefer to eat what most people eat. However, studies on the effect of social norms on entomophagy acceptance has not been widely documented. One study has been done to correlate the subjectivity of social norms with insect-eating behaviour at a subsequent bug banquet by Menozzi et al. [95]. The study resulted in no significant effect which might be due to bug banquet being an individual choice rather than a social norm.

Until now, there have not been any reports on the relationship between socio-demographic factors and consumers' insect eating preferences [96]. However, the most significant factors in relation to consumers' willingness to eat insects are neophobia, convenience, familiarity and dependence on meat [85,97]. In terms of familiarity, consumers are more likely to accept insects as a food source if they are presented in a more familiar way, such as in powdered form [98]. The support from Western countries for insects as human food might be low, but support for insects as animal feed is significantly greater. In one survey, approximately two thirds of farmers in

Belgium accepted insects as animal feed [88]. The increase in support for insects as animal feed might be an initial step towards global acceptance of insects as human food.

Before certain insects can be considered a sustainable food source, the environmental impact of such consumption must be studied. The life cycle of the insects along with assessments on greenhouse gas production, energy use and land use should be quantified. Mealworms are comparable to other animal products such as milk, pork, beef and chicken. Eating insects were suggested by previous research as a more environmentally friendly alternative compared with conventional stock [99]. Conventional stock sector contributes about 15% of total emission of anthropogenic greenhouse gases (GHG) [100]. Insect has lower (2-122 g kg<sup>-1</sup> mass gain) husbandry contribution towards GHG emission when compared to cattle and pig which are 2850 g kg<sup>-1</sup> and 80-1130 g kg<sup>-1</sup> mass gain respectively [84]. As for mealworms, many factors should be taken into account in considering the contributing factors to GHG emissions. The emission does not only come from respiration, it is also related to feed production as well as the heating of the climate-controlled-rearing facility that are attributed to the product.

There are three main factors that influence the environmental impact of mealworms and other animal products. The enteric methane production, the reproduction rate and the feed conversion efficiency of each source. In terms of methane production, the use of mealworms as an alternative food source has an advantage over other animal products because mealworms do not produce methane gas [84]. The reproduction rate for mealworms is also high compared to other animals. A female mealworm of the *T. molitor* produces approximately 160 eggs in a 3-month lifetime. The maturation period is also considered to be short, as they reach adulthood in approximately 10 weeks [101]. As for the feed conversion efficiency ratio (FCR), mealworms' FCR was similar to that reported for chicken but lower than that of pigs and beef cattle [102]. In year 2002, Finke reported that all invertebrates possess sufficient quantity of protein to meet the National Research Council (NRC) recommendations [103]. Therefore, mealworms can be developed as a potential source of sustainable protein to overcome the limitation of feeding the world's growing population. The total crude protein content found in mealworms fed with bacterial cells was 71-79% of dry weight, resulted in higher protein value and at the same time with reduced fat content (8-19% of dry weight) (unpublished data).

## 6.2 Culture, religion and the history of entomophagy

Compared to tropical countries, insects are not commonly consumed in western countries. Throughout the world, large terrestrial mammalian herbivores are the most likely animals to be consumed by majority of the human

population. Most westerners prefer these kinds of food sources not only because they provide a considerable amount of meat, but also because they provide dairy products, leather, wool and many other materials. It is often thought that the utility of these animals is the main factor that has caused the use of insects to fail to create demand in western countries [70]. Another theory is that urbanization, which is more extensive in most western countries compared to tropical countries, has led to a lower consumption of insects in the sense that people live a more rural life in tropical countries [104]. Essentially, the consumption of insects will change depending on the urbanization of developing countries. For example, the consumption of locusts in the Fertile Crescent has decreased in most areas that have been influenced by strong westernization [105]. Generally, in many cultural spheres in East Asia, Africa and South America, insects are available in the wild and are part of a traditional diet [70]. However, in western countries, people do not find insects to be palatable.

It is undeniable that food practices are partly influenced by culture, which has been historically relevant to religious beliefs. In Christian, Jewish and Islamic religious literature, there are plenty of excerpts referring to the tradition of eating insects [70]. Most of the literature highlights the availability of locusts as a food source, but very few texts have discussed mealworms as a food source. The property that makes insects preferable for eating is their size, as they should be large enough to easily locate and catch. Figure 2 depicts the percentages of the most commonly eaten insects around the globe. Based on the data, it is evident that most of the insects that are eaten are

medium-sized. In addition to the spread of Western views of insects as something to fear and repulse, insects are also often considered to be ‘starvation food’, eaten only during extreme food shortages [106].

