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FUNCTIONAL CHARACTERIZATION OF PUTATIVE *LATE ELONGATED HYPOCOTYL* (*LHY*) GENE IN *Stevia rebaudiana* MS007 via *IN SILICO* ANALYSIS

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ABSTRACT: *Stevia rebaudiana* is a medicinal herb that has been known as a low-calorie sweetener. It has been recognised as an artificial sweetener that is 300 times sweeter than sucrose. It is a commercially important sweetener that has been consumed as food and medicine, due to its ability to reduce blood sugar levels. Stevioside is one of the important compounds that give food a sweet taste. Previous studies showed that high amount of stevioside could be produced by delaying the flowering process of stevia. The aim of this study is to characterise the putative *Late Elongated Hypocotyl* (*LHY*) gene that is involved in the flowering process by using bioinformatics tools. Both analyses by using BLAST and domain search found that *LHY* gene is involved in the flowering process. These domains are SANT/Myb domain (IPR001005/SM000717), TEA domain (SM00426), Myb domain (IPR017930), and Myb domain plants (IPR006447). The phylogenetic tree was built using 20 out of 100 protein sequences from BLAST. The physico-chemical properties of putative LHY protein had been conducted through ProtParam and TMHMM, which showed that the protein is a globular protein. The phylogenetic tree construction indicated the common ancestor for the putative LHY protein, which is from the same family, i.e., Asteraceae. In summary, this study improves our knowledge of Stevia MS007 *LHY* gene by *in-silico* analysis. Therefore, future research should focus on determining the precise function of the protein in regulating the blooming stage of the stevia plant.

KEY WORDS: *Stevia rebaudiana, Late Elongated Hypocotyl, LHY, steviol glycosides.*

1. INTRODUCTION

One of the alarming illnesses that had been around since ancient Egypt around 3,000 years ago is diabetes mellitus. In 2019, according to The Star Online, the Health of Minister Malaysia stated that there were 3.6 million people suffering from diabetes, and approximately 31.3% of adults aged 18 years and older will have the disease by 2025 [37]. In addition, the World Health Organization (WHO) estimates that 108 million people worldwide have diabetes, and the number will continue to rise in 2024. Nonetheless, according to the International Diabetes Federation (IDF), the number of individuals with diabetes is rising steadily [1]. Due to its reputation as a low-calorie sweetener, *Stevia rebaudiana* offers several options to control the regulation of sugar. *S. rebaudiana* is a member of the Asteraceae family and is a popular artificial sweetener that is roughly 300

times sweeter than sucrose [2]. Besides that, the leaves of *S. rebaudiana* were found to contain diterpene glycosides like stevioside, rebaudioside A-F, dulcoside, and steviolbioside [3]. These substances also have diuretic, antitumor, anti-inflammatory, anti-hypertensive, anti-hyperglycemic, and anti-tumor effects [4].

Steviol glycosides are sweetener compounds that can be extracted from the leaves and are considered to contain zero calories. However, the production of steviol glycosides in *S. rebaudiana* is influenced by many factors, such as environmental conditions, geographic localities, physiological properties of plants, altitude, nutrient deficiency in the soil, and pathogenic diseases [5,6]. Besides, it was mentioned that the highest steviol glycoside content is found in the leaves by delaying the flowering of stevia, allowing more time for glycoside accumulation [7]. Stevia is a short-day plant that requires 13 hours of critical light for the growth process [8]. Research shows that the highest accumulation of stevia glycosides occurs during the budding phase, followed by an initial flowering stage with less than 10% flowers [8]. In addition, it was mentioned that when stevia plants grow under short daylight, the production of steviol glycosides is lower and the growth of the plants will be retarded [5].

Photoperiod plays a major role in floral induction. Phototropism is when the plants align their photosynthetic organs toward the direction of sunlight. The alignment of plants' organs was regulated by the circadian clock. For example, in *Helianthus annuus*, the circadian clock guides solar tracking in the plants by controlling the movement of the stem and shoot apices either facing east at dawn or facing west at dusk [9]. The researchers reported that circadian oscillators enhanced the fitness of plants by coordinating physiological processes with changes in the environment. Studies of molecular genetics and biochemical studies have shown that the *Late Elongated Hypocotyl* (*LHY*) gene regulates photoperiodic flowering and expression of photoperiodic flowering genes *via* the circadian clock in Arabidopsis mutant plants [10]. The initial studies focused on isolating and characterizing the *LHY* gene in *Arabidopsis thaliana*, a model plant. This involved identifying its sequence, understanding its genomic organization, and examining its expression patterns over a 24-hour period to establish its involvement in circadian rhythms [35]. Besides, research by Niwa (2007) [36] demonstrated that *LHY* interacts with other clock components, such as *Circadian Clock Associated 1* (*CCA1*) and *Timing of Cab Expression 1* (*TOC1*) genes. These interactions form a complex regulatory network that contributes to the precise timing of circadian rhythms. The central components of circadian oscillators consist of the *LHY* gene and the *Circadian Clock Associated 1* (*CCA1*) gene, which help plants exhibit early flowering, even in unsuitable environments [10, 11]. The circadian clock provides information and photoreceptors to integrate light signals for plants to sense photoperiodic changes [10].

Hence, identification of putative *LHY* gene that is involved in flowering process will improve stevia plant production to not only produce high yield of leaves but also steviol glycosides content. Hence, the aim of this study is to characterise the putative *LHY* gene, which was predicted to be involved in the formation of Stevia's flowers. By modifying the flowering process, the anticipated outcome of this study will assist in raising the output of steviol glycosides.

2. MATERIALS AND METHODS

2.1 Data Collection

In this study, bioinformatics studies were carried out on the transcriptome data of Stevia MS007, where the dataset consists of raw sequence data derived through transcriptome sequencing [12, 13]. Seventeen query IDs from the dataset were verified, by referring to the *LHY* gene of Stevia MS007. Only one protein Cluster-31069.11078, out of the 17 *LHY* genes, was chosen to a larger extent.

2.2 Translation of nucleotides using Expasy

In order to obtain the protein sequence, the nucleotide sequence for Cluster 31069.11078 from the stevia dataset was sent to Expert Protein Analysis System (Expasy) (https://web.expasy.org/translate/). It enables accurate translation of DNA sequences into protein sequences, providing essential information for gene identification, protein prediction, comparative analysis, and functional annotation.The longest protein sequence was chosen from the raw data of Stevia's database, which was saved from *.txt into *.fasta. This conversion facilitates compatibility, metadata inclusion, analysis, and data sharing across various research areas, and accessible way to work with biological sequence data.

2.3 Homology search by BLAST

The protein sequence corresponding to Cluster-31069.11078 was then analysed to find similarity using Basic Local Alignment Searching Tools (BLAST), which are available at (https://blast.ncbi.nlm.nih.gov). The BLAST programme is an invaluable resource for researchers in various fields of biology. It provides a user-friendly interface to perform sequence similarity searches, aiding in functional annotation, evolutionary analysis, disease research, and others [14]. There are more than 100 sequences that have been aligned with the query sequence, and only 20 sequences with high percent identities from different species were selected. Then, the 20 sequences were downloaded as FASTA complete sequences for further use in phylogenetic analysis.

2.4 Protein Domain search by InterPro

The InterPro database (http://www.ebi.ac.uk/interpro/) was then used to perform a domain search for the LHY protein sequence. The database can predict and integrate the sequence by representing protein domains, families, and functional sites. Besides, it also aids researchers to uncover information about protein domains, motifs, and functional sites, leading to insights into protein function, evolution, and structure [15]. There are other sources of databases that are joined together in the InterPro, such as Protein Families (Pfam) (https://pfam.xfam.org) and Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/). Pfam is a database of protein domain families and allows comparison between query sequences with the Pfam database. Meanwhile, SMART is intended to allow automatic identification and annotation of protein domains.

2.5 Physico-chemical Properties of Proteins using ProtParam and TMHMM

ProtParam (https://web.expasy.org/protparam/) was used to identify the molecular weight, theoretical pl and amino acid composition of a protein sequence. On the other hand, the Transmembrane Helices Hidden Markov Model (TMHMM) (http://www.cbs.dtu.dk/services/TMHMM-2.0/) is an essential resource for predicting transmembrane helices within protein sequences. This prediction provides insights into the structure, topology, and function of membrane proteins, impacting various areas of research.

2.6 Phylogenetic analysis

There are two main steps to build phylogenetic tree, which are Multiple Sequence Alignment (MSA) and tree construction using Molecular Evolutionary Genetics Analysis (MEGA) [16]. The Multiple Sequence Alignment (MSA) will be generated using the MUSCLE software, which is available at (https://www.ebi.ac.uk/Tools/msa/muscle/) that is used for aligning multiple biological sequences, such as DNA, RNA, or protein sequences. This is essential for understanding sequence relationships, identifying functional domains, and performing comparative analyses. Then, Molecular Evolutionary Genetics Analysis (MEGA) software is designed for comparative analysis of the query sequence with the reference sequence. MEGA software can be downloaded from (https://www.megasoftware.net). The software is available for installation on Microsoft systems. Then, a phylogenetic tree was constructed by using 20 sequences from different plant species with 1000 replicates of bootstrap.

3. RESULTS AND DISCUSSION

3.1 Homology search

The BLAST programme was used to evaluate the information that was taken from the dataset of stevia MS007. There are 100 sequences matching hits with the putative *LHY* gene according to the homology search done using blastP. Nonetheless, only 20 sequences from various species with high percentages of identity were chosen. The maximum E-value in the study is between 10^{-30}. In order to establish the sequences, the E-value computation was important. Sequences were considered homologous if they have low E values with both protein sequences and sequences from the protein database [17]. According to study data presented in Table 1, all protein sequences have scores larger than 115 and percentage identities greater than 80%. *Helianthus annuus* has the highest total score and percentage identity, with scores of 134 and 94.12%, respectively. Hence, it is concluded that all proteins were closely related to this stevia protein sequences.However, protein LHY from [*Cynara cardunculus* var. *scolymus*] shows high identity but lower score value. In BLAST analysis, both "percentage identity" and "total score" are important metrics used to assess the similarity between a query sequence and a database sequence. However, they capture different aspects of the alignment and should be interpreted differently. A high percentage identity indicates a strong sequence similarity in the aligned regions, but the total score considers various factors including gaps and mismatches. If it shows high identity but a lower score, it suggests that while there are matching segments, there are also differences or gaps in the alignment that affect the overall alignment quality.

3.2 Domain search analysis

A crucial component that is stable and encapsulates the entirety of a protein's structure is a domain [18]. One or more domains make up the majority of the protein. Also, these conserved areas can be found in various types of proteins, which aids in the activity of the protein and are not just unique to one family of genes [18]. Thus, InterPro programme was used in this study's analysis of domain searches. The outcome of the domain search investigation was displayed in Table 2.

In addition, the prediction from the InterPro analysis showed that there were three types of domains present. The length of the query protein sequence is 67 amino acids, and the location of those domains is between 21 to 67 amino acids.

Table 1: The top ten hit sequences from homology search using blastP.

Accession	Description	Percentage Identity (%)	Total Score	E-value
XP_021983251.1	protein LHY-like [*Helianthus annuus*]	94.12	134	5.3e-35
XP_016735595.1	PREDICTED: protein CCA1-like [*Gossypium hirsutum*]	86.57	119	1.6e-33
XP_023753679.1	protein LHY isoform X3 [*Lactuca sativa*]	92.54	129	2.3e-33
XP_007139035.1	hypothetical protein PHAVU_009G259600g [*Phaseolus vulgaris*]	85.07	118	2.5e-33
XP_021896768.1	protein CCA1-like [*Carica papaya*]	85.07	115	3.3e-32
XP_024996248.1	protein LHY [*Cynara cardunculus var. scolymus*]	98.28	125	5.3e-32
XP_030535347.1	protein LHY isoform X1 [*Rhodamnia argentea*]	88.06	124	2.3e-31
NP_001296649.1	protein LHY [*Cicer arietinum*]	91.80	123	3.7e-31
XP_016463073.1	PREDICTED: protein LHY-like [*Nicotiana tabacum*]	89.55	123	4e-31
XP_020211115.1	protein LHY isoform X2 [*Cajanus cajan*]	90.16	122	6.1e-31

Table 2: Result of domain search analysis through InterPro, SMART and Pfam

Accession	Domain	Function of Domain
IPR001005 or SM000717		SANT domain • Present in chromatin-remodeling enzymes and histone acetyltransferase. • It has a strong structural similarity to the DNA-binding domain of Myb-related proteins. • It plays role in helix packing due to the presence of three α helices in the domain [19]. • The SANT domain is stabilizing the histone binding conformation by directly interacting to histone tail [19]. • SANT domain is functionally divergent from the canonical Myb DNA-binding domain. • Mostly found in eukaryotes, bacteria, viruses and archaea kingdom.
SM00426		TEA domain • The domain is a DNA-binding region of about 66 to 68 amino acids that has been named after the two proteins that originally defined the domain: TEF-1 and ABAA [20]. • TEF-1 can control transcription of genes while ABAA can regulate gene in asexual spore differentiation [20]. • This domain appears to be conserved throughout evolution and it is predicted to contain three alpha-helices which are involved in DNA binding [20]. • Mostly found in eukaryotes kingdom.
IPR006447	Myb domain, plants (24-67 amino acids)	• DNA-binding domain is restricted to plant proteins and contain a response regular domain [21]. • The domain related to Myb-like DNA-binding domain.
IPR017930	Myb domain (21-67 amino acids)	• The myb-type helix-turn-helix (HTH) domain approximately 55 amino acids occur in a tandem repeat in eukaryotic transcription factors [22]. • The 3-D structure of domain forms three alpha-helices which Helix 3 is a recognition helix that binds DNA major groove.

3.3 Prediction of Physico-chemical Properties of Putative LHY Proteins

The presence of the transmembrane helices is important and useful for functional annotation analysis. The prediction of transmembrane helices is much easier than that of helices in globular domains, where the prediction of transmembrane helices is based on the hydrophobicity properties of the transmembrane towards the query protein [23].

Furthermore, it was mentioned that the "Hidden Markov Model" (HMM), which provides information on the overall topology of the protein, can be used to predict the

topology of proteins [23]. In addition, TMHMM can predict all membrane proteins in full sequenced genomes and present statistics on the frequency of proteins with different topologies. Fig. 1 illustrates the result of TMHMM for accession of *LHY* gene for Cluster-31069.11078.

The graph shows three different lines with different colours: the bold pink line means the residues are outside the cell, the pink line shows whether the residues are inside the cytoplasm or not, and the bold blue line means the residues are inside the cell. Thus, it can be concluded that the protein is a globular protein and does not have transmembrane helices because the protein is located outside of the transmembrane.

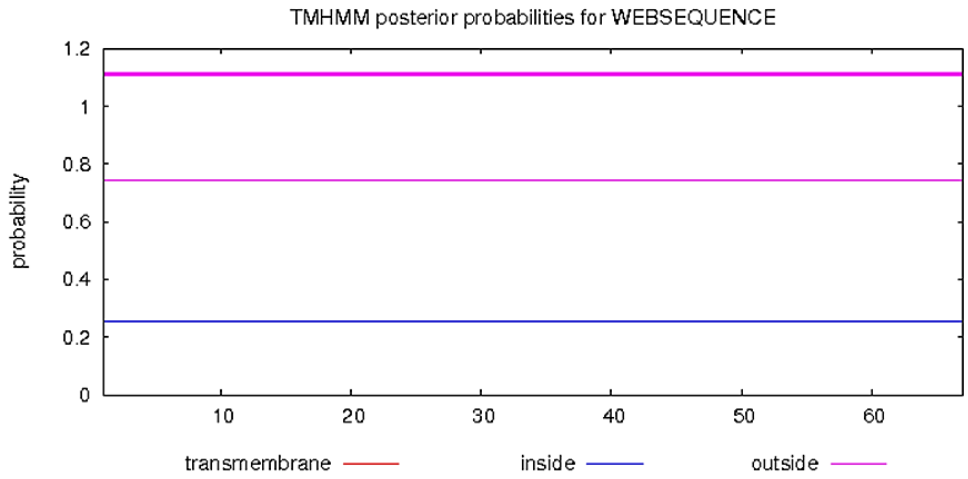

Fig. 1. Result of TMHMM for accession Cluster-31069.11078. The above bold pink line depicts that this protein is a globular protein without the presence of transmembrane.

Following that, Table 3 provided examples of predictions for the physical and chemical characteristics of putative LHY proteins. The analysis provided information on the quantity, molecular weight, and isoelectric point of the amino acids (pI). In addition, the instability index could also be obtained from this analysis, which indicates whether the protein is stable or unstable. If the instability index is lower than 40, the protein is stable, but if the index value is higher than 40, the protein is known as an unstable protein. LHY proteins are unstable proteins, so they cannot be easily extracted in the laboratory. All information from ProtParam analysis is handy, such as the molecular weight of amino acid, which is needed during electrophoresis analysis. In addition, incorporating analysis of physico-chemical properties adds depth and context to LHY proteins. The changes in properties across different species can provide insights into the adaptive evolution of proteins [38].

Table 3: Analysis of physico-chemical properties of putative LHY proteins.

Accession	Number of amino acids	Molecular weight (Da)	Isoelectric point (pI)	Instability index
Cluster-31069.11078	67	7829.75	9.52	46.73

3.4 Multiple Sequence Alignment

A vital initial phase in creating phylogenetic trees is multiple protein sequence alignments. In 2018, it was said that protein alignment is essential for identifying a protein's family, domain, and functional site as well as for predicting the protein's structure and function [24]. Local and global sequence alignment are the two different types [25]. Multiple sequence alignment was performed to align each sequence in the query set. In this study, MUSCLE was applied to align protein sequences. Fig. 2 presents the analysis of multiple sequence alignments. The different colours of proteins depict the different physico-chemical properties of proteins [26]. From the figure, it can be seen that at the C terminal end of proteins, there is not much variation based on the colour of the protein. Based on Edgar (2004), he said the conserved residues denoted with an asterisk (*). With the N- and C-termini of the sequences removed, a solid phylogenetic tree was created using the alignment of the sequences utilised in the analysis [26].

Fig. 2. Analysis of multiple sequence alignment using MUSCLE. In the protein sequences, the domain region is shown by a red box, and identical amino acid residues are marked by an asterisk (*).

3.5 Phylogenetic analysis

The putative LHY protein in this study is predicted to be involved in regulating the circadian clock of plants based on phylogram tree that contained bootstrapping values greater than 50% [10]. The maximum likelihood algorithm was chosen to construct this phylogenetic tree. These algorithms used a statistical approach to infer the probabilities of sequences in a model tree. The phylogenetic tree generated using the maximum likelihood tree approach is depicted in Fig. 3. The phylogenetic tree can be separated into three subgroups based on the "bootstrapping value," which is more than 70% with 1000 replicates of the tree.

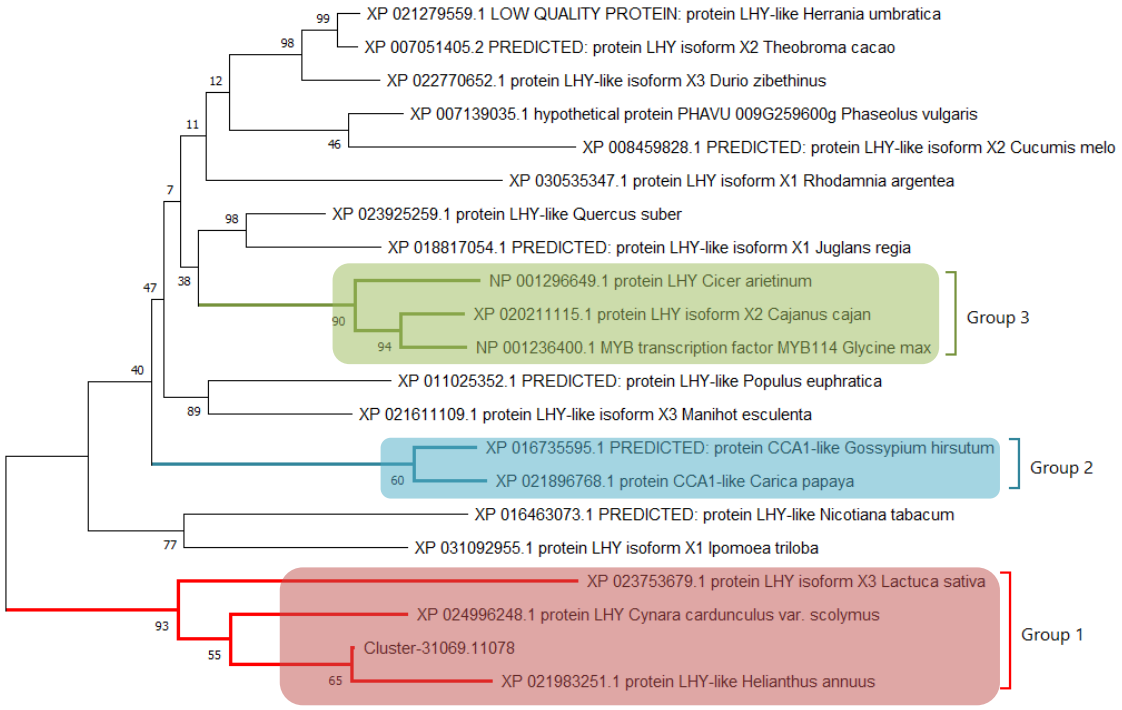

Fig. 3. Phylogenetic tree construction by the maximum likelihood tree method. Parameter: JTT model with amino acid changes per site, the branch lengths are measured in the same units as the developmental distances used to infer the evolution of the phylogenetic tree.

According to the phylogenetic tree, sub-group 1 includes *Lactuca sativa*, *Cynara cardunculus*, and *H. annuus* from the Asteraceae family, as well as putative LHY protein. It can be assumed that the putative LHY protein also comes from the same family, i.e., Asteraceae. Based on a study in 2016, it was stated that asterid species are known as ancestral genome duplications that contribute to the evolution of the Asteraceae family [27]. These species shared approximately the same ancestor, with bootstrap value higher than 90%. The rapid diversification of the Asteraceae is associated with the polyploidization in this family [27].

CCA1 and LHY have been shown to support physiological processes in plants, including plant growth and biotic and abiotic stress response [28]. Besides, it was identified that the LHY protein acts as a central player in the circadian clock of plants that controls the gene expression rhythm and also the photoperiodic induction in plants [29]. In addition, *H. annuus* depicts that *LHY/CCA1* expression can suppress the expression of PRR proteins in the morning [30].

On the other hand, sub-group 2 consists of different plant families, i.e., *Gossypium hirsutum* and *Carica papaya*, which came from the Malvaceae and Caricaceae families with 'bootstrap' values equal to 60%. Mainly, this group contained CCA1-like proteins with a conserved domain. Gossypium species appear to have divergent protein sequences due to duplication events [31]. The substitution of an amino acid leads to paralog, which appears in the linkage over a long period of time. When paralogous occurs, the composition and function of sequences or genes will be changed. In addition, families of Caricaceae undergo

duplication events in their chromosome numbers and appear to have stable genome sizes [32].

The sub-group 3 of the Fabaceae family contains the HTH (helix-turn-helix) myb-domain, which plays a role in DNA binding during the transcription process. This subgroup has a high 'bootstrap' value of more than 90%. According to the study, MYB is one of the largest gene families for plant transcription factors and has a very conservative DNA binding domain [33]. They play an important role in the regulation of hormones, organogenesis, leaf morphogenesis, and seed germination [34]. LHY with CCA1 and TOC1 located in the centre of the circadian clock is a MYB protein transcription. Additionally, the amino acid sequences of LHY and CCA1 are remarkably similar and control the circadian rhythms in plants [33].

4. CONCLUSIONS

From the analysis that has been carried out, the putative *LHY* gene of Stevia MS007 was successfully identified and characterised through an *in-silico* method. The LHY protein shows high percentages of identity with *H. annus*, *Gossypium hirsutum*, and *Cynara cardunculus* var. *scolymus*. Besides, the discovery demonstrates that the SANT domain, TEA domain, and Myb-like DNA-binding domain are present in the *LHY* gene and are important in stabilising the histone binding conformation by directly interacting with the histone tail during the transcription process. Nonetheless, based on a physicochemical property investigation, it was shown that LHY proteins are unstable proteins that cannot be easily isolated in the laboratory. The relationship between Cluster-31069.11078 of Stevia MS007 LHY and the other homologous proteins was determined using phylogenetic tree analysis. The evolutionary tree shows that the Stevia MS007 LHY protein shares a common ancestor with individuals from the Asteraceae family. *S. rebaudiana* and *H. annuus* were closely linked since the clade is well supported and the bootstrap values were greater than 50%. While they share the same plant family, they are distinct species with their own characteristics and attributes. It would be a huge benefit to be able to modify this information, in order to manufacture artificial sweeteners.

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TECHNO-ECONOMIC AND ENVIRONMENTAL ANALYSIS OF MELIPONICULTURE IN BUKIT SANDY, BANDUNG

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ABSTRACT: Propolis produced by stingless bees has a high economic value in Indonesia. Bukit Sandy is a plantation area in Cimenyan, Bandung which is famous for citrus picking tourism and has the potential to be developed for meliponiculture. Currently, there are stingless bee colonies in Bukit Sandy, but the colonies are not growing well. This research aimed to examine environmental conditions and techno-economic analysis of meliponiculture in Bukit Sandy. This research used descriptive analysis, vegetation analysis, colony carrying capacity analysis, and techno-economic analysis methods. The results show that environmental conditions in Bukit Sandy are suitable for meliponiculture. Dominant forage vegetation potential for stingless bees in Bukit Sandy are mahogany (tree), pine (pole), lemon (sapling), and paspalum (seedling). The estimated potential for production of honey in Bukit Sandy ranges from 2.25-30.21 kg/month and the carrying capacity of stingless bee colonies in Bukit Sandy is 4-54 colonies. This study proposed several scenarios for techno-economic analysis for cultivation of *Heterotrigona itama*; i) 4 colonies with raw propolis and honey as products, ii) 4 colonies with propolis extract, honey and propolis residue as products, iii) 54 colonies with propolis extract, honey and propolis residue as products. Based on the techno-economic analysis, meliponiculturein Bukit Sandy is technically feasible and profitable for scenario 2 and 3, while scenario 1 is not profitable. Scenatio 2 is proposed for early stages of meliponiculture in Bukit Sandy and later transformed to scenario 3 for higher profit and benefits to the society.

KEY WORDS: *Colony carrying capacity, Heterotrigona itama, Meliponiculture, Techno-economic analysis, Vegetation analysis.*

1. INTRODUCTION

Propolis is one of non-timber forest product that has a high economic value derived from bees. Propolis has many beneficial properties such as antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant [1], fungicide [2], anti-protozoa, anti-cancer, anti-tumor, and hepatoprotective [3] and can be used as an ingredient in cosmetics, food, and medicine [4,5]. Stingless bees are one of the bees that produce propolis. These bees produce more propolis but produce less honey than *Apis* bees [6,7]. The propolis produced by stingless bees has been reported to contain a higher economic value because of a higher bioactive content compared to the propols produced by *Apis* bees [8].

Stingless bees can be cultivated like honeybees. The advantages of meliponiculture are that it does not need to be maintained intensively, it does not sting, it does not need special equipment, relatively resistant to pests and diseases, and it does not have famine periods [9]. In addition, stingless bees can increase the productivity of plantations because they are pollinating insects [10,11]. However, meliponiculture is less desirable because of low honey productivity but recently, meliponiculture has begun to be considered for development because the honey produed by stingless bees has abetter quality than produced by honeybees. In addition, stingless bees also produce other pdocucts particularly propolis and pollen [9].

Bukit Sandy is a plantation area in Cimenyan, Bandung which is famous for citrus picking tourism and has the potential to be developed for meliponiculture. The area has many varieties of plants besides citrus such as butter avocados, lemon, guava, and many more [12]. The vegetation diversity is one of the potentials for meliponiculture in Bukit Sandy. Moreover, the presence of several colnies of stingless bees in Bukit Sandy can help pollinate the plants in that area and the products from meliponiculture can also be profitable for Bukit Sandy. However, the colonies are not growing well under current conditions in Bukit Sandy.

Previous studies conducted by Ichwan et al. [13] and Yanto et al. [14] showed that suitable environmental conditions for stingless bee colonies are very important for survival. Carrying capacity of the colony is alse another important factor that need to be considered to ensure availability of sufficient nutrients for stingless bee colnies to grow well [15,16]. If the factors are managed well, meliponiculture is technically and economically feasible as previously investigated by Adam et al. [17]. Systematic studies that investigate environmental and techno-economic analysis of meliponiculture are still very scarce. Therefore, this study aimed to analyze environmental conditions, carrying capacity of stingless bee colonies, and techno-economic analysis of meliponiculture in Bukit Sandy.

2. METHODS

This study was conducted in the Bukit Sandy located at Ciharalang Kulon No.9, Mekarsaluyu Village, Cimeyan District, Bandung Regency, West Java. Geographically, Mekarsaluyu Village is located at 900 – 1100 m above sea level (masl) altitude that is categorized as highland with an average air temperature of 26˚C-29˚C and rainfall of 1,500 mm/year [18]. The total area of Bukit Sandy is ± 9 Ha but only approximately ± 7 Ha that can be easily accessed in this study. The selection of sampling plots in this study was done purposively and carried out from May 2021 to June 2021.

2.1 Sampling and Data Collection Methods

The sampling methods used in this study were purposive sampling and intensity sampling. Purposive sampling was used to collect data interview from the Bukit Sandy management as respondents while the intensity sampling was used to collect vegetation data (5%) from Bukit Sandy area based on Hadjar et al. [19]. The sample plots were shown in Fig.1 with a size criterion of 20 x 20 m for the tree level, 10 x 10 m for poles, 5 x 5 m for saplings and shrubs, and 2 x 2 m for seedlings and herbs and the placement of sample plots as shown in Fig. 2 in the foraging range of 200 m from the point of the colony [20].

Measurement of temperature and humidity were carried out using data loggers. Other data collection was carried out throughout observations, interviews, inventory, Google imagery, GPS trackers, and literature study. Field observations and interviews with

employees and management of Bukit Sandy were carried out in March 2021 to gather information about Bukit Sandy's environmental conditions. Inventory of vegetation was used to collect species identity, density, frequency, and diameter at breast height to describe the vegetation in Bukit Sandy. Google imagery and GPS tracker were used for mapping the plantation areas in Bukit Sandy.

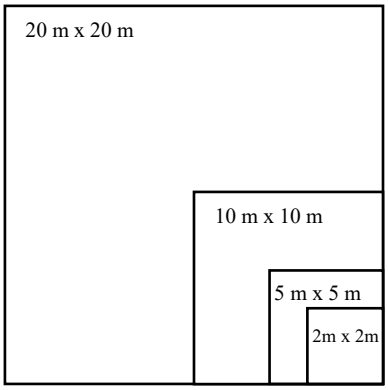

Fig.1.Sample plots for vegetation analysis in Bukit Sandy

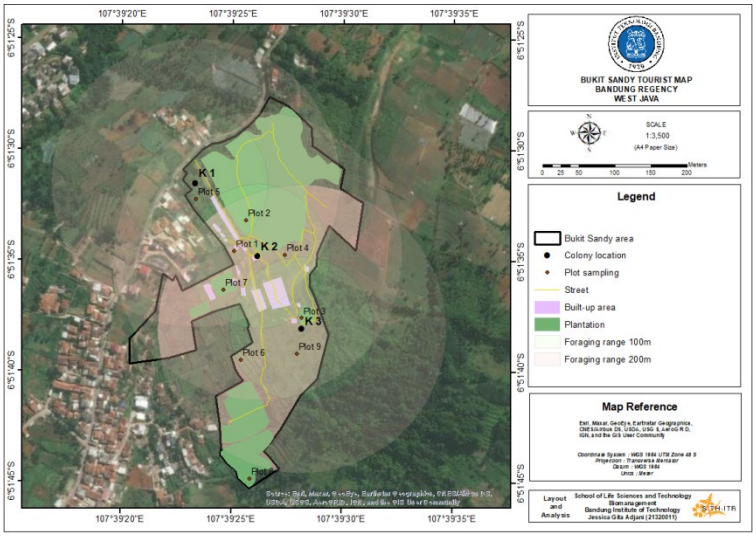

Fig.2.Location of stingles bee colnonies and sample plots in Bukit Sandy

2.2 Methods Analysis

2.2.1 Vegetation Analysis

Plants that have been inventoried from each sample plot were matched with the literature suitable for vegetation of stingless bees. After that, the data was tabulated to calculate the importance value index (IVI) based on relative density (RD), relative frequency (RF), and relative dominance (Rdom) for the level of trees and poles using Eq. (1) to (4) [21] and Eq. (5) for sapling and seedling levels [22].The values of IVI can be used to express the level of dominance of species of a plant community in Bukit Sandy.

$$RD\ (\%) = \frac{species\ density}{total\ species\ density} \times 100\% \quad (1)$$

$$RF\ (\%) = \frac{species\ frequency}{total\ species\ frequency} \times 100\% \quad (2)$$

$$Rdom\ (\%) = \frac{species\ dominance}{total\ species\ dominance} \times 100\% \quad (3)$$

$$IVI\ (\%)_{tree\ and\ pole} = RD + RF + Rdom \quad (4)$$

$$IVI\ (\%)_{sappling\ and\ seedling} = RD + RF \quad (5)$$

2.2.2 Colony Carrying Capacity Analysis

Calculation of feed availability was based on the nectar availability in Bukit Sandy. Amulen et al. [23] estimated the potential of beekeeping based on the nectar availability sources. The calculation model requires data on number of flowers per plant, nectar volume per plant, and sugar concentration. The data of sugar concentration obtained from the literature was then converted to total sugar using the equation suggested by Chamberlain and Rajaselvam [24]. Honey production potential (HPP) and modified colony carrying capacity (CCC) can be calculated based using Eq. (6) and Eq. (7) as suggested by Bareke et al. [25] where f is the average number of flowers per plant, s is the average amount of sugar per flower, t is the time of flowers secrete nectar (days), c is bee consumption, and p is the productivity of harvested honey.

$$HPP\ (kg\ sugar/plant) = f * s * t \quad (6)$$

$$CCC\ (colony) = \frac{HPP}{c\ +\ p} \quad (7)$$

2.2.3 Techno-economy Analysis

Technical analysis for meliponiculture in Bukit Sandy was carried out based on the availability of vegetation of stingless bees to produce various products particularly honey, raw propolis, propolis extract, and propolis residue. Meanwhile, the economical analysis in this study includes calculation of Total Capital Invetsment (TCI), Total Production Cost (TPC), total revenue, Break Even Point (BEP) unit with FC as fixed costs, p as price per unit, and v as variable cost per unit; BEP price; Net Present Value (NPV) with r as an interest rate of return; Internal Rate of Return (IRR) with i as interest rate, Benefit-Cost Ratio (BCR); and Payback Period (PP) where n as the last year of cash flows has not covered the investment, a as the amount of investment, b as cumulative cash flows in the n year, and c as cumulative cash flows in the n+1 year [26-28]. The value of money and interest rates used in this study were based on the values during the investigated period.

$$BEP\ (kg) = \frac{FC}{p - v} \quad (8)$$

$$BEP\ (US\$) = \frac{FC}{1 - \left[\frac{v}{p}\right]} \quad (9)$$

$$NPV\ (US\$) = \frac{net\ cash\ flow\ 1}{(1+r)} + \frac{net\ cash\ flow\ 2}{(1+r)^2} + \cdots - Invesment \quad (10)$$

$$IRR\ (\%) = i_1 + \frac{NPV_1}{NPV_1 - NPV_2} \times (i_2 - i_1) \quad (11)$$

$$BCR\ (\%) = \frac{\sum PV\ net\ cash\ flow}{\sum PV\ invesment} \times 100\% \quad (12)$$

$$PP\ (year) = n + \frac{(a-b)}{(c-b)} \times 1\ year \quad (13)$$

3. RESULTS AND DISCUSSION

3.1 Environmental Conditions in Bukit Sandy

During the investigation period, there were 18 colonies of *Tetragonula laeviceps* producing a limited amount of honey and propolis. Based on the interviews and observations, the condition of 15 colonies did not develop properly while 3 colonies had bee fleas within the hive with 1 colony full of stingless bees but no honey meanwhile the other colonies were almost abandoned by the colony. Only 16 colonies can be saved for further survival.

Bukit Sandy is topographically categorized as a hilly area with different slopes and altitudes. Research from Sabila [11] shows that the slope in Bukit Sandy is dominated by the steep category with range of 25-45% and several areas that have extremely steep slopes (> 45%) because Bukit Sandy management wants to maintain in the natural state of that location. Beside that, Bukit Sandy is 987.5 – 1125 masl altitude which is categorized as highlands. The altitude will affect the types of plants that grow in that area which impacts the availability of feed for stingless bees [29].

Bukit Sandy area is not only used for tourism but also for plantations of local commodities and academic research. Based on the observation results, pesticides were applied at nearby asparagus plantation area rented by local farmers within Bukit Sandy. This condition does not meet the requirements for meliponiculture which prohibits the use of pesticide within the areas of stingless bee clonies because it can cause decrease growth and may even lead to death for the colonies [9].

According to Kwapong et al. [30], meliponiculture near pesticide areas can be overcome by coordinating with local farmers regarding the timing of pesticide spraying so that the Bukit Sandy manager can close the hives of stingless bee colonies before the spraying begins. Furthermore, there is ascreen house dedicated for cultivation *Hermetia illucens* (Black Soldier Fly, BSF) within the area of Bukit Sandy. The BSF is known asa predator for stingless bees, so meliponiculture needs to be far from the secreen house [31]. The minimal exposure to pesticide and predator is the foraging range from the point of the colonies.

The mapping of land area in Bukit Sndy from Google imagery is shown in Fig. 3 and Table 1. Total area in Bukit Sandy is 7.60 Ha. The built-up area and plantation in Bukit Sandy are 3.27% and 37.96%, respectively. Land potential in Bukit Sandy is around 58.77% which indicates that there is still a large area for meliponiculture. According to Ichwan [13],

meliponiculture does not require a large area of land. Large areas of land can be used to provide necessary vegetations for stingless bees.

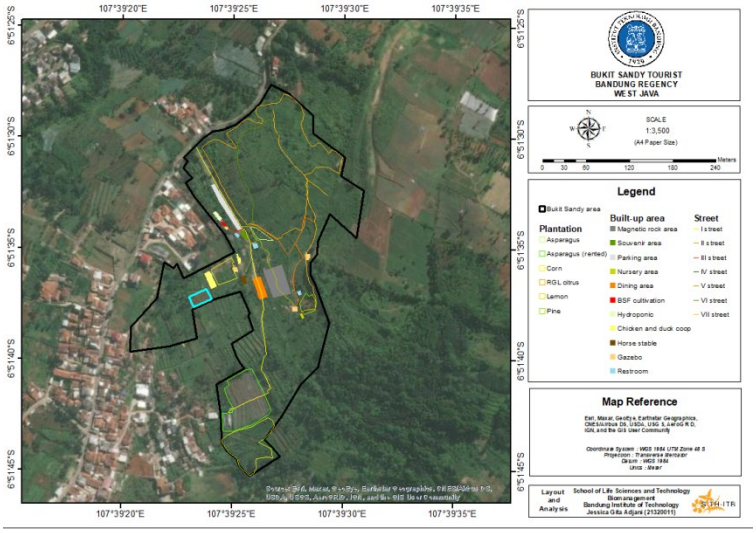

Fig.3.Mapping of built-up and plantation ares in Bukit Sandy

Table 1: Recapitulation of land area in Bukit Sandy

Name	Area (Ha)	Percentage (%)
Bukit Sandy	7.60	100
Plantation	2.89	37.96
Built-up area	0.25	3.27
Potential land	**4.47**	**58.77**

Microclimate is one of the requirements for stingless bees to develop properly [13]. Based on the observation results, the air temperature in July 2021was around 18-27˚C with 70-95% humidity in Bukit Sandy. Stingless bees can live and develop at temperatures of 23-30˚C and relative humidity of 77-94% [32]. Other research mentions that *Tetragonula sp.* can live at 22-28˚C temperatures with 55-88% humidity [33]. The results shows that the microclimate at Bukit Sandy is still within range that is suitable for meliponiculture.

3.2 Vegetation Analysis

Availability of feed is the key to the success of stingless beekeeping [34]. Based on interview results, Bukit Sandy has many types of plants besides citrus variety Rimau Gerga Lebong (RGL) such as lemon, buddha's hand lemon, avocado, passion fruit, jackfruit, sunflower, lemongrass, cogon grass, pine, lantana, asparagus, and may more. The natural disaster which occurred for the first time at Bukit Sandy in 2020 is a tornado. This disaster caused some of those plants and some areas in Bukit Sandy extreamly damaged.

Based on the vegetation analysis, 31 types of plants were identified, and the results were shown in Table 2 and Table 3. Plants that have the potential as feed for stingless bee were around 67.74%. Five plants are identified as tree level, three plants as pole level, eight plants as sapling level, and seventeen plants as seedling level (herbs and liana). Plants at pole level are tree except pine and at sapling level are shrubs except mangoes. Highest IVI for each level is suren, pine, lemon, and cogon grass, respectively. Suren from tree level and cogon grass from seedling level not categorized as stingless bee feed. Therefore, the highest IVI for tree and seedling level as stingless be feed is Mahogany and paspalum, respectively. Mahogany as stingless feed can provide nectar and resin [29,35], pine (the only shrub at

pole level) provide resin [36], lemon provide nectar and pollen [37], and paspalum provide pollen [38].

Table 2: Vegetation analysis in Bukit Sandy

No	Name	Scientific name	RD (%)	RF (%)	Rdom (%)	IVI (%)	Feed
Tree							
1	Avocado	*Persea americana*	11.11	16.67	4.93	32.71	√
2	Suren	*Toona sureni*	33.33	33.33	46.27	112.93	-
3	Mahogany	*Swietania macrophylla*	33.33	16.67	12.77	62.77	√
4	Rambutan	*Nephelium lappaceum*	11.11	16.67	15.17	42.95	√
Pole							
1	Matoa	*Pometia pinnata*	14.29	33.33	10.34	57.96	√
2	Pine	*Pinus merkusii*	71.43	33.33	62.76	167.52	√
3	Suren	*Toona sureni*	14.29	33.33	26.90	74.52	-
Sapling							
1	Lemon	*Citrus limon*	31.43	25.00	-	56.43	√
2	RGL citrus	*Citrus reticulate var RGL*	28.57	18.75	-	47.32	√
3	Caliandra	*Calliandra calothyrsus*	5.71	6,25	-	11.96	√
4	Lantana	*Lantana camara*	22.86	25.00	-	47.86	√
Seedling							
1	Touch-me-not	*Mimosa pudica*	6.17	10.81	-	16.98	√
2	Cogon grass	*Imperata cylindrica*	52.77	21.62	-	74.39	-
3	Paspalum	*Paspalum sp.*	29.87	21.62	-	51.49	√
4	Redflower ragleaf	*Crassocephalum crepidioides*	3.94	8.11	-	12.05	-

Table 3: The floral calender and type of vegetation feed in Bukit Sandy

No	Name	Type of feed			Month											
		N	P	R	1	2	3	4	5	6	7	8	9	10	11	12
1	Avocado	√	√													
2	Pine			√												
3	Mahogany	√		√												
4	Mangoes	√	√	√												
5	Matoa	√														
6	Rambutan	√	√	√												
7	Lemon	√	√													
8	Water apple	√	√													
9	RGL citrus	√	√													
10	Caliandra	√	√													
11	Lantana	√	√													
12	Cassava	√	√	√												
13	Touch-me-not	√	√													
14	Paspalum		√													
15	Corn		√													
16	Sweet potato	√														
17	Goat weed	√	√													
18	Snakeweed		√													
19	Clidemia		√													
20	Orosne		√													
21	Kirinyuh	√	√													

Feed availability compositions based on the plant type in Bukit Sandy are 76.2% nectar (N), 81.0% pollen (P), and 23.8% resin (R). Bees use nectar as source of energy while pollen as source of protein [39]. Lack of nectar sources will affect the hygiene behavior of the bees

which affects the growth of the colony and make the bees want to look for pollen [40]. The composition of nectar and pollen is slightly different, but the ratio of nectar and pollen is almost equal. Feed distribution based on plant type was highest in March and lowest in February and November (Fig.4). The average distribution of feed per month is 8.33% ± 1.20%.