### 6.3 Are insects Halal to be consumed according to Islam?

The term ‘Halal’ is always connected to what Muslims are authorized to eat. The Muslim community comprises approximately 24% of the global population and may be considered the second largest religious group [108]. When discussing insects for human consumption, Muslims are always baffled as to whether they are halal and can be consumed.

There is no universal halal status that can be found in the Islamic context, as it differs for every species of insects and between schools of Sunni fiqh (Maliki, Hanbali, Shafi’I and Hanafi). Some Hanafi scholars have mentioned that it is prohibited to eat insects, while Maliki scholars have the opposite belief, allowing the consumption of insects with the condition that the insect must be dead by any means. These differences in beliefs have been debated by Iman Ibn Rushd in his book [109] that explains the definition of ‘filth’ when God said in the Quran, ‘and unlawful all that is filthy’ (7:157). Based on these verses (ayah), many scholars believe that the consumption of insects is prohibited. However, in Islam, every Quranic verse must be properly interpreted before it can be practised in reality. In this case, most scholars find it unacceptable to eat insects due to the nature of insects themselves, being filthy and disgusting.

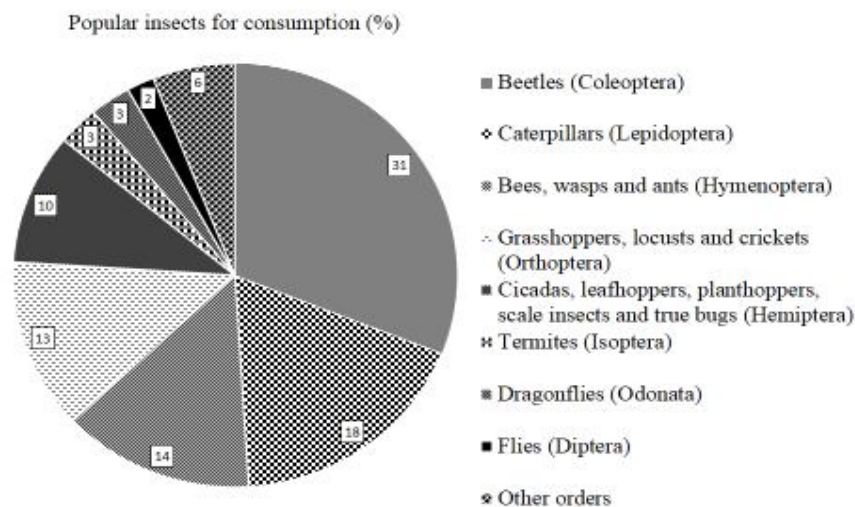


Figure 2. Popular insects for consumption [70,107]

On the permissibility of eating locusts, it is mentioned in the scriptures 'we went on seven expeditions with Messenger of Allah and we ate locusts.' [110]. So, it is clearly mentioned that Muslims are permitted to consume locusts. However, it is not clear whether the use of the word 'locusts' includes other kinds of insects.

Maliki scholars state that every dead insect can be eaten. As stated by Ibn Rushd in his book, the scholars are debating the belief that there is no need to slaughter the locusts and it is permitted to eat them if they are dead. There are some other scholars who mention that locusts need to be slaughtered by cutting off their heads or poking them with pins [111].

Another debate is regarding to whether snails, which are also categorized as insects, can be eaten. There are two types of snails: land snails and sea snails. The act of eating land snails is categorized as eating insects and vermin under the terms of Halal eating practices in Islam. Another reason why insects and vermin such as geckos, cockroaches, ants, bees, flies, worms and many others cannot be eaten is because it is not possible to slaughter them; this is a reference to (interpretation of the meaning) 'Forbidden to you (for food) are: Al-Maitah (the dead animals)' and 'unless you are able to slaughter it (before its death)' [al-Maa'idah 5:3]. According to Islamic law, slaughtering must be done between the neck and the upper chest [112].

With these disparities in beliefs, it is quite difficult to decide whether mealworms can be considered halal food. This issue is also related to scientific research that indicates whether insects are providing benefits or harm to those who consume them. Fly larvae (maggots) were formerly used to treat wounds by stopping or preventing gangrene [113]. If it were proven that insects could be used for medical purposes without resulting in any harmful side effects to humans, they would be considered edible food. If insects were known to cause harm to human beings, it would be prohibited to eat them. These new protein-rich foods need to be rigorously evaluated by responsible parties, particularly together with religious scholars, in order to affirm a common understanding that can be accepted by the global Muslim community.