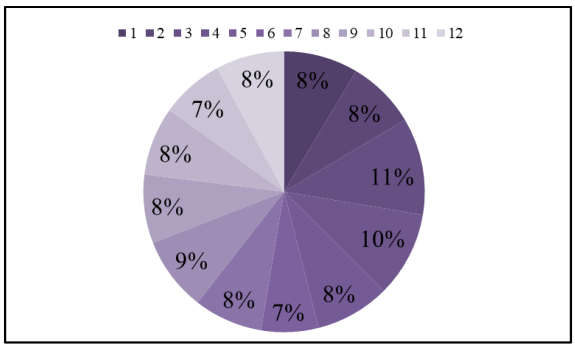

Fig. 4. Feed distribution permonth available for stingless bees in Bukit Sandy

3.3 Carrying Capacity of Stingless BeeColonies in Bukit Sandy

K3 is a meliponiculture location in Bukit Sandy that meets the requirements based on the environmental description. Estimation of feed availability uses the honey production potential (HPP) approach with total sugar content units in plants [23, 25]. However, there are competitors with other insects such as butterflies, ants, bees, etc. so the HPP decreased by 40% [23]. To convert total sugar mass into honey, it was assumed that the acceptability of honey on the market is 18% water content so 82% total dissolved sugar of 82% is obtained from 1 kg of honey [25]. Stingless honey has a water content of 28.60% so the dissolved sugar of stingless honey is 71.40% [41]. The results of HPP per month are shown in Table 4.

Table 4: Honey production potential and colony carrying capacity per month in Bukit Sandy

Month	HPP (kg)	HPP after competiton (kg)	Sugar to honey (kg)	CCC
January	6.28	3.77	5.28	10
February	6.28	3.77	5.28	10
March	35.95	21.57	30.21	54
April	32.27	19.36	27.11	48
May	27.61	16.57	23.21	42
June	2.69	1.61	2.25	4
July	2.69	1.61	2.25	4
August	2.69	1.61	2.25	4
September	25.85	15.51	21.72	39
October	25.85	15.51	21.72	39
November	25.85	15.51	21.72	39
December	6.28	3.77	5.28	10

The calculation of colony carrying capacity (CCC) assumed that the stingless bees collect 16 mg nectar per day with a sugar content of 0.4 mg sugar/mg nectar [42] and the number of individual stingless bees in the colony is 2000 [1] so 1 colony needs 0, 96 kg of nectar/month or equivalent to 0.38 kg of sugar/month or 0.54 kg of honey/month. Honey productivity varies between 49.2-66.6 ml per 2 months [43], 200 ml/colony/year [44], 0.3-

0.4 kg/colony/year [45], and 600-700 gr/colony/year [46]. Hence, it can be inferred that stingless bees produce less than 1 kg and the honey productivity used in this study was estimated at 300 g/colony/year or 25 g/colony/month.

Based on the analysis, the carrying capacity of stingless bee colonies in the K3 location varies from 4 to 54 colonies each month (Table 4). The minimum carrying capacity of stingless bee colonies in the K3 location makes the feed sufficient every month. If Bukit Sandy manager wants to maximize the carrying capacity of stingless bee colonies, it is necessary to provide additional feed support and requires good feed management for the survival of colomies.

3.4 Techno-economy Analysis

The techno-economic analysis carried out in this study was based on the estimation of the carrying capacity of stingless bee colonies in Bukit Sandy. The main product of the meliponiculture is propolis. Hakim and Abduh [47] reported that *Tetragonula laeviceps*can produce 2-2.4 g/week/colony propolis while Pribadi [48] reported that *Heterotrigona itama* can produce 34.97-37.20 g/colony/month. As such indicates that *H. itama* can produce more propolis than *T. laeviceps*. Moreover, the microclimate conditions in Bukit Sandy are more suitable *H. itama* with average temperature and humidity of 28.81°C and 83.06%, respectively [49]. Hence, this study investigates a techno-economic analysis for meliponiculture in Bukit Sandy using *H. itama* with three proposed scenarios:

1. Scenario 1 (SC 1): meliponiculture based on minimum CCC (4 colonies) with raw propolis and honey as products.
2. Scenario 2 (SC 2): meliponiculture based on minimum CCC (4 colonies) with propolis extract, honey and propolis residue as products.
3. Scenario 3 (SC 3): meliponiculture based on maximum CCC (54 colonies) with propolis extract, honey and propolis residue as products.

3.4.1 Technical Analysis

The calculation of production capacity uses the estimated productivity of raw propolis and honey that have been calculated. The productivity of the raw propolis used in this study is 0.42 kg/colony/year [48] and 0.30 kg/colony/year for honey [45]. The estimated production capacity for scenarios 1 and 2 is 1.68 kg raw propolis/year and 1.2 kg honey/year. Meanwhile, the production capacity for raw propolis is 22.66 kg/year and 16.20 kg/year honey for scenario 3.

The production process started with meliponiculture using MOTIVE (Modular Tetragonula Hive) made of wooden boxes with dimensions 20 cm x 18 cm x 18 cm [47,48], to production of meliponiculture products in Bukit Sandy. The overall process was divided into two types of process such as the production process of raw propolis and honey for scenario 1 and the production process of propolis extract, honey, and propolis residue for scenarios 2 and 3 as shown in Fig. 5.

The implementation of meliponiculture for scenarios 1 and 2 needs to reduce the current number of colonies on Bukit Sandy because it exceeds the estimated CCC. In contrast to scenario 3 that needs to increase the number of colonies to maximize the CCC of the existing colonies. It is aligned with increasing the amount of feed and good feed management for stingless bees. The additional feed used for scenario 3 is sunflower [50] and coral vine [51]

because of stingless bees like both flowers. Both flower plants produce nectar and pollen [50].

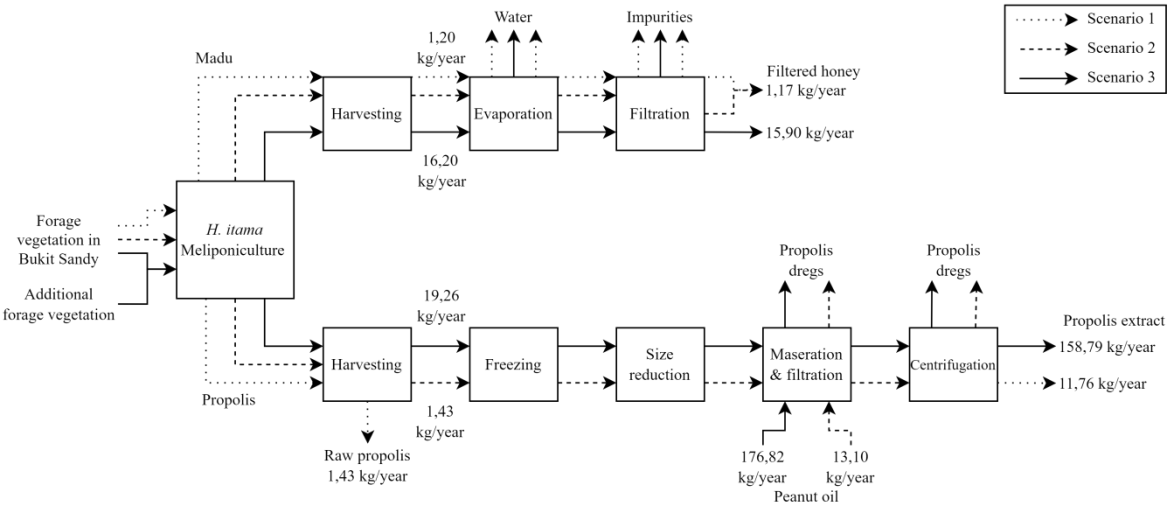

Fig. 5. Block flow diagram and mass balance for scenario 1,2, and 3

The honey production process started from the harvesting process. It was assumed that honey from *H. itama* meliponiculture in Bukit Sandy could be harvested 4 times per year [8]. The next process was to filter honey to separate impurities from the honey and evaporated the honey to reduce the moisture content of honey for all scenarios [17]. The final target of the expected moisture content in *H. itama* honey was 27% according to the criteria of SNI 2018 [52] and the Department of Malaysian Standards [53] while the targeted impurities were 0.7% [52]. The process of reducing the moisture content of *H. itama* honey uses a simple tool with the dehumidification method [54,55]. The dehumidification process was set at 40˚C for 1 hour 30 minutes.

The propolis production process started with harvesting of raw propolis once a month [48]. It was assumed that 85% of raw propolis that could be separated from the propolis frame [1]. Raw propolis from scenario 1 could be packed immediately while for scenarios 2 and 3, were stored in the refrigerator for 24 hours [56] to make it easier for size reduction [57]. The next processes were the extraction process by maceration and the purification process by filtration and centrifugation. Peanut oil was chosen as the solvent because there are additional benefits from the oil and have prospects in the field of health and halal food supplements [4,57]. The ratio between raw propolis and peanut oil was as suggested by Firmansyah et al. [57] particularly 1:10 w/v and the maceration process was carried out for 7 days to obtain a high content of antioxidant compounds with an estimated yieldof 89.98%. Purication was carried out using centrifugation with to produce propolis extract as the main product and propolis residue as the co-product.

3.4.2 Economical Analysis

The investment cost for meliponiculture of *H. itama*in Bukit Sandy is shown in Table 5 which comprises of *H. itama* colonies, total cost of equipment, distribution and electrical installation, water installation, land and building costs, and unexpected costs [1]. The total investment cost for scenario 3 is much higher than the other 2 scenarios due to higher amount of colonies and consequently more investment is needed. The total production cost of proproplis for all scenarios are shown in Table 6. The total production cost consists of

fixed costs (FC) and variable costs (VC). Fixed costs are costs that are relatively fixed and are not affected by the amount of production. Fixed costs for *H. itama* meliponiculture include labor salaries, equipment depreciation, electricity utilities, internet, equipment maintenance, and marketing costs. Variable costs are costs that will change according to the amount of production and can be affected by changes in the market. Variable costs include raw materials for production and packaging.

Table 5: Total capital investment for meliponiculture of *H. itama* in Bukit Sandy

Component	Cost (US$)		
	Scenario 1	**Scenario 2**	**Scenario 3**
***Direct Cost* (DC)**			
H. itama colonies	41.26	41.26	556.99
Total cost of equipment	148.60	939.39	3.727.92
Distribution and electrical installation	0	127.21	433.22
Water installation	0	0	398.83
Land and building	0	687.64	3,060.02
Total DC	189.86	1,795.51	8,176.98
***Indirect Cost* (IC)**			
Unexpected costs (0,05 DC)	9.49	89.78	408.85
Total IC	9.49	89.78	408.85
***Total Capital Investment* (TCI)**			
TCI = DC + IC	199.35	1,885.28	8,585.83

Table 6: Total annual production cost of propolis for meliponicultre of *H. itama* in Bukit Sandy

Parameter	Cost (US$)		
	Scenario 1	**Scenario 2**	**Scenario 3**
Total Fixed Cost (TFC)	1,538.28	5,961.22	26,450.65
Total Variabel Cost (TVC)	7.23	691.79	9,782.38
Total Co-credit (CC)	51.00	136.00	1,838.00
Total Production Cost (TPC)			
TPC = FC+VC	1,545.51	6,653.02	36,233.03
TPC/kg propolis	1,080.77	565.73	228.18
TPC - CC	1,494.51	6,517.02	34,395.03
(TPC-CC)/kg propolis	1,045.11	554.17	216.61

From Table 6, it can be obserbed that TPC per kg of propolis for each scenario decreases when the sales of co-products such as honey and propolis residue were considered. The TPC-CC per kg for scenarios 2 and 3 are still acceptable for the Indonesian and international markets that still allow for a profit margin [58,59] whereas for scenario 1, the TPC-CC per kg is too high for commercial sales. Therefore, scenario 1 was not analyzed further. The Break-even point (BEP) for scenarios 2 and 3 are 7.72 kg (US$ 6.415,19) and 100.45 kg (US$ 32.639,18), respectively as illustrated in Fig. 6 and Fig. 7.

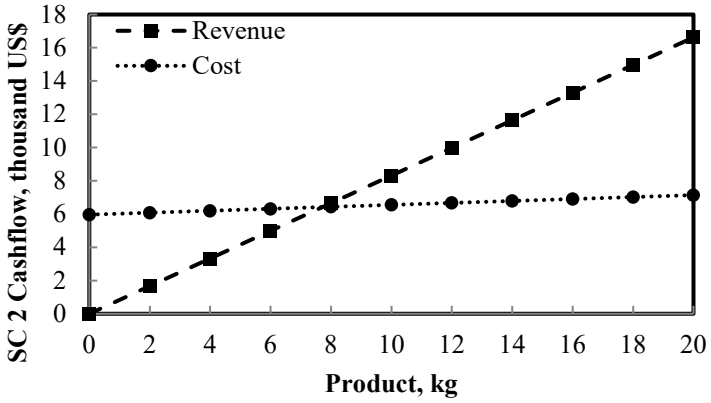

Fig. 6. BEP of scenario 2 for meliponiculture of*H. itama*in Bukit Sandy

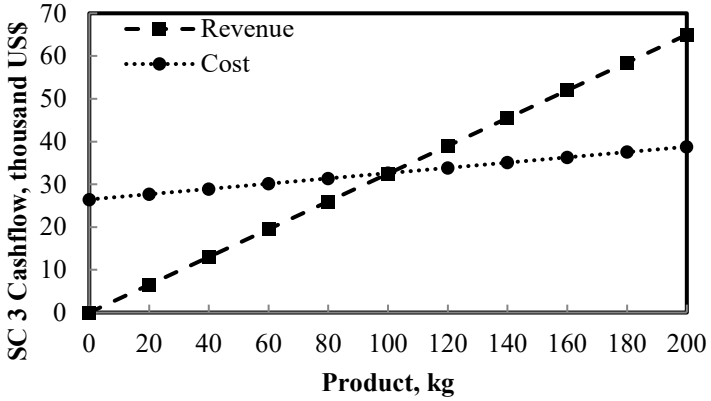

Fig. 7. BEP of scenario 3for meliponiculture of*H. itama* in Bukit Sandy

The cumulative cash flows for scenarios 2 and 3 are shown in Fig. 8. The preparation process for meliponiculture at Bukit Sandy was estimated around 6 months. In addition, the sales of propolis for scenarios 2 and 3 in the 1st year were assumed 85% and will increase by 3.5% [1] in the 2nd - 5th year and reached 100% in the 6th year. Sales of honey for scenario 2 were assumed to be 100% annually while for scenario 3 was 85% in the 1styear and will increase by 6.5% [1] in the 2nd - 3rdyear and reached 100% in the 4th year. The production capacity of the products was assumed to remain the same. Income tax for scenario 2 was subjected to a 0% rate while for scenario 3 was 0.5% based on Indonesian regulation No. 7 year 2021. The discount rate was assumed to be 15% [60].

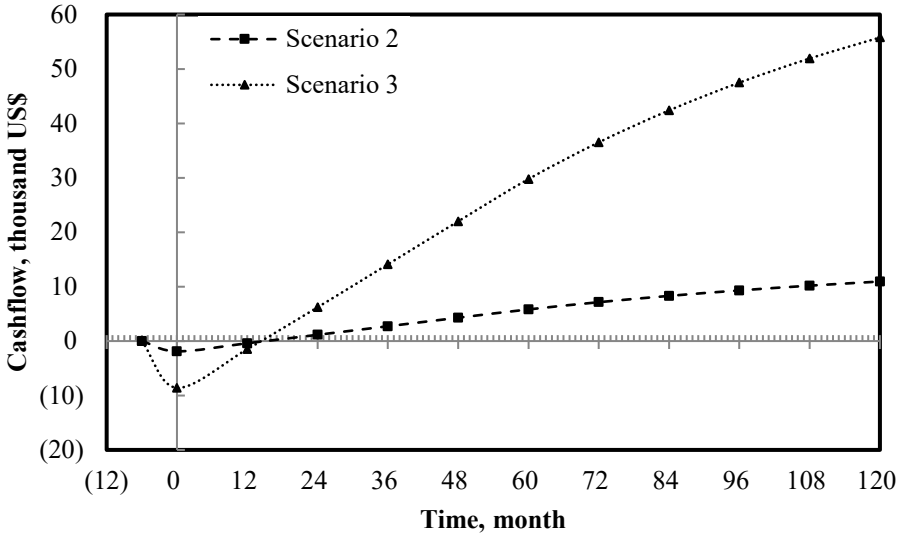

Fig. 8. Cumulative cash flow for *H. itama* meliponiculture in Bukit Sandy

Table 7: The investment criteria of meliponiculture in Bukit Sandy

Criteria	Scenario 2	Scenario 3
NPV	US$10.909,85	US$116.208,62
IRR	105.19%	121.15%
PP	1.3 years	1 year
BCR	4.02	4.49

The recapitulation of the investment criteria is shown in Table 7. The investment is considered feasible if the NPV > 0, IRR > 3.75% (Bank of Indonesia reference rate), BCR > 1, PP is faster than the project lifetime, BEP unit < production unit [61]. Based on that criteria, scenarios 2 and 3 maybe considered feasible with is scenario 3 is more profitable as compared to scenario 2. However, scenario 2 maybe a good option for early stages of meliponiculture in Bukit Sandy due to easier operation for managing only 4 colonies of *H. itama* and yet to achieve a PP after 1.3 years. After the establishment of meliponiculture in Bukit Sandy, the number of colonies can be increased up to 54 colonies of *H. itama* for higher profit and benefits to the society.

4. CONCLUSION

This study has investigated the environmental conditions, carrying capacity of stingless bee colonies, and techno-economic analysis of meliponiculture in Bukit Sandy. The findings suggest that environmental conditions in Bukit Sandy are suitable for stingless beekeeping with dominant forage vegetation potential for stingless bees in Bukit Sandy are mahogany, pine, lemon, and paspalum. The estimated potential for production of honey in Bukit Sandy ranges from 2.25-30.21 kg/month and the carrying capacity of stingless bee colonies in Bukit Sandy is 4-54 colonies. Meliponiclture based on minimum carrying capacity (4 colonies) with propolis extract, honey and propolis residue as products is proposed for early stages implementation in Bukit Sandy and gradually transformed to meliponiculture based on maximum carrying capacity (54 colonies) with propolis extract, honey and propolis residue as products for higher profit and benefits to the society.

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AI-BASED WASTE MANAGEMENT OPTIMIZATION IN THE HALAL FOOD INDUSTRY OF MALAYSIA: A MINI REVIEW

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ABSTRACT Solid waste management (SWM) has become a critical issue in Malaysia, with increasing amounts of waste generated every year and limited resources available to manage it effectively. Additionally, the halal food industry is rapidly growing and expanding globally due to the rising Muslim population, predicted to reach 2.2 billion by 2030 at an annual growth rate of 1.5 percent. This increasing production and consumption of halal food has an impact on the environment. Artificial intelligence (AI) has the potential to revolutionize solid waste management by improving efficiency, reducing costs, and optimizing waste management processes. This mini review provides an overview of the impact of AI on solid waste management in Malaysia, focusing on the current trends, challenges, and opportunities in the industry, particularly in the halal food sector. The review offers insights into the potential of AI in enhancing waste collection, optimizing waste management processes, improving resource recovery and recycling, and reducing waste to landfill. Additionally, the review explores the current initiatives, projects, and developments in the field of AI and solid waste management in Malaysia and identifies areas for future research and collaboration. The review concludes that AI has a significant role to play in improving solid waste management in Malaysia, and continued investment and development in this area is necessary to achieve sustainable waste management practices. Furthermore, its findings have the potential for wider applications and inspire future research in AI-based waste management solutions across various industries. The findings and recommendations of this review have the potential to be adapted and implemented in other industries facing similar waste management challenges.

KEY WORDS: *Artificial intelligence, Machine Learning, Halal food waste, Sustainable waste management, Malaysia.*

1. INTRODUCTION

The increasing population in Malaysia and worldwide has led to a significant rise in solid waste production, posing a critical challenge for many countries to manage effectively. While Muslims represent a substantial market segment for halal food, it is imperative to recognize that they are not the exclusive consumers of such products. The halal food industry is a rapidly growing global market, driven by the expansion of the Muslim population, which is predicted to reach 2.2 billion by 2030, growing at a rate of 1.5% annually [1]. With the increasing demand for halal food, there is an opportunity for food manufacturers to expand and introduce new sustainable products to the market. Currently, the halal food market is estimated to be worth over $2 trillion, up from $1.5 trillion in 2018 [1]. Worldwide, many approaches have been tested to effectively manage the increasing volume of waste. Several innovative solutions were tested to optimize waste management

processes and reduce the amount of waste sent to landfills. Nevertheless, it is noteworthy that these endeavors often neglect to specifically target the halal food sector. In Malaysia, the most commonly used method of solid waste management is landfilling disposal [2]. The process involves collecting and transporting waste to designated landfill sites, where it is deposited and compacted in a controlled manner. While landfilling is considered the easiest and cheapest method of managing solid waste, it has significant environmental and health effects, namely the release of methane and other greenhouse gases, as well as the potential for groundwater contamination. Alternatively, solid waste management in Malaysia includes other methods such as recycling, composting, and incineration. Nevertheless, these methods are not adopted as widely as landfill disposal.

Many programs have been implemented or planned by the Malaysian government, with a main aim to promote sustainable solid waste management practices, including reducing waste generation, encouraging waste reduction and recycling, and improving waste management infrastructure. Artificial intelligence (AI) and machine learning (ML) have the potential to offer sustainable solutions to solid waste management challenges by using advanced algorithms and data analytics. These algorithms can analyze vast amounts of data, such as waste generation patterns, collection, and resource recovery and recycling efforts, to optimize solid waste management processes. Table 1 summarizes the findings from the chosen articles.

Table 1. Summary of findings from related articles using AI and ML

Models/Technology	Application	Results	Ref.
Artificial intelligence and Deep Learning	Visual inspection process within the manufacturing process	Model inspected 715 images and achieved an accuracy of 99.86% in inspecting products, outperforming all the other existing models from published works. AI image recognition can help design out food waste by ensuring the packing of only quality products, reducing return rates and subsequent waste.	[3]
ML algorithms	Quality control for food items during manufacturing and packaging.	ML algorithms successfully predicted deviations in production, thereby offering the potential to reduce food wastage by ensuring the packing of only quality products, reducing return rates and subsequent waste.	[4]
	To predict the unexpected level of hazards in a food supply chain network.	predict the unexpected level of hazards in a food supply chain network.	[5], [6]
Blockchain and AI	Sustainability and data monetization in the food supply chain.	A combination of AI and blockchain can ensure traceability in the food supply chain to decrease food waste.	[7]

The objective of this mini review is to explore the use of AI in solid waste management and the halal food industry in Malaysia and to provide a concise assessment of the current trends, challenges, and opportunities. The review aims to explore how AI can be utilized to address waste management challenges throughout the process, from waste generation to resource recovery. By analyzing these issues, the review aims to identify areas for future research and improvement.

2. CURRENT STATE OF SOLID WASTE MANAGEMENT AND HALAL FOOD INDUSTRY

Despite efforts by the government and private sector to improve waste management systems and infrastructure, many areas in Malaysia still rely on traditional methods such as landfill disposal and open dumping. Currently, there are 146 non-sanitary operating discharges in Malaysia, including landfills, open dumps, and other non-sanitary sites [3]. Several research has discussed the significant environmental and health impacts of using such disposal methods. This section will discuss an overview of the solid waste generation and management in Malaysia and the challenges and limitations of traditional solid waste management methods.

2.1. Overview of the solid waste generation and management in Malaysia

Solid waste generation in Malaysia has increased significantly in recent years, attributed to population growth and economic development. Recently, Malaysia generates approximately 39 million tons of waste per year [4], 65% of which is household solid waste which has increased twofold in the last two decades due to population growth and urbanization [5]. Waste per capita also doubled, increasing from 0.90 in 2005 to 1.17 in 2022 [6]. The majority of this waste is generated in urban areas, including household waste, industrial waste, construction, and demolition waste. The latest statistics indicate that Malaysia generated almost 3 million tonnes per year of household food waste. As per the World Bank, urban areas generate two to three times the amount of solid waste compared to their rural counterparts [7].

Malaysia has set a goal to reduce the amount of solid waste sent to landfills from 82.5% in 2021 to 40% by 2023 [5]. Despite efforts by the government to promote waste reduction and recycling, and the potential to recycle up to 80% of the collected dry waste, the current recycling rate in Malaysia is still low, estimated at around 20-22% [8]. The need for sustainable and efficient solid waste management in Malaysia is critical and has been widely discussed. Furthermore, there is a pressing need for initiatives dedicated to promoting waste reduction, recycling, and the integration of advanced technologies such as AI. However, there are still significant challenges to improving solid waste management in Malaysia, including a lack of consistent and comprehensive data, limited public awareness and participation, and a need for further investment and innovation in solid waste management technologies and practices.

2.2 Current state-of-art for AI in Halal Food industry Waste Management

The essential need for sustainable and efficient food systems has underscored the urgent necessity for improvements in the area of solid waste management in the food industry [9]. Furthermore, studies have explored different approaches to improve waste management practices in the industry, including reducing waste generation [10], improving segregation and collection of waste [11], and implementing innovative waste treatment and disposal technologies, and implementing innovative waste treatment and disposal technologies.

Tseng et al. [12] has focused on identifying innovational solutions and areas of improvement in food chain using data-driven of sustainable food supply chain for reducing food waste and enhancing waste management practices. The study also provided a comparison between halal and non-halal food supply chains. Additionally, the study highlights the most significant indicators for both halal and non-halal sustainable food supply chain, food safety was fundamental for both supply chains, food consumption,

security, and food waste management were the mostly important in non-halal food supply chain. Whereas, Islamic values, halal certification, and halal supply chain trust were the most crucial indicators in a halal food supply chain (HFSCs). The literature review has concluded that supply chain management represents a critical aspect of halal food production [13], and therefore, integrating eco-friendly and sustainable practices into supply chain management constitutes an effective approach.

Similarly, Rejeb [13] has conducted a review and bibliometric analysis on 74 journal articles. Despite a growing number of publications in the last two decades, the analysis suggests that halal food supply chains remain a relatively new area of research. The main focus of the review was to identify trends in publications on HFSCs, including the top contributing countries, publishing journals, and research methods employed in the collected studies. It could be clearly noticed that currently there is limited systematic data-driven approaches exists to identify the most suitable indicators for promoting sustainability in both halal and non-halal food supply chains [12-13]. Moreover, halal food supply chains (HFSCs) are becoming more complex, uncertain, and fragmented.

Out of the 74 chosen papers, Malaysia is the predominant country with 37 publications. The article highlights the increasing global demand for Halal food products and the need to ensure their sustainability [13]. However, there is a lack of research on the sustainability of HFSCs compared to non-halal food supply chains. Moreover, the existing literature on HFSCs focuses mainly on the certification process and the religious requirements of halal food, rather than the sustainability issues. Besides, HFSCs face similar sustainability challenges as non-halal food supply chains, including food safety, environmental impact, and social responsibility. The review also indicated that it was not until 2007 that literature regarding the creation of sustainable HFSCs appeared. From then, the research on sustainability in the context of halal food gradually gained momentum, and by 2014, it had made significant progress.

Furthermore, Bux [14] provided a comprehensive review of the literature on the intersection of halal food sustainability, certification, and blockchain technology. The authors examine the challenges faced by the halal food industry in ensuring sustainability and ethical production practices and explore the potential of blockchain technology to address these challenges. Authors elaborated that blockchain technology has the potential to enhance transparency and traceability in the halal food supply chain, which can help to prevent fraud, ensure ethical production practices, and increase consumer trust. However, the article primarily focuses on the potential of blockchain technology to enhance transparency and traceability in halal food certification. Yet, blockchain technology has also been explored in the context of waste management.

França et al. [15] has proposed a system that utilizes blockchain technology to provide financial management for waste collection in the municipality. The objective of this system is to promote health, environmental education, and socio-economic inclusion of citizens, with the use of social currency. Additionally, the author concluded that the restructuring and supervision of existing solid waste management models through blockchain technology can potentially decouple economic growth from the depletion of non-renewable resources. This approach may lead to the conservation of non-renewable resources and the retrieval of materials through an improved solid waste management system.

Lastly, Reynolds et al. [10] presents an overview of 17 practical measures for preventing food waste during the consumption and consumer phase of the supply chain. The

measures were identified by conducting a rapid review of literature published between 2006 and 2017. The review revealed that certain interventions, such as using color-coded refrigerator, product labelling, and providing information, could be implemented on a large scale in households. Similarly, plate and portion size adjustment, modification of menus and nutritional guidelines, and changes in class curriculum could be effective in preventing food waste outside of the home.

Overall, the current halal food supply chain and production systems in Malaysia lack the presence of AI technology, presenting a significant challenge that requires attention. Implementing AI technology in the halal food industry presents various opportunities to achieve a sustainable production system, minimize waste, and ensure food safety. Studies have shown that the incorporation of AI technology in the food industry can improve quality control, reduce production time, and minimize food waste [11-12], [16-17].Therefore, it is crucial for the Malaysian government, private sectors and Muslim community to invest in AI technology to enhance the Halal food production system and promote sustainable practices.

3. THE ROLE OF AI IN SOLID WASTE MANAGEMENT

AI is the use of technology to mimic the human brain. Recently, AI has gained popularity as a computational approach to address various issues across numerous fields. AI has been utilized in several aspects of Solid Waste Management (SWM) to enhance the performance and productivity of waste management operations [18-19]. It has been discovered that AI is better equipped to handle complex, unpredictable, and missing data, and it is able to continuously improve based on experiences [18]. Some of the key areas in which AI has been used in solid waste management include waste sorting and recycling [20] waste collection optimization [21] environmental monitoring [22], and public awareness and education (Fig. 1).

Likewise, ML has emerged as a transformative technology with broad applications across various industries, including the realm of artificial intelligence (AI) [16]. ML, a subset of AI, encompasses a diverse set of techniques that enable computer systems to learn from data and make predictions or decisions without being explicitly programmed.

While numerous research efforts have concentrated on the application of AI and ML technologies in solid waste management, there is a notable scarcity of research in the specific domain of the halal food industry. Sinthiya et al. [24] has conduct a systematic literature review on AI-based smart waste management covering between 2001 to 2021, the research analyzed 40 research papers. Each has utilized different types of AI models - including pure ML and deep learning models in addition to hybrid models - for different application to enhance SWM systems. Mostly, the articles have focused on the municipal solid waste management field, with limited discuss for organic food waste. Nevertheless, the systematic literature review has only used one database, Scopus, which has limited the research outcomes.

Fig. 1: Applications of AI in Food Waste Management adopted from Owen et al. [27].

Similarly, Abdallah [18] provided an extensive analysis of how artificial intelligence is utilized in managing solid waste worldwide. The main focus of the review was to evaluate different AI models and tools utilized in this field, in addition to highlight the advantages and drawbacks of each. He studied 84 research studies worldwide, and 8 in Malaysia covering from year 2004 to 2019. Similar to a review by Sinthiya et al. [24], most of the articles has focused on municipal solid waste management applications including waste generation, waste classification, and system improving models. However, organic waste was rarely discussed with no mention to halal food.

Despite the growth in research in this area, artificial intelligence systems are mainly still at the research and development stage [18]. Furthermore, the findings from the previously discussed systematic literature review and other literature reviews indicate that the majority of articles have predominantly concentrated on municipal solid waste (MSW). In contrast, there has been relatively less attention given to addressing other fields [18-19], [24]. Although municipal waste consists mainly of food waste, in Malaysia, food waste is approximately 37% of municipal waste composition [25].

4. OPPORTUNITIES AND CHALLENGES OF ARTIFICIAL INTELLIGENCE IN SOLID WASTE MANAGEMENT

The application of artificial intelligence (AI) presents numerous opportunities, while on the other hand, it also poses global challenges [18-19]. The employment of AI and ML

has been widely discussed among governments, organizations, and researchers for an extended period, across diverse fields.

Kutty and Abdalla [16] provided a macroscopic overview of the tools and techniques used in sustainable food security-related projects, with a focus on data management systems and food waste management. According to the authors, achieving global food security requires addressing a series of challenges, including food waste accumulation in the food supply chain, tackling gender disparities, and climate-related concerns to promote sustainable consumption and production practices at several stages of the food supply chain. The findings could also be applied in the halal food sector by customizing database management systems, tools, and data visualization platforms. Moreover, proper knowledge and understanding of the existing tools and techniques for tracking and assessing food waste at several stages of the halal food supply chain is also essential.

4.1. Opportunities of AI in Halal food industry

Since the implementation of the Third Industrial Master Plan 2006-2020 (IMP3), Malaysia has aimed to become a worldwide Halal centre, Malaysia has seen significant investments, both from the public and private sectors, towards the development, execution, and acceptance of AI technologies [13]. AI could be utilized in several areas of such systems, namely, Quality Control, Traceability, Demand forecasting, Resource Optimization, and Risk Management.

4.1.1. Quality Control

Quality assurance is critical in the halal food industry for several reasons. However, in the context of waste management, quality assurance is essential to ensure limited food waste during the production stage. AI technologies could reduce food waste by identifying potential issues early and enabling proactive intervention, thus preventing food spoilage, extending the shelf life of food products, and reducing food disposal [16-17].

Maiyar et al. [17] has examined "REAMIT" project, which stands for "Remote Evaluation of Ambient Intelligence Technologies in Intelligent Food Procurement" project. The REAMIT project is an EU-funded research project that aims to develop a system that utilizes Internet of Things (IoT) sensors to monitor food quality in real-time throughout the entire supply chain. The project's goal is to improve food safety and reduce food waste by providing better quality control and traceability of food products. The system uses a combination of sensors, including temperature, humidity, and gas sensors, to monitor food conditions. The collected data is then analyzed using AI and ML algorithms to detect anomalies and predict potential issues.

While the REAMIT project's primary goal is broader in scope, its advancements in real-time food quality monitoring, data analysis, and predictive capabilities have the potential to benefit various sectors, including the halal food industry. The application of these technologies can enhance the quality assurance and traceability of halal food products, ensuring compliance with Islamic dietary laws and meeting the growing demand for high-quality halal products. Thus, the innovations stemming from projects like REAMIT open up exciting opportunities for improving the halal food industry's quality control processes and overall sustainability.

Likewise, an AI-based image recognition system has been developed for efficient sorting of apple fruits [26]. The system has shown remarkable results with an average

accuracy of 99.70%. Additionally, the accuracy of the Convolutional Neural Network (CNN) based apple sorting system was observed to be 99.38%. These results demonstrate the sorting image recognition system's ability to accurately classify apples based on their characteristics, allowing for non-destructive testing and grade classification of the fruit. Such a system could be developed with further research and development of automated fruit sorting systems and other food types.

This technology's potential extends to addressing food waste in the context of halal food production and supply chains. By adapting and expanding this system to halal food products, it could play a crucial role in minimizing waste and improving quality control. With further research and development of automated sorting systems for halal food and other food types, we can enhance the efficiency of waste reduction efforts and ensure compliance with Islamic dietary laws, thereby promoting sustainability in the halal food industry.

Overall, AI has the ability to create a feedback loop for existing food safety and quality programs to evaluate their performance in meeting the expectations and goals of business quality assurance management [14-17].

4.1.2. Traceability

Food waste tracking throughout the supply chain is essential to reach a sustainable halal food waste management system. This helps identify the causes of waste and allows for more efficient management and reduction of waste. Both AI and ML could be used to analyze data and provide insights into where waste is occurring and how to prevent it. For instance, AI models can analyze data from sensors placed on food packaging to determine when food is likely to spoil, allowing for more accurate shelf-life predictions and reducing the likelihood of food being thrown away.

Additionally, ML can also be used to analyze data during food production and distribution stages [27] identifying inefficiencies and areas for improvement. It also helps provide a detailed record of waste, allowing for more targeted interventions to reduce waste.

One established application is the use of RFID, or radio frequency identification, which is a technology that uses IoT. It involves the use of a tag, which is a small electronic device that contains an integrated circuit and an antenna. The integrated circuit is responsible for storing, processing, and modulating/demodulating the radio frequency signal that is used to communicate with the reader. The tag is also able to transmit the signal via the antenna.

Zuo et al. [28] proposed the use of Radio Frequency Identification (RFID) tags in sensor applications such as humidity, temperature, gas, pH, integrity, and traceability in connection with food packaging. The authors suggest that RFID sensors offer significant advantages in terms of sensing ability and data transmission for smart packaging solutions. The future development of smart packaging is focused on simpler, low-cost, robust, and less power-demanding sensor networks, and chip-less RFID sensors have the potential to achieve these functions.

Overall, traceability is a key element in halal food waste management. Using AI and ML can help organizations to identify and reduce waste, leading to more sustainable and efficient halal food production and distribution. However, there are still challenges to be overcome, such as biocompatibility, cost, multi-tag collision, multi-parameter sensors, recycling issues, security, and privacy of RFID systems [28].

4.1.3. Demand forecasting and Resource optimization

As the population increases, so does the demand for food. According to the United Nations' Food and Agriculture Organization, approximately one-third of the world's food production, equivalent to 1.3 billion tons of food annually, is lost or wasted. Demand forecasting is crucial for reducing food waste as it helps companies to accurately predict the necessary amount of food to produce or stock to meet customer demand. Food loss and waste can happen at various stages of the food supply chain, ranging from the harvest to the retail phase. While food loss occurs primarily during production and distribution, food waste occurs mainly at the retail and consumer levels. In fact, consumer food waste accounts for the largest share of food waste throughout the entire chain, encompassing farming, processing, distribution, and retail. Estimates suggest that consumer waste ranges between 40% and 60%, whereas retail waste is typically around 10% [11] .

By utilizing AI-based demand forecasting models, companies can predict future demand for their products accurately, allowing the adjust of their production and inventory levels accordingly. Hence, it reduces the risk of overproduction and subsequent waste, while also ensuring that enough food is available to meet customer needs. In addition to cost savings and improved efficiency in the supply chain. Moreover, AI can be used to optimize the use of resources in the Halal food industry, such as water, energy, and raw materials. By using predictive analytics and ML algorithms to analyze data on resource usage and production processes, AI can help identify opportunities for efficiency improvements and cost savings.

Lutoslawski et al. [11] has proposed using a nonlinear autoregressive exogenous neural network to predict demand for processed foods, the models' showed high levels of accuracy with coefficient of determination (R^2) measure ranging from 0.962 to 0.996 depending on the product. The study suggests that such models can be used in intelligent management systems to support more efficient and sustainable food production and distribution.

Kotaro [29] has developed a “heterogeneous mixture learning” technology that employs algorithms for short-term demand estimation in food grocery stores. This technology relies on two crucial factors: (1) the automated discovery of optimal patterns and (2) the utilization of a prediction model with high readability. Its comparison with the conventional technology is shown in Fig. 2. This technology has proven to be effective in reducing unsold items, thus contributing to the reduction of food waste.

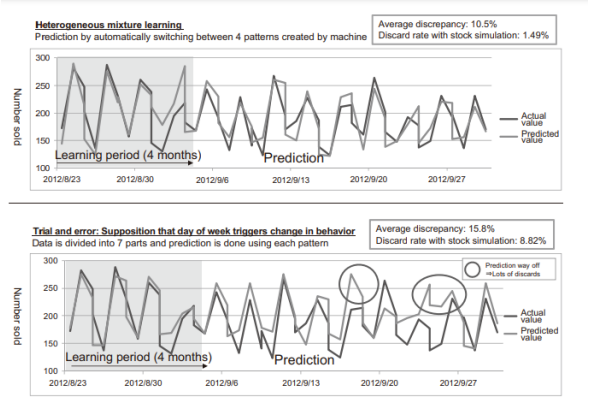

Fig 2. Comparison of results with conventional statistical algorithms.

Additionally, big data analytics and predictive analysis offer valuable benefits to the food waste industry. They enable precise predictions and informed decision-making based on data-driven insights, helping organizations optimize waste reduction, streamline resource allocation, and reduce costs. Real-time insights allow for timely responses to changing conditions, while the identification of hidden patterns and trends leads to innovative solutions. Furthermore, predictive analysis helps in mitigating risks associated with food waste. These findings suggest that the application of advanced technology and data analytics can play a crucial role in managing halal food waste, enhancing overall efficiency and sustainability in the sector.

Overall, traditional data science forecasting models have been used in the past, such as multiple regression, exponential smoothing, the Holt-Winters model, ARIMA, supervised regression and classification models, random forest, gradient boosting, and stochastic optimization. However, these methods have several limitations, including a short "life cycle", inability to learn, and lack of generalizability. AI and ML can be utilized to overcome these limitations, including deep learning models [18], [30]. AI-based demand forecasting models can predict future demand for food products accurately, allowing companies to adjust their production and inventory levels, accordingly, thus reducing the risk of overproduction and subsequent waste, while also ensuring enough food is available to meet customer needs.

4.2. Challenges for AI Implementation

AI provides numerous opportunities and benefits to improve halal food waste management in addition to the above-mentioned areas. However, there are also some challenges that need to be considered and further studied. Based on a review of previous studies and research the following challenges have been identified.

To begin with, few studies dedicated to developing AI applications consider the unique features and qualities of food waste management systems with nearly none examining halal food waste systems. Creating AI applications that are tailored to the distinctive characteristics of food waste management systems requires collaborative research between halal food scientists, computer scientists and waste management experts from different disciplines. It is essential to emphasize the need for a highly skilled AI technical team in this collaborative effort, as highlighted in the works of Xia et al. [31] and Yigitcanlar & Cugurullo [32].

Additionally, the lack of sufficient data is considered one of the major challenges in implementing AI systems for halal food waste management. AI models require extensive datasets for training and calibration [33], but current research is hindered by incomplete or inadequate waste data. This is mainly because SWM industries, particularly in developing countries, have outdated systems with limited reliable records and insufficient sensory data [18].

Furthermore, the fast-evolving and diverse range of AI models can hinder the integration of AI in managing halal food waste. While many studies have reported successful results in organic waste management, the overall progress in halal food waste has been slow, and there is a lack of focused research on halal food waste management using AI. Lastly, "The black box nature" of AI models is a significant challenge in the implementation of such approach. AI models rely on large datasets, which are often hidden

or protected in publications, making it difficult to replicate or standardize the models across various products or industries.

5. CONCLUSION REMARKS

In conclusion, the implementation of AI and ML technologies in solid waste management has shown promising results in enhancing the efficiency and productivity of waste management operations. As we reflect on these advancements, it becomes evident that AI has the potential to revolutionize Halal waste management by optimizing waste reduction strategies and streamlining supply chain operations. This promising trajectory aligns with the broader goals of sustainable and efficient waste management practices. The Islamic community can take advantage of the current rapid development and explore the utilization of AI. With adequate financial support, regulation, and certification, it is possible to commercialize tailored AI models, leading to significant economic and environmental benefits.