## 7-Conclusion

*Cupriavidus necator* is not only popular as a PHA-producing bacterium, but also as an SCP after the discovery of its potential was made in 1964. Due to the high protein supply that can be provided by *C. necator*, it is fed to yellow mealworms to enhance its protein content and simultaneously purify the PHA granules via a single step process. An ideal PHA production process will not only produce high yield of PHA but at the same time able to supply high cell biomass that could provide sufficient nutritional protein for animals and insects. Various

challenges faced in the usage of insects as food source could be dependent on the permissibility of government policies and regulations. In addition, there are also religious concerns about the intake of insects as an alternative protein source, issue of consumer acceptance and public controversy associated with insects as sustainable food source.

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## 8. Conflict of interest

The authors declare no conflict of interest.

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## کاربرد بالقوه کاپریاویدوس نکاتور به عنوان تولیدکننده پلی هیدروکسی آلکانواتها و پروتئین تک یاخته‌ای: مروری بر چشم‌اندازهای علمی، فرهنگی و مذهبی

چین وی چی<sup>1</sup>، مانوج لکشمانا<sup>1,2</sup>، ایفا فراهین جیپری<sup>1</sup>، نابیلا هسنا محمدهاپروودین<sup>1</sup>، کومار سودش<sup>1,2\*</sup>

1- دانشکده علوم زیستی، دانشگاه سایز مالزی، 11800 میندن، پنانگ، مالزی.

2- مرکز USM-RIKEN علم پیری (URICAS)، دانشگاه سایز مالزی، 11800 میندن، پنانگ، مالزی.

### تاریخچه مقاله

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### واژگان کلیدی

- خوراک حیوان
- کاپریاویدوس نکاتور
- پلی هیدروکسی آلکانواتها
- پروتئین تک یاخته
- پذیرش جامعه

### \* نویسنده مسئول

کومار سودش، دانشکده علوم  
زیستی و مرکز USM-RIKEN علم  
پیری (URICAS)، دانشگاه سایز  
مالزی، 11800 میندن، پنانگ، مالزی.

تلفن: +604-6534367

پست الکترونیک:

ksudesh@usm.my

### چکیده

**سابقه و هدف:** پلی هیدروکسی آلکانواتها زیست‌پلاستیک‌هایی (bio plastic) سازگار با محیط زیست می‌باشند که به روش میکروبی تولید می‌شوند و به علت پایداری حرارتی و دوام قابل مقایسه با پلاستیک‌های مصنوعی می‌توانند جایگزین آنها شوند. اگرچه، هزینه بالای تولید به جهت تامین منبع کربن برای ریزاندامگان‌ها و فرایند بازیابی پایین دست استفاده از پلی هیدروکسی آلکانواتها در زمینه‌های گوناگون را محدود می‌کند. تبدیل فرآورده‌های جانبی صنایع غذایی و کشاورزی مانند ضایعات روغن‌های پخت و پز، گلیسرول، روغن ضایعات پالم، شیر تنه درخت پالم روغنی و ضایعات سویا به PHAs رویکردی جالبی است که می‌تواند ضایعات را به حداقل برساند و یا ارزش آنها را افزایش دهد.

**یافته‌ها و نتیجه‌گیری:** به تازگی، نه تنها به پلی هیدروکسی آلکانواتها، به عنوان فرآورده‌هایی با ارزش افزوده، بلکه به باکتری‌های تولیدکننده PHA به عنوان یک غذای مغذی یا منبع خوراک توجه زیادی شده است. قبلاً گزارش شده بود که باکتری تولیدکننده PHA، کاپریاویدوس نکاتور، می‌تواند به عنوان پروتئین تک یاخته به علت دارا بودن مقادیر بالای پروتئین در خوراک حیوان مورد استفاده قرار گیرد. سوسک کرم خوراکی (تنبریو مولیتور) نیز به عنوان حشره مدل برای ارزیابی کارایی یاخته‌های کاپریاویدوس نکاتور به عنوان هم منبع پروتئین و هم بازیابی دانه‌های پلی-هیدروکسی آلکانواتها مورد استفاده قرار گرفته است. اتحادیه اروپا مقررات به منظور اطمینان از ایمنی زنجیره غذایی سختگیرانه‌ای را برای نوع منبع مورد استفاده وضع کرده است. به علاوه، در این زمینه به جنبه‌های فرهنگی و مذهبی نیز باید توجه داشت. این مقاله مروری بر ارزش تغذیه‌ای کاپریاویدوس نکاتور به عنوان SCP و ایمنی آن به عنوان خوراک حیوان تمرکز دارد. همچنین، اثر استفاده از فرآورده‌های جانبی صنایع کشاورزی و غذایی، به عنوان منبع کربن برای تولید پروتئین تک یاخته و در کنار آن پذیرش جامعه این محصول را مورد بحث قرار می‌دهد.

**تعارض منافع:** نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافی مرتبط با انتشار این مقاله ندارند.