However, most of the current research in this area has focused on municipal solid waste management, with limited discussion on organic waste and no mention of halal food waste. There is a great opportunity for AI to be implemented in the Halal food industry to tackle waste management challenges, particularly in areas such as quality control, traceability, demand forecasting, resource optimization, and risk management. Nevertheless, there are also challenges and limitations in the implementation of AI in waste management, such as the lack of data and the excessive cost of implementing AI technologies.

Therefore, further research is needed to explore the potential of AI in managing solid waste in the Halal food industry and to address the challenges and limitations in its implementation.

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ENHANCED HYBRID SYSTEM OF MICROBIAL ELECTROLYSIS CELL AND ANAEROBIC DIGESTER (MEC-AD) PERFORMANCE THROUGH MODIFIED ELECTRODES WITH MULTI-WALL CARBON NANOTUBES

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ABSTRACT: The hybrid system of microbial electrolysis cell (MEC) and anaerobic digester (AD) has been a promising approach for sustainable energy production and waste treatment. The integration of MEC and into the AD digester offers multiple advantages over conventional AD systems. The study was conducted on the modification of carbon felt (CF) anode, and stainless-steel mesh (SSTM) cathode with multi-wall carbon nanotubes (MWCNT), to facilitate the biomethane production and upgrade within the hybrid system. The microbial attachment to the electrodes was analyzed, the substrate concentration, current density, and biogas composition and volume were monitored. The SEM imaging of the electrodes showed that the microbes followed a different growth behaviour in modified and unmodified electrodes. In addition, MWCNT modified SSTM showed a potential hydrogenotrophic growth selectivity, unlike unmodified SSTM, which had a more syntrophic microbial community. Stainless steel mesh-modified cathode showed the highest biogas and methane production with a value of 14.4 ml CH_4/g glucose. In addition, the carbon-felt modified electrodes showed a maximum substrate degradation value of 93% and a current density of 4.5 mA/m^2.

KEY WORDS: *MEC-AD hybrid system, Stainless steel mesh with MWCNT electrode, Carbon felt with MWCNT electrode, and Modified electrodes.*

1. INTRODUCTION

The hybrid system of microbial electrolysis cell and anaerobic digester has been a promising approach for sustainable energy production and waste treatment [1].The approach has leveraged the microbial communities' capabilities of both systems to produce electricity and biomethane with the enhanced breakdown of organic waste [2].

The integration of MEC and AD into digester offers multiple advantages over conventional systems, anaerobic digestors are responsible for breaking down complex substrates into fermentable sugars and acids, and finally converted to mainly biomethane and carbon dioxide, while MEC's is responsible for the production of hydrogen utilized for the upgrade of CO_2 to biomethane within the system [3]. To commercialize this hybrid system, researchers have turned to modifying commercially available, cheap carbon electrodes as a replacement for precious metal electrodes currently used in the hybrid system, like palladium and platinum electrodes [4]. Multiple modifications to carbon-electrodes are made to improve bacterial adhesion and conductivity specifically, and overall

performance in general, like the modification of carbon fibre with self-supported N-doped C/Fe_3O_4-nanotube composite arrays [5], carbon black with humic-acid [3], carbon felt with carbon derived from mango wood biomass [6], preparing porous carbon cloth using Nickel (N-doped). While previous research on MEC-AD electrode's modification demonstrate promising results in terms of biogas enhancement, the research gap remains in finding modifications that not only enhances the biomethane production, but also the overall performance of the fermentation stages, in terms of substrate fermentation and utilization, along with methanogenic microbes' enrichment, and biomethane upgrade. Among the promising approaches in electrode's modification is the incorporation multiwall carbon nanotubes (MWCNT's).

MWCNT's exhibits an expectational electrical conductivity, speeding up the electron transfer efficiency within the biofilm [7]. In addition, the modification of electrodes with MWCNT's increases the electrode surface area, biocompatibility, hence increasing the colonization of microorganisms, facilitating an efficient fermentation process, and biomethane production [8]. The study focuses on the modification of carbon felt (CF) anode, and stainless-steel mesh (SSTM) cathode with MWCNT, to facilitate the biomethane production and upgrade within the hybrid system. The MWCNT growth on electrodes and microbial attachment were observed through scanning electrode microscopy (SEM), substrate degradation in terms of glucose utilization, current density, and biogas volume and composition was monitored.

2. MATERIALS AND METHODS

2.1. Electrodes Modification

Stainless steel mesh grade (304) with mesh size (1 mm), was used as cathode. Carbon felt research grade was used as the anode. The electrode's thickness, width, and length were fixed at 0.1, 4, 9 cm, respectively.

Carbon felt and stainless-steel mesh electrodes surface were washed with ethanol and acetone prior to modification. Carbon felt was modified using carbon ink prepared by mixing distilled water, MWCNT with a purity of (99%) and polyvinyl pyrrolidone (PVP) binding agent in the ratios of (1:2:0.4) (ml/mg/mg), respectively were mixed and vortexed for 1 min to insure material dispersion. Then, CF was submerged in the carbon ink and sonicated for 1 h to ensure ink dispersion within the fiber's strands, then the electrode was removed and dried in the oven at 170 °C for 20 min.

Stainless-steel mesh was modified using carbon ink prepared by mixing 95% ethanol and MWCNT with a ratio of 1:2 (ml/mg), respectively. Then, the mesh was submerged in the carbon ink and mixed at 100rpm for 1h, removed and dried at 170 ºC for 20min, the process was repeated until the mesh was completely coated.

2.2. Synthetic Substrate and Inoculation

The reactor was fed with modified growth medium was prepared by adding glucose 5 g/l; peptone 10 g/l; yeast extract 5 g/l; starch 1 g/l; sodium chloride 0.5 g/l; sodium acetate 0.5 g/l; cysteine hydrochloride 0.5 g/l [9]. The pH of the substrate was adjusted to 7 with every feeding.

The initial microbial source was collected from an anaerobic digester at the Sime Darby plantation at Carey Island, Selangor. The samples were kept in the chiller until further usage.

The effluent of previous anaerobic digester was centrifuged at 8000 rpm for 5 min. Then the supernatant was discharged, and precipitate was used as the seeding sludge for the systems.

2.3. Set-Up of MEC-AD System

Three systems were set up, two MEC-AD hybrid systems and one anaerobic digester. The first hybrid system was equipped with modified carbon felt and unmodified stainless-steel mesh (MEC-AD-CF), while the other hybrid system was equipped with unmodified carbon felt and modified stainless-steel mesh (MEC-AD-SSTM). The hybrid systems were connected to a power supply with an applied voltage of 0.9 V. All three systems' temperature were maintained at 37 °C, with an initial pH of 7. The biogas volume was monitored using water displacement method [10]. The water's pH was adjusted to 3 to avoid CO_2 solubilization. Fig .1 shows the MEC-AD hybrid system set-up.

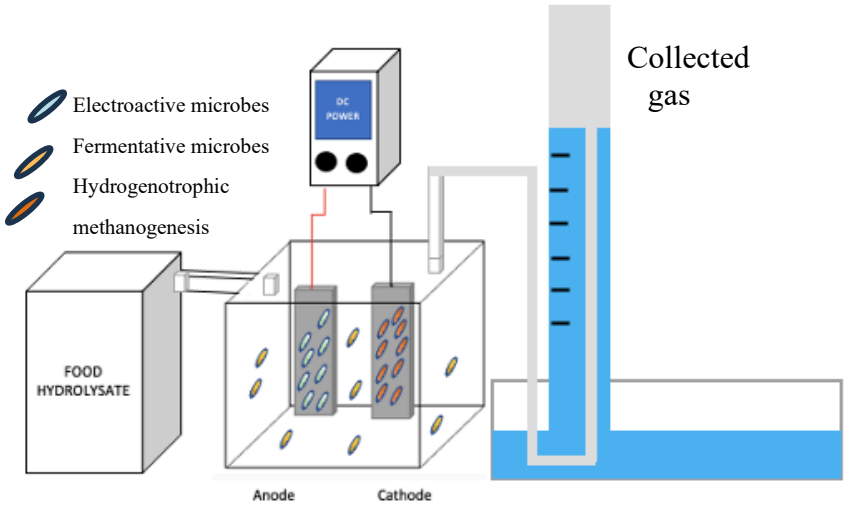

Fig. 1. MEC-AD hybrid system set-up.

2.4. Sample Preparation for Scanning Electrons Microscopy (SEM) Imaging

A piece with the dimension of 0.5X0.5cm of electrode was cut, washed with phosphate buffer, then fixed using 2.5% glutaraldehyde for 4 h. Then the samples were washed and dehydrated with ethanol (50%, 75% and 100%), respectively for 15 min. Then, they were analyzed using scanning electron microscopy (SEM).

2.5. Biogas and Sample Analysis

The biogas composition of all four systems was monitored using CH_4, H_2 and CO_2 multi-gas analyzer (PG810) daily. To measure the amount of reducing sugar consumed, 1 ml of reactors media was mixed with 1 ml of 3,5-Dinitrosalicylic acid (DNSA) solution, placed in boiling water for 15 min, and then was analyzed at 540 nm. The current of the hybrid systems were monitored using an ammeter connected to both ends of the anode and cathode.

3. RESULTS AND DISCUSSION

3.1. MWCNT Growth on Electrodes and Microbial Attachment

3.1.1. SEM Imaging Carbon Felt Electrodes

Microbes had different behavioral growth on modified and unmodified electrodes based on SEM's electron images as shown in Fig. 2 (A, D) of unmodified, and modified CF respectively. Fig. 2B and 2E show the general distribution, bacterial growth, and colonization of microbes on modified and unmodified electrodes, respectively. In contrast, Fig. 2C and 2F are closer images of microbial growth behavior of modified and unmodified electrodes, respectively. Based on Fig. 2B of unmodified CF, the microbial behavior was a big lumpy biofilm formation and growth in certain fiber regions, rather than a full coverage. On the other hand, the microbial growth on CF exhibited a different growth behavior in which the microbes thoroughly covered the fiber's surface and the MWCNT in between the fibers with the distribution of irregular individual colonies on different areas on the fibers as shown in Fig. 1E, the microbial density on the unmodified CF was much less than the modified CF.

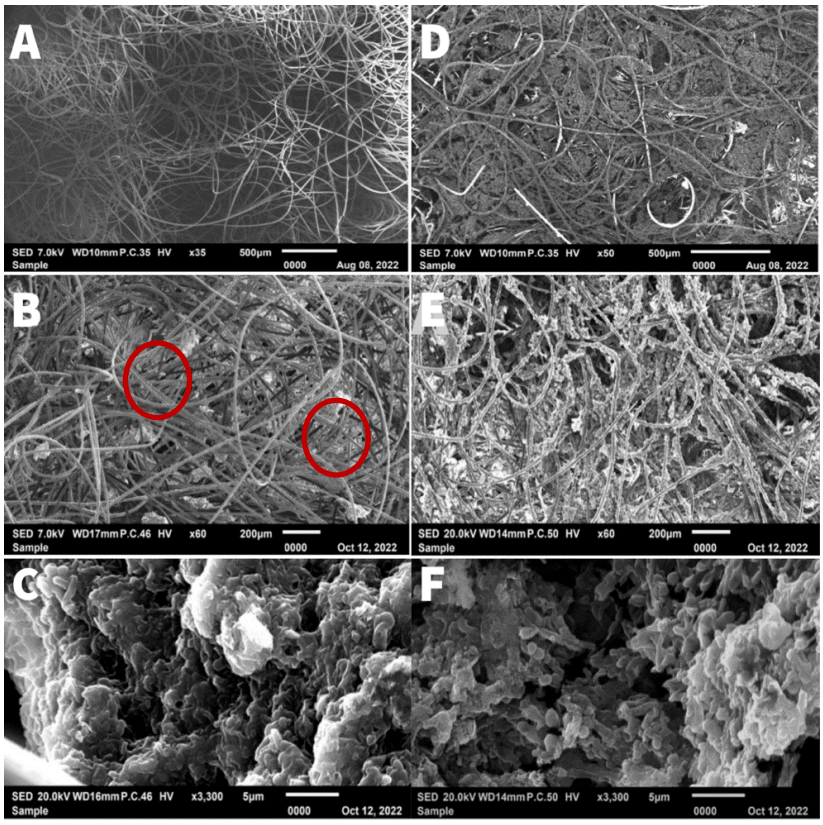

Fig. 2. SEM Images of Unmodified CF(A), Microbial growth on Unmodified CF (B, C), and Modified CF (D), Microbial growth on modified CF (E, F).

Fig. 2F of modified CF shows the direct growth of microbes on MWCNT-covered surface, offering a higher surface area for microbial growth. In addition, MWCNT, a conductive material, has also affected the electron transfer behavior of the microbes. From the same image, MWCNT facilitated the electron transfer directly from the microbe's surface to the electrode as can be seen in Fig. 2F of modified electrodes. To elaborate, a

study by Kadier et al. reported that electrons generated from the oxidation of organic materials by a single microbe are directly transferred to the anode [11]. On the other hand, the microbial community growing on unmodified electrodes, as shown in Fig. 2C had a denser EPS secretion and formation, which could suggest that the method of electron transfer through conductive biofilm was dominated. Microbes in the unmodified electrodes secreted extracellular polymer matrix (EPS) to help them attach themselves to the electrode and facilitate the electron and substrate transfer. In the modified CF, EPS density was lesser compared to the unmodified electrodes since the microbes have a lesser secretion of substance in the reactor equipped with CNT. Fig. 3 shows an illustration of electron transfer mechanism in unmodified and modified electrodes. It explains the microbial behavior with and without modification. In the modified CF, the extracellular polymeric substance density was lesser compared to the unmodified electrodes and this has been reported previously by Andrea et al., owing to CNT, microbes have lesser secretion of substance in reactors equipped with CNT [12]. There is no direct explanation to the correlation between the amount of electron produced and transferred directly, or through EPS. However, electrons transferred directly from the surface of organism to the electrodes use less energy than electrons transferred through electron transfer chain, namely microbes and EPS [13].

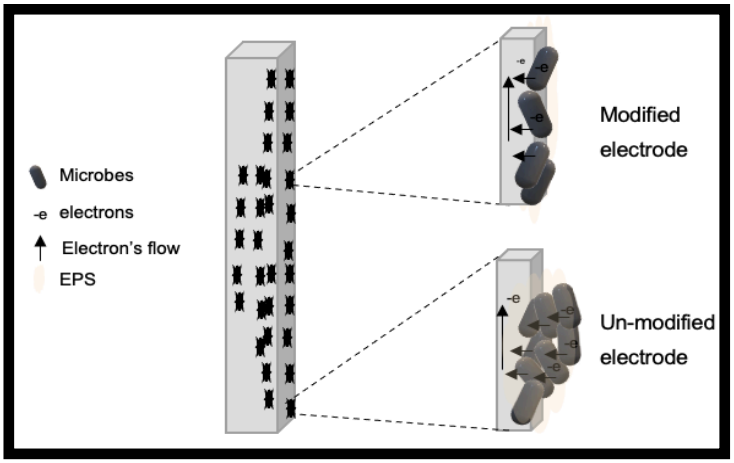

Fig. 3. Electron transfer mechanism in the unmodified and modified electrodes.

3.1.2. SEM Imaging of Stainless-Steel Mesh

Fig. 4(A, D) shows the images of unmodified and modified SSTM, respectively. The microbial growth on the modified and unmodified stainless-steel mesh had similar behavior to the microbial growth in the modified and unmodified CF electrodes. The unmodified electrodes had a cluster growth behavior, as shown in Fig. 4B. In the modified electrodes, microbes grew directly on the surface of the mesh and MWCNT, as shown in Fig. 4D. In Fig. 4C of the unmodified SSTM, and Fig. 4F of the modified SSTM, a different microbial community growth and distribution was observed. In the unmodified electrodes (Fig. 4C), a variety of different microbial shapes existed i.e., rod, long rods, cocci- and di-cocci-shaped microbes. In the modified SSTM (Fig. 4F), rod-shaped microbes were of significant population, followed by cocci and di-cocci-shaped microbes. Hydrogenotrophic methanogenesis has rod-long shapes, while acetolactic methanogenesis has cocci and di-cocci shapes. The same results were reported by studies conducted by Greesh & Pratishka and Sylvia et al. [14-15], where long-rod shapes microbes were identified as hydrogenotrophic, and cocci, di-cocci shaped microbes were identified as acetolactic methanogens. This is an evidence of the effect of MWCNT in enriching the methanogenic

community, as reported previously by Andreia et al., the addition of CNT has accelerated the population of hydrogenotrophic methanogenesis culture in the digester [16].

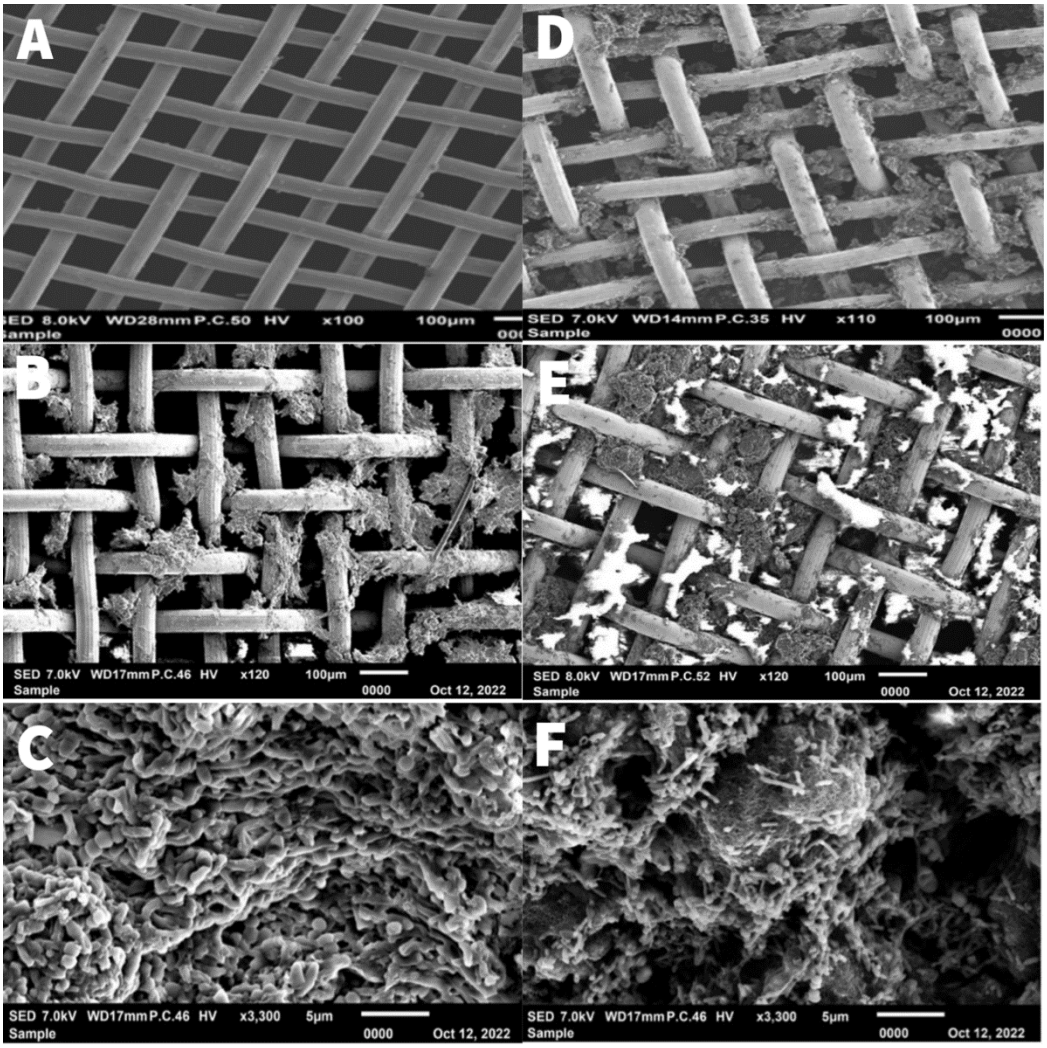

Fig. 4. SEM Imaging of Unmodified SSTM(A), Microbial growth on Unmodified SSTM (B, C), and Modified SSTM(D), Microbial growth on modified SSTM (E, F).

3.2. CURRENT DENSITY

The anode is responsible for the oxidation reaction of the substrate, producing acids, electrons, and hydrogen. The current density indicates the activity of electrogenic bacteria. A higher current density results in the more active and higher population of electrogenic microbes [17]. Fig. 5 presents the data on the current density of two different hybrid systems, equipped with the unmodified and modified CF electrodes. Based on the systems equipped with unmodified carbon felt anode, and modified stainless steel mesh cathode, it can be observed that no current was generated in the first few days. Starting from the sixth day, a small current volume was generated. The current volume increased up to day 10, and then a drop of 50% was observed on the following day. The increase in the current volume implies the growth and increase in the electroactive microbial community on the anode. This could be owing to the depletion of the substrate. The fluctuation in the current throughout the 20 days could also be owed to the microbes developing the extracellular polymer matrix on the electrodes [18].

On the other hand, reactors equipped with a modified carbon felt anode, and unmodified stainless steel mesh cathode showed a relatively high current density on the first cycle with a current density of 2.67 mA/m^2 compared to 0.0 mA/m^2 for reactors with unmodified carbon felt anode. A study by Ludovic et al. suggested that modifying porous electrodes with MWCNT increased carbon electrodes' biocompatibility and electrode's microbial density and thus generating the current density [19]. Coating with MWCNTs improves the electrochemical communication between the microbes and improves the conductivity of the materials [20]. Moreover, Mohita et al. reported that MWCNT modification reduces the inner resistance of the electrodes and increase the active surface area, which reduces the ohmic loss, hence improving the current density [21].

Besides, the increase in current density could be attributed to a novel type of microbe called Geobacter which are electroactive that coexists with the fermentable microbes[22]. Geobacter produces high current densities in the MFC and MEC systems [23]. They utilize VFAs like acetate using extracellular, insoluble Fe (III) and Mn (IV) oxides as terminal electron acceptors [24]. A similar study with the anode of graphite felt modified with MWCNT to treat landfill leachate showed high current density production of 4.2 mA/m^2 [25].

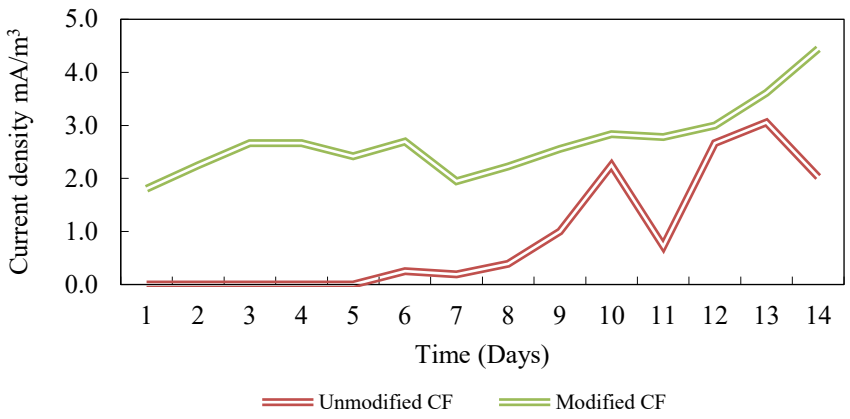

Fig. 5. Current density of system equipped with unmodified and modified CF.

3.3. SUBSTRATE DEGRADATION

The anode is responsible for the substrate degradation. The substrate degradation rate was monitored in terms of glucose consumption. Fig. 6 illustrates glucose degradation of two different hybrid systems, equipped with unmodified and modified CF electrodes. AD reactors showed no significant substrate degradation in the first five days. The degradation value was lower than 55% throughout the analysis. The increase in degradation rate for the digester was faster in the first four days compared to the hybrid system with unmodified CF electrodes. However, the substrate consumption was higher in the hybrid system and increased throughout the analysis with microbial adaptation to the anode. The unmodified CF systems achieved a high percentage of 83% towards the end of the analysis. It can be attributed to the larger surface area for microbial growth hence, a faster substrate consumption. In addition, this could be attributed to the enhancement of performance by degradative and oxidative microbes by carbon felt anode. A study by Luo and co-workers suggested that carbon felt anodes with an applied voltage above 0.5 V highly enhance degradative microbes in MEC-AD hybrid systems, along with the oxidative microbes [26].

However, the modified CF systems showed the best substrate degradation performance throughout the analysis, maintaining a value over 80% and achieving a maximum percentage of 92.55%. In addition to the enrichment effects of carbon felt, MWCNT modification has a wider porous surface area with high biocompatibility for oxidative and degradative microbes to grow. The increase in substrate removal efficiency can also be attributed to the carbohydrate's bioconversion through the favorable redox potential between the electrodes, hence enrichment of functional degradative microbes [27]. The results align with a similar study by Mansoorian on the treatment of landfill leachate using MEC showed that the substrate degradation of systems equipped with MWCNT modified CF had a high substrate degradation value of 97%, compared to the control with a value of 72% only [25].

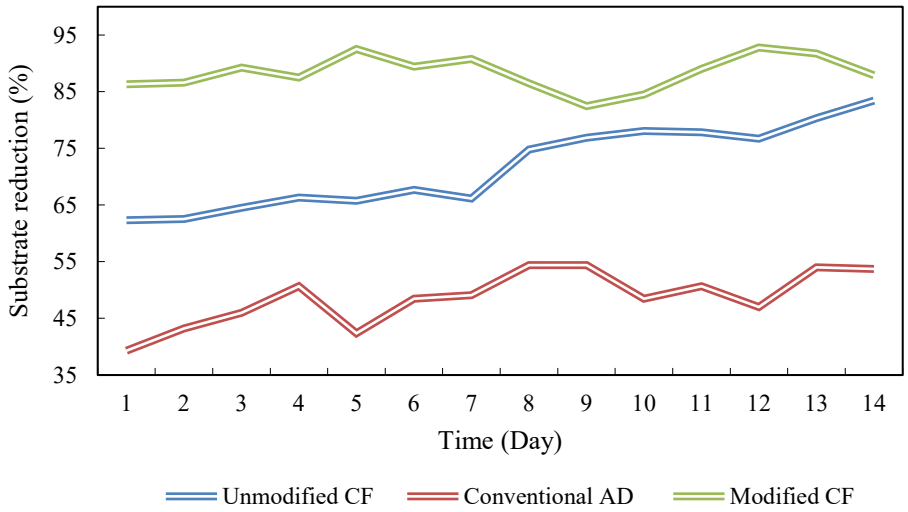

Fig. 6. Glucose reduction in semi-batch systems of unmodified, modified CF, and conventional AD fed with 50 ml/day substrate.

3.4. BIOGAS AND COMPOSITION

The cathode is responsible for biomethane production and upgrade within the system. Fig. 7 displays CH_4 and CO_2 volume. From the biogas production rate, the hybrid system with modified SSTM has substantially outperformed systems with the unmodified SSTM and conventional digester. In the modified reactor, the biomethane production substantially increased on the sixth day onwards, achieving a value of 287 CH_4/g glucose while only producing 12 ml CO_2/g glucose. On the other hand, unmodified reactors gradually increased biomethane throughout the cycle, outperforming conventional digesters with a cumulative biomethane value of 57.7 ml/g glucose and 26.8 CO_2 ml/g glucose. The digester had the lowest biomethane production of 37 mL/g glucose, yet the highest cumulative CO_2 with a value of 41 mL/g glucose. It was reported previously that conventional digesters' biomethane only accounts for 50-60%, and the remaining is CO_2 [28] compared to integrated systems. Integrating electrodes into the system gives a higher surface area for microbial growth. Hence, a higher volume of the substrate is available for faster consumption. Modifying the SSTM cathode with MWNT has increased the surface area and biocompatibility of the mesh, as observed in the SEM images. In addition, MWCNT and conductive materials have been reported previously to improve direct interspecies electron transfer (DIET) reactions between fermentative and methanogenic microbes [29].

Moreover, Andreia et al. reported that CNT increases the population and selectivity of the hydrogenotrophic and electroactive methanogenesis community [16]. Unlike acetolactic methanogenesis, which consumes acetate to produce CH_4 and CO_2, hydrogenotrophic methanogenesis produces methane through the consumption of H_2 and CO_2 in the production of biomethane, thus, reducing the CO_2 concentration while increasing the biomethane volume.

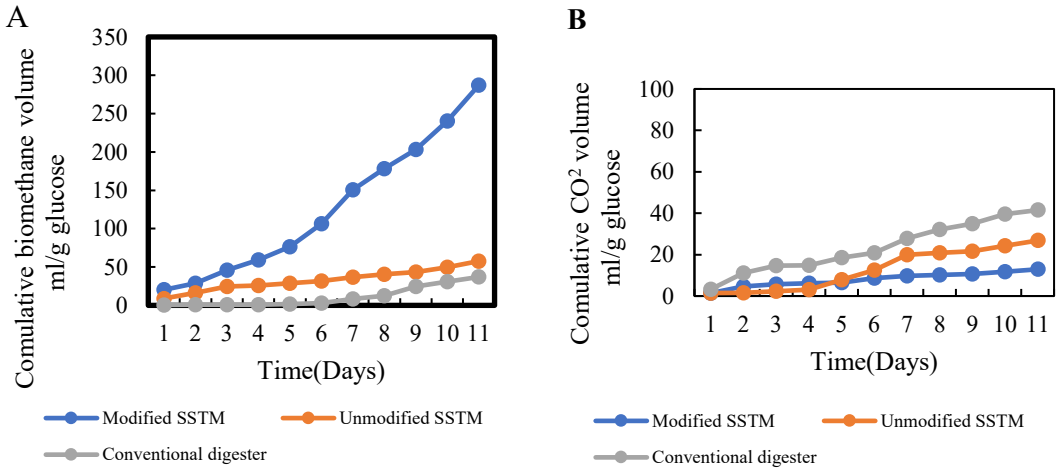

Fig. 7. Cumulative biomethane(A) and CO_2 production(B) of conventional digester, Modified system, and unmodified system.

4. CONCLUSION

The electrode's modification with MWCNT on the carbon felt and stainless-steel mesh highly improved the microbial attachment and behavior. High current density and substrate degradation indicates the elevated performance of fermentative microbes. In addition, the increase in biomethane and decrease in CO_2 values compared to the conventional AD digester and unmodified systems shows that the biomethane upgrade within the system was successful.

ACKNOWLEDGEMENT

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CARBON FIBER AEROGEL FROM NANO-FIBRILLATED CELLULOSE OF SUGARCANE BAGASSE AND WASTE ENGINE OIL RESIDUE FOR OIL SORPTION

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ABSTRACT: The oil adsorption method is one of the best approaches that can be used to treat an oil spillage on the water surface. Researchers have moved forward in the development of highly efficient and low-cost oil absorbent materials for the oil adsorption process, in addition to having environmentally friendly properties. Following this, combination of two different wastes has been used as raw material to prepare oil sorbent material with such characteristic in this study. Nano-fibrillated cellulose was extracted from sugarcane bagasse and was combined with carbon residue from waste engine oil to produce carbon fiber aerogel (CFA). Different ratio of cellulose to waste engine oil residue, WER (0.5, 1.0, 1.5) at different carbonization temperature (500ºC, 600ºC, 700ºC) was used to develop CFA and the oil sorption capacity was investigated. It was observed that ratio of cellulose to WER of 1.5 and carbonization temperature of 600 ºC achieved the highest oil sorption capacity of 5.47 g/g. The fabricated sample CFA possessed highly fibrous and rough surface structure as observed by FESEM, low density (0.17 g/cm^3) with high carbon content (C-C and C≡C) as confirmed by the FTIR. The results of this study are expected to encourage the use of sustainable waste sources as raw materials for the development of oil sorbent materials.

KEY WORDS: *Sugarcane bagasse, Waste engine oil residue, Porous carbon fiber aerogel, Nano-fibrillated cellulose, Oil sorption*

1. INTRODUCTION

Lately, the demand for petroleum products is increasing rapidly, parallel to the rapid development of the world economy. However, the expanding process of oil exploration and transportation for meeting the high demand of customers has led to the frequent occurrence of large and small oil spills around the world [1]. Without immediate action, oil spillage will heavily impact lives around us which will cause economic losses and threaten the environment. In addition, clean water is vital for our bodies and a resource where we benefit it in our daily life. According to the latest UNICEF & WHO (2015), report on sanitation and drinking water worldwide indicates that over 663 million individuals still lack access to safe

drinking water [2]. Hence, this pollutant source needs to be treated very well from time to time because the water needs to be used daily.

Physical water treatment through physical adsorption by porous materials, is regarded as the most successful and promising strategy to tackle oil spill problem [3]. An excellent oil sorbent material should present the following characteristics: high adsorption capacity, high selectivity, low density, and environmental friendliness. These characteristics are essential to separate oil-water mixtures effectively. Recently, 3D carbon-based aerogel has been investigated as an oil sorbent material. Aerogel, as a kind of porous material with a spatial mesh structure, has attractive characters including multi-level pore structure and high specific surface area [4, 5]. Various synthetic carbon-based aerogels, such as carbon nanotube sponges, graphene and carbon nanofiber, have been established and showed very high adsorption capacity as oil sorbent [2, 6]. However, the method used to synthesis these materials involved high cost and complicated steps. Moreover, they are not environmentally friendly and sustainable [4].

As alternative, researchers have investigated the use of organic waste such as agricultural biomass to produce carbon-fiber aerogel for oil sorption through pyrolysis process. Micro-fibrilated and nano-fibrillated cellulose were extracted from agricultural waste sources such as peanut shell [4] or banana peel [7] which were later converted to carbon fiber aerogel. Similarly, porous carbon material has also been prepared from oil sludge residue [2] however its oil sorbent property has not been studied. The combination of different type of waste sources for the preparation of carbon aerogel is yet to be investigated. Thus, this paper describes the development of carbon fiber aerogel through green approach using nano-fibrillated cellulose extracted from sugarcane bagasse and waster engine oil residue (WER) as oil sorbent material. The physicochemical properties as well as oil sorption capacity of the novel carbon fiber aerogel were studied. The fabricated carbon fiber aerogels found to possess unique characteristics such as being lightweight, hydrophobic, and recyclable.

2. METHODOLOGY

2.1. Pretreatment of waste engine oil carbon residue

Waste engine oil residues (WER) were obtained from Pentas Flora Sdn. Bhd. and were pre-treated before use. The WER were washed few times with ethanol to remove oil residue and were dried overnight in an oven at 80℃. The dried WER were further crushed using mortar and pestle to obtain small size particles. Then, the small size WER was proceeded with sieving process to obtain powdered carbon residue of less than 100 μm.

2.2 Synthesis of RF polymer

Resorcinol formaldehyde (RF) resins were synthesized using resorcinol and 37% formaldehyde solution as precursors. Firstly, 1.2 mL of aqueous ammonia solution (NH_4OH, 25%) was mixed with solution containing 48 mL absolute ethanol (EtOH) and 288 mL of deionized water (DI). After stirring it for more than 1 h, 0.84 mL formaldehyde solution and 1.2 g resorcinol was added to the stirred solution and stirred again for another 24 h at 30 ℃.

Next, the solution being slow centrifuged at 4750 rpm at 30 min and the pellet were air-dried at 100 ℃ for 48 h.

2.3 Synthesis of nano-fibrillated cellulose

The method to prepare the nano-fibrillated cellulose was obtained from Asem et al., [8] with slight modification. Sugarcane bagasse (25 g) was added into the mixture of 500 mL of distilled water and 10 g NaOH in 1L beaker in which, it placed in water bath for 5 h at temperature 80 ºC. Then, the sample is washed and filter with distilled water at least three times using vacuum pump. Next, the sample is bleached with 250 mL of water and 250 mL of hydrogen peroxide. The sample is washed and filtered again with distilled water at least three times using vacuum pump. After that, the sample is then bleached with the mixture of 60 g of NaOH and 500 mL distilled water in 1 L beaker for 1 h at temperature 80ºC. After that, the sample is washed and filtered with distilled water at least three times using vacuum pump. Then, 2.5 g of isolate sample is added into 5 mL of 1% sulfuric acid at temperature 80ºC for 1 h. Next, the sample is washed and filtered many times (at least 10 times) with distilled water using vacuum pump. After that, the sample is mixed with 20 g of NaOH with 400 mL of water for 24 h. Then, treat crude cellulose into 400 mL distilled water, 8g of sodium chlorite and 3 mL acetic acid for 3 h. Lastly the sample is washed and filtered with distilled water at least ten times using vacuum pump.

2.4 Preparation of Carbon Fiber Aerogel (CFA)

Pre-treated WER and nano-fibrillated cellulose (NC) were mixed with cross linker recorsinol formaldehyde (RF) polymer, maleic acid, and polyethyleneimine at different ration. The mixture was blended well in ultrasonic bath sonicator for 30 min. Then, the mixture was poured into the mold before freezing it at -80 ℃ for 24 h. Next, the mold was put in freeze drier machine to remove water from the sample for about 3 d. Next, the sample was subjected to carbonization where the samples were activated using tube furnace with flowing nitrogen gas (N_2) at different temperature in a ceramic boat. The temperature inside the tube furnace was programmed to increase linearly at ~5 ºC/min, dwelling hold at 600 ℃ for 30 min and ramping down ~30 ℃/min to the room temperature. To study the response towards oil adsorption capacity, two parameters were varied which include ratio of NC to WER which is from 0.5 until 1.5 and activation temperature from 500 ℃ until 700 ℃ for carbonisation process.

2.5 Engine oil Sorption Measurement

The CFA aerogel was weighed before being immersed in engine oil (liquid moly) around 15 min to let oil sorption into the sample and then removed for another weight measurement [3]. The process for oil adsorption can be calculated by using Equation 1. To ensure the recyclability of the PCF aerogels, the sorbents were extensively washed with hexane following oil absorption and oven-dried at 80°C.

The process can be calculated by using the following equation:

$$OA = (m_f - m_i)/m_i \quad (1)$$

where,
OA = oil adsorption capacity
m_i = weight before adsorption
m_f = weight after adsorption

2.6 Characterization of PCFA

The CFA was characterized by using Fourier transform infrared (FTIR) spectroscopy to measure the availability of functional group present on the sample and to confirm complete conversion of raw material to carbon aerogel and field emission scanning electron microscope (FESEM) is used to observe the surface microstructure and porosity. The bulk density (ρ) was calculated from the mass and volume measurements. The water contact angle (Θ) on the surface of the aerogel was also measured to support the water sorption test.

3. RESULTS AND DISCUSIONS

3.1 Physicochemical Properties of Carbon Fiber Aerogel (CFA)

3.1.1Physical Properties of CFA

As shown in Fig. 1a, white colored nano-fibrillated cellulose was successfully extracted from sugarcane bagasse. Fig. 1b displaying the nanocellulose fiber-WER (NCFW) composite revealed fair distribution of carbon residue of waste engine oil around the white nanocellulose fiber. After the carbonization process at different carbonization temperature and nanocellulose to WER ratio, carbon fiber aerogel (CFA) cubes were successfully prepared as shown in Fig. 1c.

The physical characteristic of CFA such as weight, density and diameter were measured before oil adsorption tests were performed. Density of the samples were calculated based on the weight and volume of the sample. All measurements of the CFA sample before and after carbonization are tabulated in Table 1. It was observed that the weight, thickness, diameter, and density of the sample was reduced following the carbonization process. The weight of the sample is reduced by half of its initial weight where the weight was reduced from 0.5 g to 0.2 g while the thickness of the sample was reduced from 1.5 cm to 1.13 cm after carbonisation process giving the slight reduction in the density of the sample from 0.15 gcm^{-3} to 0.14 gcm^{-3}. The dimensional changes and mass loss were influenced by carbonization temperature for nano-fibrillated cellulose of sugarcane bagasse. The mass loss was due to removal of water and degradation of small molecules in the NCFW to become carbonaceous material [9]. The density of the obtained CFA sample is higher than the previously reported carbon fiber aerogel from agricultural waste due to integration of the WER with much heavier weight.

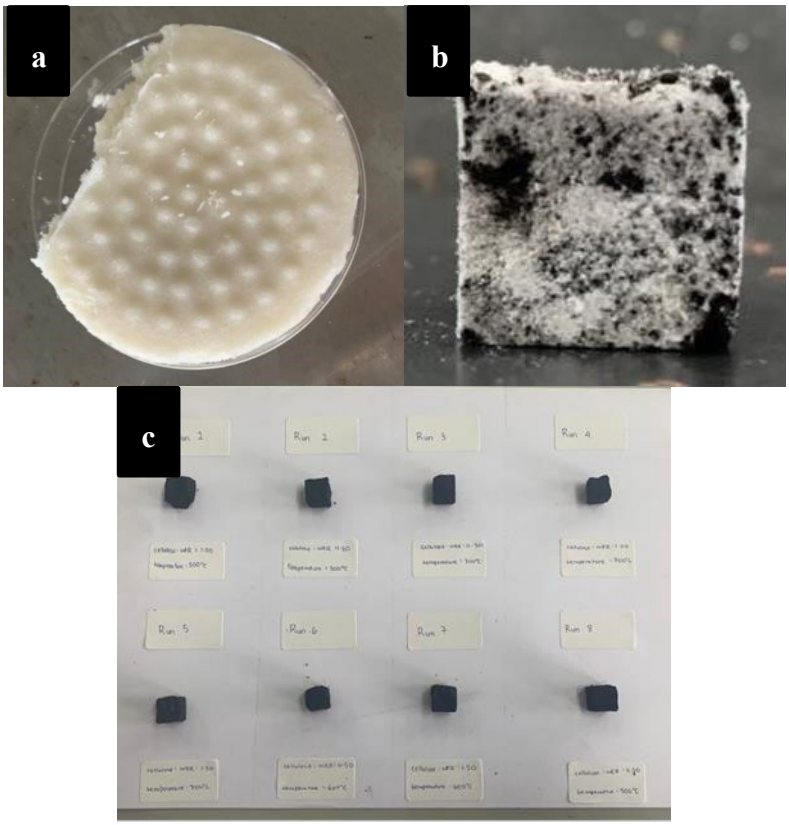

Fig. 1. Nanocellulose prepared from sugarcane bagasse (a), NCFW sample (b) and the CFA sample carbonized at 600 ℃ (CFA-600) (c).

Table 1: Physical properties of NCFW and developed CFA-600

Properties	**Before carbonization process**	**After carbonization process**
Weight (g)	0.50± 0.02	0.20 ± 0.02
Thickness (cm)	1.50 ± 0.03	1.13 ± 0.03
Density (gcm^{-3})	0.15 ± 0.03	0.14 ± 0.006

3.1.2 Microstructure of carbon fiber aerogel

The morphologies of the NCFW and CFA-600 are presented in Fig 2. From Fig. 2a, it was observed that the morphology of NCFW is having rod-like whisker shape with interconnected structure of nano-fibrillated cellulose (diameters < 200 nm) [8, 10]. Meanwhile, WER having random geometry of solid granules and RF polymer in microspherical shape. The RF polymer particles were added into the sample to bridge the nano-fibrillated cellulose to the WER [11]. It was observed that the surfaces of nano-fibrillated carbon fibers in Fig 2b which were converted from the nanocellulose fiber having rough surface structure. Irregular porous structure in between the carbon fiber was also observed in the CFA-600 sample (Fig. 2b) is assumed as able to provide large free space volume for oil uptake. No obvious aggregation of the WER is observed, suggesting that the fabrication method is appropriate in preparing the cabon fiber aerogel. Fig. 3 shows XRD

pattern of the CFA-600. It is observed that sample exhibits a broad hump at around 22°, a signature for amorphous carbon [12] which confirms complete conversion of the nano-fibrillated cellulose to carbon.

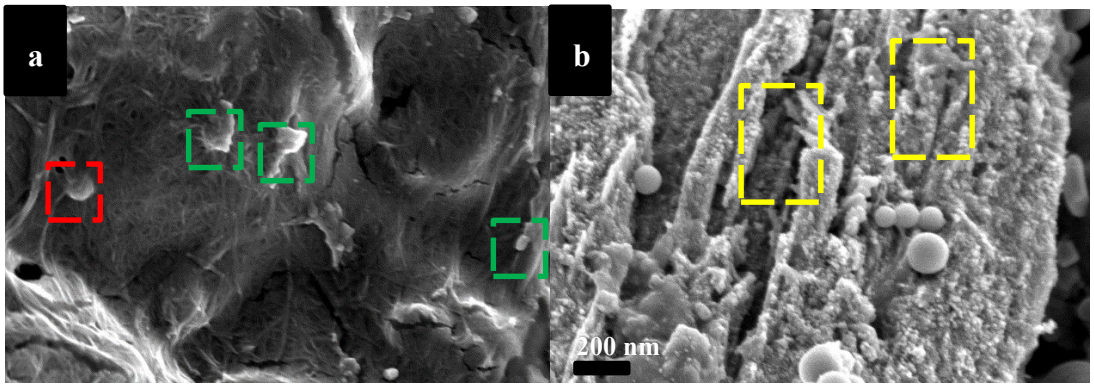

Fig. 2. FESEM images NCFW (a) and CFA-600°C (b) at 30000x magnification. Red box indicates the RF microsphere, green box indicates the WER and yellow box indicate the porous structure.

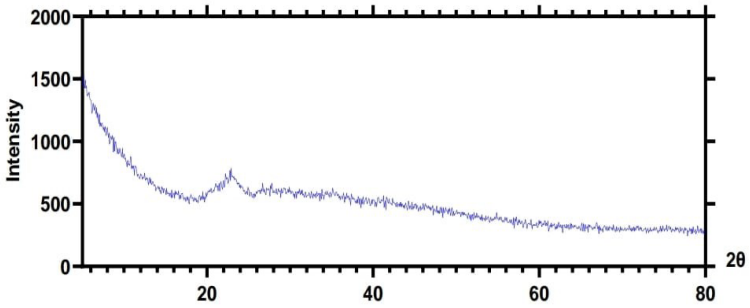

Fig. 3. XRD patterns of the CFA-600.

3.1.3 Surface functional group of carbon fiber aerogel

Fig. 4 shows the FTIR spectra of NCFW, NC, WER and CFA-600 respectively. The characteristic functional groups of NC are present for the NCFW sample but is absent for the CFA-600 i.e. the C-O bond at 1027 cm^{-1}. The transmittance band of CH group is also reduced at wavenumber 3442 cm^{-1}. The characteristic peaks of C−C at 1100 cm^{-1}, C═C at 1580 cm^{-1} and C≡C bonds at 2070 cm^{-1} are observed in the CFA-600, indicating the formation of a full carbon structure after the carbonization process. Wan et al. [13] reported that by exposing the NC at 600°C in N_2, the reduced atmosphere transforms the cellulose to a carbon fiber. Similar functional groups are found in WER, but the peak intensities are much weaker for C-C bond but much higher for C=C bond. The 3518 cm^{-1} bands that shows in the NC and NCFW illustrate that O-H group from nanocellulose fiber (NC), and RF polymer reduced after carbonization. It is reported that the conversion of the cellulose fiber to carbon through pyrolysis process will remove active hydrophilic group [14, 15].

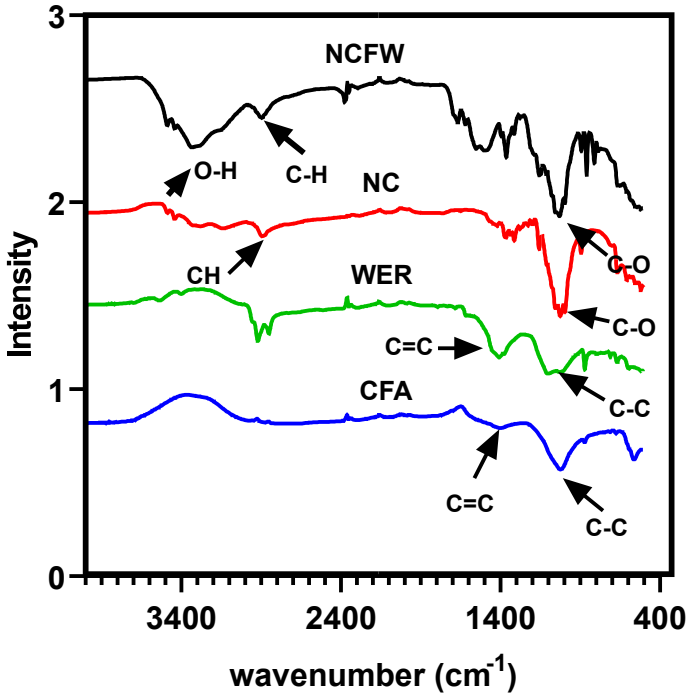

Fig. 4. FTIR spectra NCFW, NC, WER and CFA-600 samples

3.1.4 Water sorption test of carbon fiber aerogel

The study of hydrophobicity of CFA-600 can be performed by analyzing the CFA600 on the water surface. Fig. 5b shows that the CFA-600 sample remains floating on water surface after 24 h at room temperature compared to when it is initially added into the water (Fig 5a) even with slight agitation of the sample in the water. This is probably due to its ultra-lightweight and hydrophobic properties. The contact angle (Θ) of CFA-600 with water was measured as ~101° which support its hydrophobic properties. For selective oil sorption application, the ultra-lightweight and hydrophobic properties of the aerogel is of great importance. This observation suggested that the prepared sample is an ideal candidate as oil sorbent material with could eliminate competition of oil sorption with water.

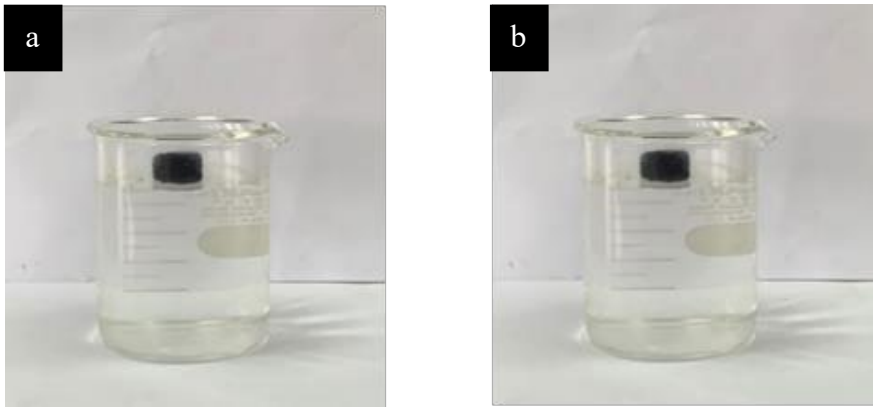

Fig. 5. Water sorption test of CFA-600 sample at initial condition of 0 h (a) and after 24 h (b)

3.2 Oil sorption behavior of CFA

As supported by the FTIR result, the pyrolysis condition of 600°C in an N_2 atmosphere is sufficient to convert the NCFW composite to a full CFA matrix with optimal oil sorption capacity. It is interesting to further investigate the effect of the carbonization temperature on the engine oil sorption behavior of the CFA. As shown in Fig. 6, increasing the temperature from 600°C to 700°C at ratio of the NC to WER 1 slightly decreased the oil loading capacity from 4.66 g/g to 4.40 g/g meanwhile reducing the temperature to 500 ℃ slightly increased oil loading capacity to 5.33 g/g. It is reported that increasing the pyrolysis temperature would increases the surface area of the biochar sample along with lower water content and higher fixed carbon amount [16]. However, greater surface area sometimes is not much desirable because it increases water retention capacity to a greater extent. The absorption capacity varied between the samples could be due to different pore morphologies of the aerogels induced by different carbonization temperature [17]. The loading capacity achieved by this CFA is not as high as the one achieved by the Ieamviteevanich et al., where bacterial nanocellulose were employed to prepare the carbon aerogel at 500℃.

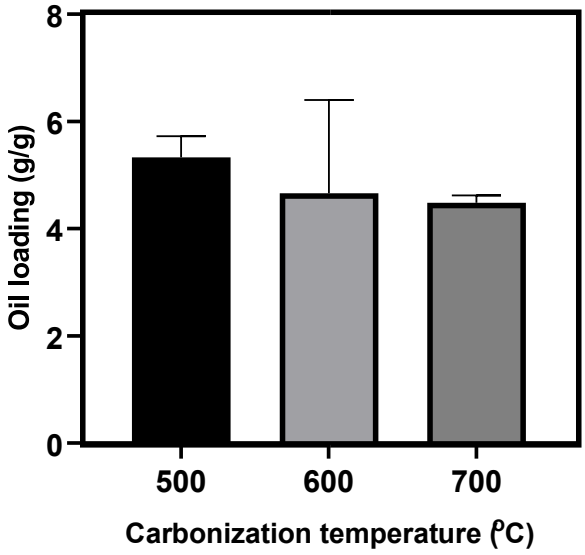

Fig. 6: Oil loading behavior relationship with carbonization temperature of the sample at ratio of cellulose to WER 0.5.

Oil sorption test was also performed for the CFA-600 sample by changing the ratio of the NC to WER from 1 to 0.5 and 1.5. It was observed in Fig. 7a that reducing the ratio of the NC to WER to 0.5 slightly reduced the oil sorption capacity from 4.49 to 3.48 g/g. Meanwhile, further increasing the amount of NC to WER to 1.5 increased the oil sorption capacity to 5.47 g/g. Thus, the result suggested that higher amount of NC compared to WER at carbonisation temperature of 600℃ will help to synthesis CFA with good oil adsorption capacity. Even though previous studies reported that carbon produced from agricultural waste tend to have hydrophilicity behavior [6], adding the hydrophobic WER particles into the 3D aerogel framework could reduce the total surface area of the final sample due to possible aggregation behavior of the WER. Fig. 7b, demonstrated that a

piece of CFA-600 with NC to WER ratio of 1 placed in a beaker filled with fresh engine oil can fully absorbed the oil within less than 2 min.

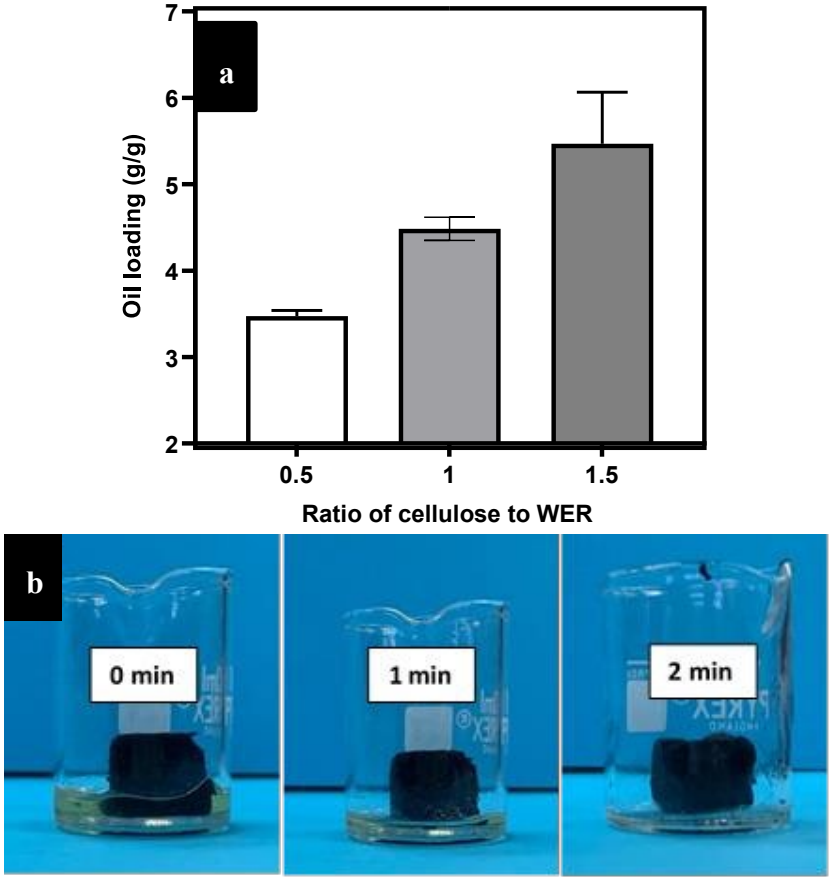

Fig. 7: Oil loading capacity relationship with the ratio of cellulose to WER at 600℃ (a) and the oil sorption behavior of the CFA-600 with time (b)

3.3 Recyclability test of PCFA

CFA-600 sample at ratio of the NC to WER 1.5 is used to do the recyclability test. The oil-absorbed aerogel is washed with hexane before drying at 80 °C to remove the absorbed oil. It is observed that the 3D structure of the sample is still intact after washing and drying and carbonaceous surface of the fibers of the aerogel are preserved. However, the CFA-600 sample capacity to absorb the oil reduced by 80% after the second cycle with 1 g/g oil sorption capacity compared to the first cycle (Fig. 8a). The CFA-600 sample take slightly longer time to absorb the oil compared to the first cycle where 30 min is needed to absorb the engine oil as shown in Fig. 8b. It takes longer time to absorb the oil compared to the first cycle might be due to some of the oils were still trapped in the pores of the sample which suggests that more washing cycles with hexane is needed [18]. Overall, the experiment shows that the sample is capable to be recycled back for adsorption process.

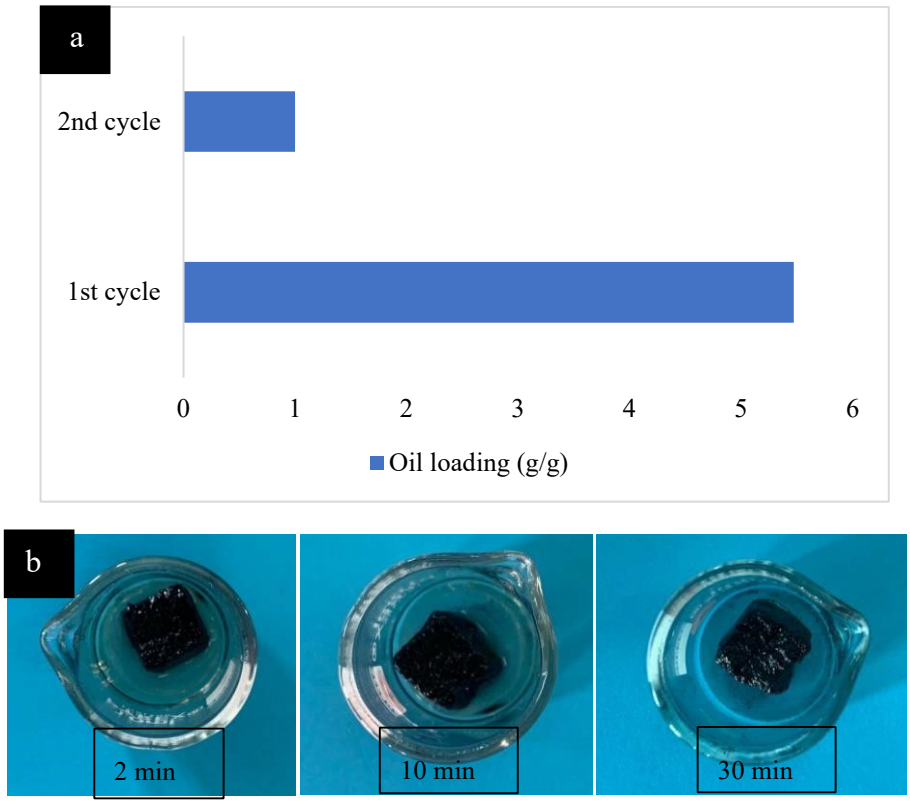

Fig 8. Oil loading capacity of CFA-600 at two different cycles (a) and demonstration of oil sorption behaviour of CFA-600 sample with time during the second cycle (b).

4. CONCLUSION

In summary, 3D carbon nanofiber aerogel (CFA) derived from nano-fibrillated cellulose and WER as oil sorbent material has been successfully developed by carbonization method. The CFA sample consists of porous network of the fibrillated carbon and exhibits ultralighweight and high selectivity towards oil. The CFA has an adsorption capacity of 5.47 g/g achieved at nanocellulose to WER ratio of 1.5 and carbonization temperature of 600 °C. The CFA can be recycled due to its intact structure with proper removal of the absorbed oil after previous oil sorption. The present work demonstrates an exciting prospect from using combination of different waste materials to alleviate oil spills incidents, that will have positive benefits to the environment.

ACKNOWLEDGEMENT

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A BIBLIOMETRIC ANALYSIS OF *CLITORIA TERNATEA*

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ABSTRACT: *Clitoria ternatea* L. has gained more attention from the scientific community in the recent years due to its potential biological properties including antidiabetic, anticancer, anti-inflammatory and antimicrobial activities. This explains the increment in the number of scientific studies done on the plant. However, there is no available paper found on the bibliometric analysis of *C. ternatea* specifying the important relationship between the content of the bioactive compounds, the types of research and the geographical diversity. Thus, this study aims to conduct a bibliometric analysis on the research trends, spatial distribution and related bioactive compounds of the *C. ternatea*. Search term ("*Clitoria ternatea*") OR ("Blue Butterfly Pea flower") is used as the keyword in SCOPUS database and 598 publications were found within the period of 1954 until 2020. The growth of publications showed a sharp increase in 2011 and 2018 and keep growing throughout the years since then. The VOSviewer programme was used to analyze keywords, countries, bioactive compounds, medicinal benefits and authors through visual knowledge mapping to assess the research trends. However, only 43% of the publications were selected for further analysis subsequent to screening stage. Results on the relationship between *C. ternatea* and bioactive compounds showed that antioxidant was the most frequently encountered pharmaceutical potential and anthocyanin was the most frequently encountered biological properties. The geographical distribution analyses showed that most researches were originated from Southeast Asian countries. The bibliometric analysis performed in this study has identified trends in *C. ternatea* plant from 1954 to 2020, which will guide the future directions in this research field.

KEY WORDS: *Clitoria ternatea, Medicinal plant, Bioactive compounds, Functional food, Bibliometric analysis*

1. INTRODUCTION

Nowadays, people prefer to utilize natural based products because they are safe and free from synthetic and artificial ingredients. Humans have been using natural products since prehistoric times and with limited knowledge and sources, they have done numerous trials and errors to learn how to recognize, prepare, formulate, and utilize suitable plants to be used for their ailments [1]. *C. ternatea*, generally known as blue butterfly pea flower is a herbal plant which comes from Fabaceae family. The Fabaceae is a large pea or bean family of flowering plants with medicinal benefits. The *C. ternatea* plant species is easy to maintain, and it can be widely found in Africa, Asia and Central America [2]. Flavonoids, anthocyanins, steroids, saponins, taraxerol and tannins are some of the bioactive compounds

found in in this plant [3-4]. Over the years, *C. ternatea* has gained interest within the research community due to its benefits in agriculture and medicine including the potential as anti-inflammatory, analgesic, antipyretic, antidiabetic and antioxidant activity [5].

The morphology of *C. ternatea* comprises flowers, fresh and old pods, leaves, and roots as shown in Fig. 1. There are varieties of distinct shapes of the flower and colour influenced by geographical locations that *Clitoria Ternatea* stems from. Fig. 2 showcases the different flower types and colours of *C. ternatea*. The most distinct trait of the flower, however, is the inner part which resembles that of the female genitalia, hence the name "Clitoria".

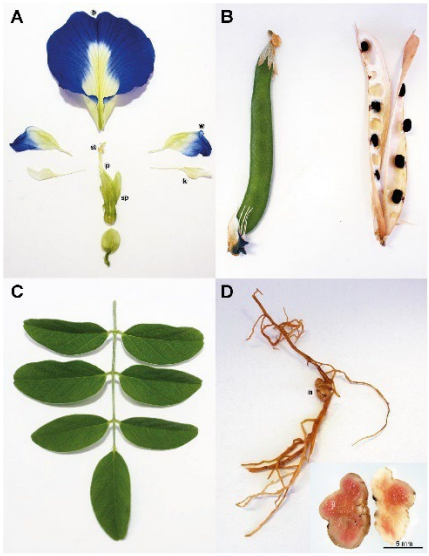

Fig. 1. Morphology of C. ternatea plant (A: flower, B: pods, C: leaves, D: roots with nodules). Adopted from Oguis et al., 2019

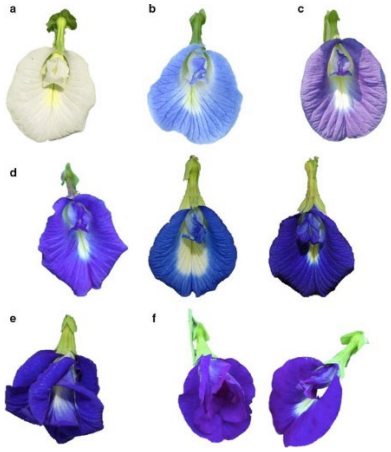

Fig. 2. Distinct flower types and colours of *C. ternatea* - a = white, single flower; b = light blue, single flower; c = light purple, single flower; d = dark blue-dark purple, single flower; e = dark blue-dark purple, double flower; f = dark blue-dark purple, mixed (single and semi-double) flower. Adopted from Havananda & Luengwilai, 2019.

Bibliometric analysis is an interdisciplinary approach to discover any research trends since it quantitatively and qualititatively analyzes the production, growth, and consumption

of scientific publications [6]. [7] reported that the term "bibliometrics" was originally coined by Pritchard (1969) to replace the classical term "statistical bibliographies".

Previously, there have been numerous scientific studies of bibliometric analysis done on the application of natural remedies on various diseases. Some of them includes study on the use of herbal medicine for obesity and rheumatoid arthritis [8] and [9] respectively, prevention and treatment of gastric ulcer [10], cytotoxicity and toxicity assays of medicinal plant [11], anticancer activity of herbs [12], and trends for stroke research [13]. From these studies, it is proven that the bibliometric analysis is helpful in the process of discovering trend of studies.

In this study, bibliometric analysis is performed to investigate the research trends of *C. ternatea* studies while analysing the spatial distribution of the study and discovering the identified bioactive compounds. This method is selected because of its ability to discover the current research gap and predict future trend [14]. The types of bibliometric network relations that are commonly studied are citation, co-citation, bibliographic coupling, keyword co-occurrence and co-authorship. In this study, analysis on co-occurrences and bibliographic coupling are performed.

2. MATERIALS AND METHODS

The techniques of performing bibliometric analysis can be categorised into review technique, evaluative technique, and relational technique [15]. The review technique is a method which collect information from the bibliographic data of published documents and statistical analyses. Systematic review, meta-analysis and qualitative study are some examples of this technique. The evaluative technique measures the academic impact on three factors: (1) productivity (number of papers per year and per individual author), (2) impact metrics (total number of citations per year and per individual author/journal and so forth, and (3) hybrid metrics (average number of citations per paper, productivity and impact indeces [16 – 18]. The third technique namely the relational technique, allows for the exploration of the relationship between categories within a research field including the structure of a research topic and patterns among authors or affiliations [15]. The analyses performed by the relational technique can be grouped into bibliographic coupling, co-authorship analysis, co-citation analysis, and co-word analysis [18]. Among the three techniques, the review and evaluative techniques do not allow the identification of networks between the authors [15, 18]. Hence, this study utilized the relational technique for its bibliometric analysis. Details of the implementation of the relational technique im this study is discussed in Section 2.4. The bibliometric analysis in this study comprises four stages, database selection, data screening, data extraction and data analysis. The flow diagram of the selection stages is shown in Fig. 3.

2.1. Research Questions

The specific objectives of this study are to discover the research trends of *C. ternatea*, to examine the spatial distributions of its studies, and to identify the types of bioactive compounds possessed by this plant species. Following these research objectives, the present study intends to answer the following research questions:

RQ 1: What are the global research trends for blue butterfly pea flower species?

RQ 2: What are the spatial distributions of *C. ternatea* studies?

RQ 3: What are the bioactive compounds in the plants?

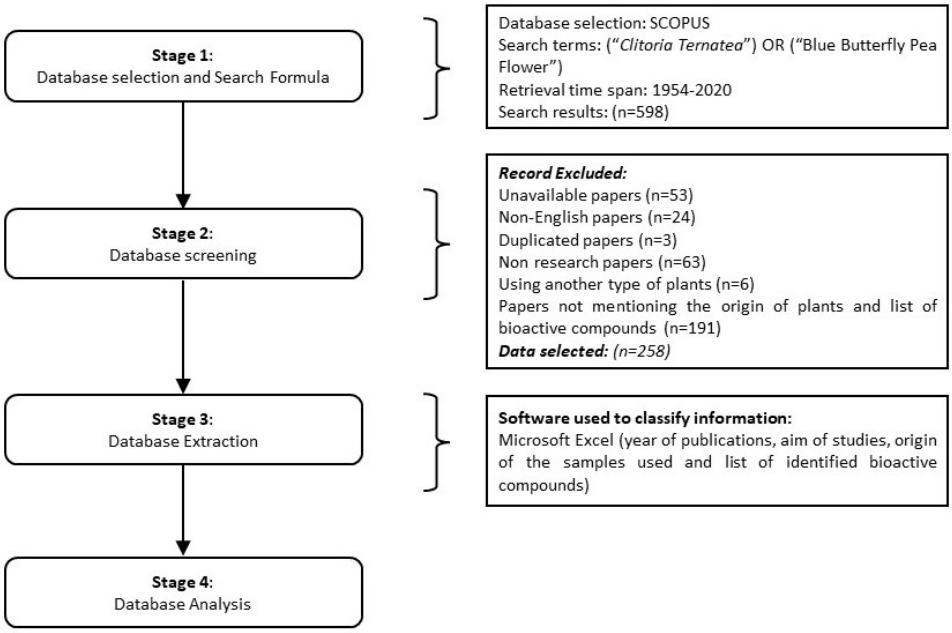

Fig. 3. Flow diagram of the selection process.

2.2. Database Selection

The first step in bibliometric analysis is to decide on the appropriate database to be used to obtain the relevant documents based on the established research questions. In this study, SCOPUS database was selected because it offers two methods of search; a basic and an advanced search in which complex and long search queries can accomplished the objective with high validity. Furthermore, this online database allows users to export the documents in various formats such as in Mendeley, RefWorks, RIS, .csv, BibTex and PlainText format.

2.2.1 Search Formula

The search applied used a Boolean operator 'OR' in which a combination of the words ("*Clitoria ternatea*") OR ("blue butterfly pea flower") is searched within article title, abstract and keywords (TITLE-ABS-KEY) field in SCOPUS website.

2.3 Data Screening and Data Extraction

The available publications among the obtained 598 number of results are downloaded in PDF format to ease further analysis. The titles and abstracts of selected papers are heavily screened. However, when the required information is not available in the title and the abstract, material and methods and conclusion sections are also screened. The whole paper screening following the inclusion and exclusion criteria listed in Table 1 is performed by six main steps. This screening result are extracted in Microsoft Excel in form of tables to classify the required information according to the year of publication, aim of studies, origin of the samples used and list of identified bioactive compounds.

Table 1: Inclusion and exclusion criteria for data screening

Criteria	Decision
Articles must be written in English language	Inclusion
Duplicated articles within the same search results	Exclusion
Reviews, conference abstract and systematic review articles	Exclusion
Articles must mention the origin of the plant sample used	Inclusion
Article must list down the bioactive compounds in the plant	Inclusion
Articles that are not available or accessible	Exclusion
Articles using samples of another type of plants	Exclusion

Firstly, within the 598 papers found, it was identified that there were 53 unavailable papers mainly because these papers did not provide the DOI numbers of the documents and most of them were old papers that were unaccessible. Secondly, there were 24 papers that did not use English language and the languages used were Spanish, Portuguese, German and Japanese languages. Next, the three duplicated papers were removed. In the fourth screening step, the non-research papers including review articles, erratum articles and letters totalled 63 papers and all of them were excluded.

Then, six papers using plant types other than *Clitoria Ternatea* were also rejected. Finally, in the final step, it is found out that there are 176 papers that do not mention the list of bioactive compounds screened, seven papers that do not state the origin of the plant samples used and there are eight papers that stated less info on the required information in which both the origin of the samples and bioactive compounds are not listed. Overall, 258 documents are qualified and selected for further data analysis. The selected publications are then analysed to obtain the visual projection of bibliometric study.

2.4 Data Analysis

The selected publications are exported from SCOPUS in a CSV format (comma separated values) to enable the visualization of data analysis through VOSviewer software version 1.6.15.

In applying the relational techniques for the bibliometric analysis, the VOSviewer was set up according to the following sequence. Firstly, in the create map pop-up, choose the create a map based on bibliographic data option. Secondly, under choose data source, select the read data from bibliographic database file. Next, select the corresponding folder or files, and finally under the choose type of analysis and counting method, choose co-occurence for type of analysis, authors for unit of analysis, and full counting for counting method.

3. RESULTS AND DISCUSSION

3.1 Distribution of Publications by Years

Search result from SCOPUS obtained 598 publications. The retrieval time span is from 1954 to 2020. This result excludes the publications from the year 2021 onwards whilst not limiting the earliest year of the publications occurrence in order to indicate the year

researchers started to discover *C. ternatea* species. Overall, there was an increasing trend in the number of publications per year from 1954 to 2020 as shown in Fig. 4. This increment indicates that people start to acknowledge the potential benefits of this plant species. From these 598 publications, 258 publications were selected after data screening is performed according to the criteria listed in Table 1.

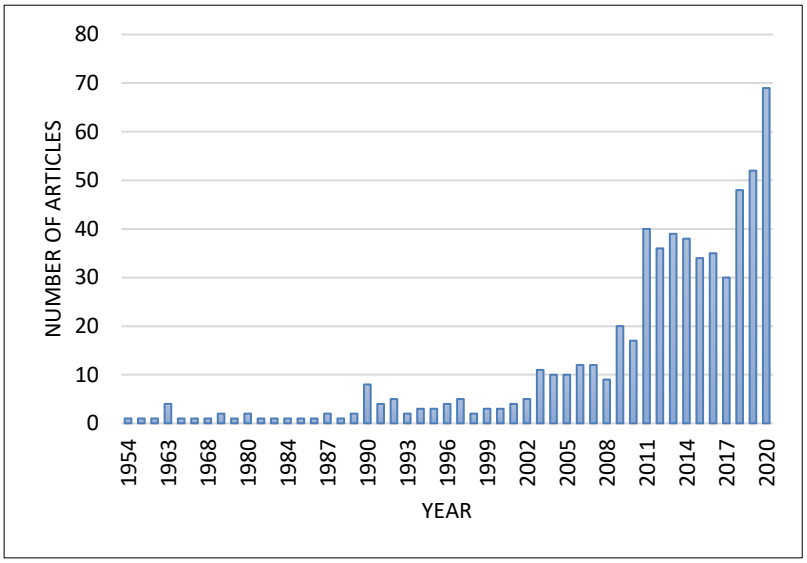

Fig. 4. Distribution of *C. ternatea* studies.

3.2 Keywords

Analysis performed on the co-occurrence of keywords identified 754 keywords used by authors in their studies. Fig. 5 shows circles of different sizes that represent the frequency of occurrences of the keywords used by the authors. A bigger circle indicates that the keywords are frequently used. The most frequent keywords used are *C. ternatea* with 93 occurrences followed by antioxidant with 20 occurrences, anthocyanin with 17 occurrences and butterfly pea with 16 occurrences (Table 2). The keywords used in the publications allow for tracking the evidence of the topics that have been studied related to *C. ternatea*.

3.3 Research Trends

Fig. 6 shows the research trends of the 258 publications which spans over 24 themes, with phytochemical and pharmacological as the two highly discussed themes. This shows that the potentials of *C. ternatea* have attracted the interest of diverse scientific fields. The scope of phytochemical researches covers the extraction, screening and identification of the bioactive compounds in *C. ternatea*. The next trending research fields are pharmacological and pharmaceutical studies. Pharmacological researches focused on how the chemical compounds affect the body systems while pharmaceutical researches is a study about drug development, physical and chemical properties of the drugs and their tolerable dosage [19]. For *C. ternatea,* its medicinal potentials were tested such for various biological properties including antidiabetic, anticancer and anti-inflammatory [20]. In agricultural sector, *C. ternatea* are tested as ruminant feeding and as pesticides [21]. For plant cell culturing, they are studied to establish a mass production of *C. ternatea* followed by testing on their antimicrobial and antibacterial activities [22-23].

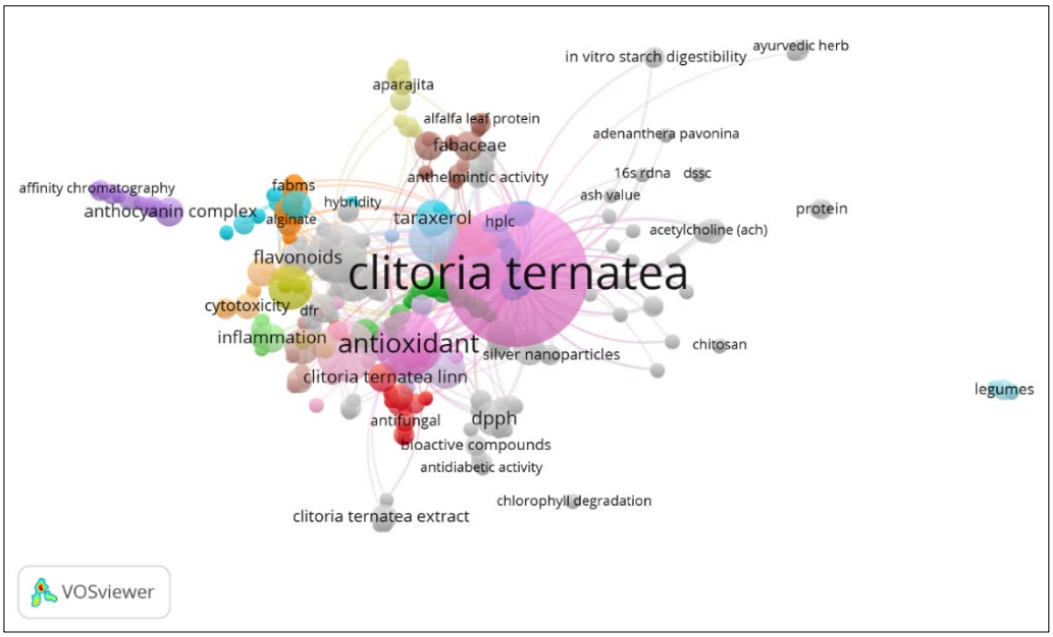

Fig. 5. Mapping on co-occurrences of author keywords related to *Clitoria ternatea*.

Table 2: Top 10 keywords used by authors in studies.

Number	Keywords	Occurrences
1	*Clitoria ternatea*	93
2	Antioxidant	20
3	Anthocyanin	17
4	Butterfly pea	16
5	*Clitoria ternatea* L.	12
6	Antioxidant activity	10
7	Anthocyanins	9
8	Antibacterial activity	7
9	DPPH	6
10	Response surface methodology	6

In the study of dye colorant, this plant is mostly tested for their applications in hair dye, textiles and food industries [24-26]. In food and nutrition study, *C. ternatea* are used not only to improve quality of food but to bring positive health effects to human bodies. Their extracts are also used to synthesize the nanoparticles in nanotechnology as they are safe to environment. In characterization scope, this plant species is characterized to determine their morphology and phenology traits [22].

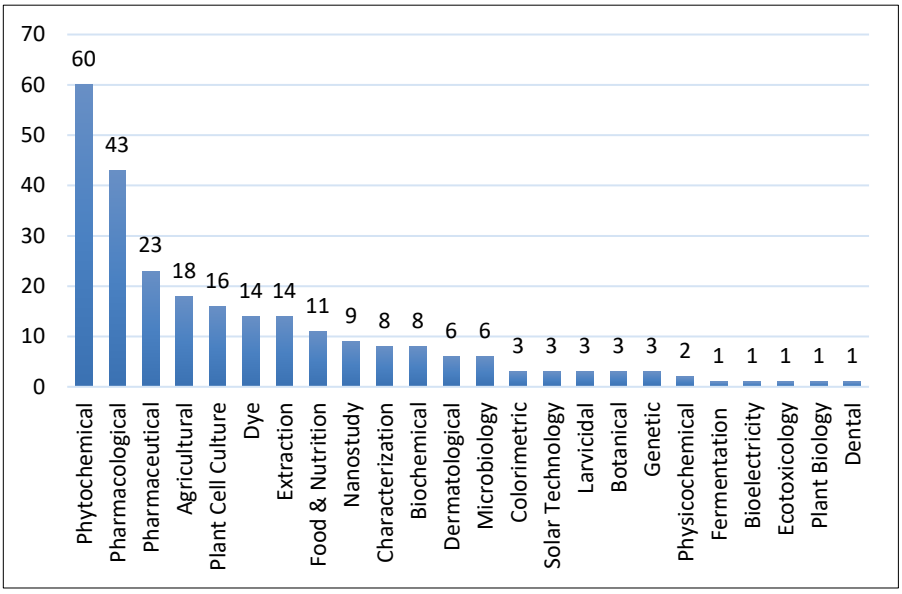

Fig. 6. Research trends of *C. ternatea* studies analysed from 258 papers.

3.4 Countries

Fig. 7 shows the visual mapping of the countries where the publications were published. The analysis is performed on the bibliographic coupling with geographical distributions. The bigger the circles is, the higher the numbers of publications published by the countries. The circles that are close together represent the publications that are citing the same publications and based on bibliographic coupling, they are strongly related to each other [24]. The countries with the most contribution to educational research literatures were made as focal points in the visual map [27]. Among 32 countries, India topped the list with 107 papers, followed by Thailand (40), Malaysia (39), Japan (18), Indonesia (16) and United States (16). Based on Fig. 8, India also topped the list of the origin that the plant samples were used by the researchers to carry out their studies. This is because, in India, *C. ternatea* is widely used as one of the Ayurvedic treatment and thus, various studies are conducted by the researchers in India including verification of its authentication in pharmaceutical industries [19, 28]. From these two results, India is possibly a country with a rich source of natural biodiversity.

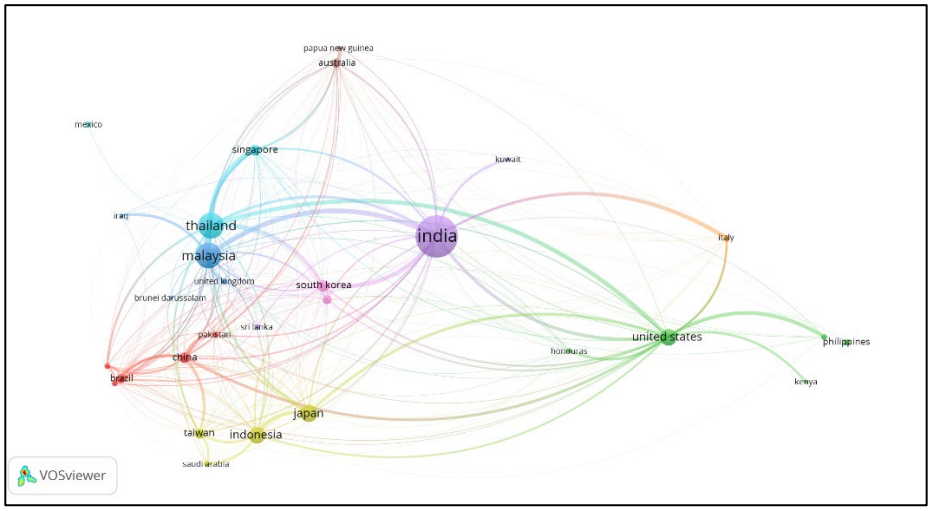

Fig. 7. Mapping on bibliographic coupling of geographic distributions of publications.

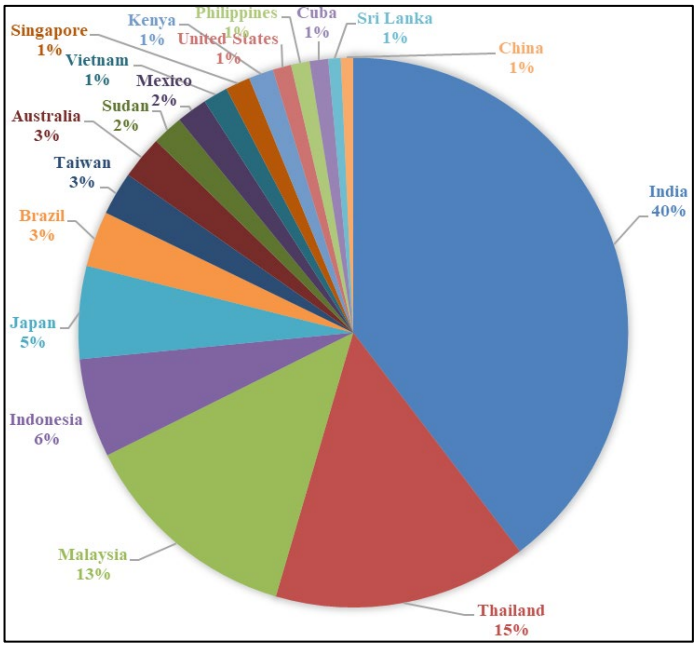

Fig. 8. Papers percentage for origin of plant samples by researchers.

3.5 Bioactive Compounds

Table 4 shows the list of some of the identified bioactive compounds from the morphology of the *C. ternatea* species. It shows that some parts of this plant have a unique bioactive compound and some parts have bioactive compounds that are similar to other parts. For example, the petals contaithocyanin which are not found in other parts of the plant whilst flavonoids can be found in both seeds and petals. The variations of the phytochemicals in *C. ternatea* explains the increment of researches performed on them in recent years.

Table 4: Morphology of *C. ternatea* and its corresponding bioactive compounds

Morphology of *C. ternatea*	**Bioactive compounds**	**Reference**
Roots	Taraxerol	[19]
Seeds	Sterols, alkaloids, glycosides, saponins, tannins,phenolic compounds & flavonoids	[29]
Whole plants	Alkaloids, glycosides, flavonoids, tannins, saponin & steroids	[4]
Roots, stems and leaves	Taraxerol and β-sitosterol	[30]
Petals	Anthocyanins and flavonoids	[3]

4. CONCLUSION

This study shows that the numbers of research performed on *C. ternatea* species has been increasing in the over the years. Recently, *C. ternatea* has been tested more profoundly in the area of solar cells, nanoparticles and colorimetric studies. This shows that the usage of this plant is not limited to only medicinal purposes and nutritional food intake. Moreover, the analysis of this study shows that phytochemical traits of *C. ternatea* are not affected by plant morphologies, but they are slightly affected by geographical distributions of the plants. The aim of identifying the spatial distributions of this study is generally achieved of which India topped the other countries by contributing the most to the publications of studies in *C. ternatea*. Bibliometric analysis supported the results of research trends and spatial distribution of studies through the mapping visualization of the studies on the author keywords and publication countries since the VOSviewer software compressed large amount of data into a result of data visualization. In conclusion, this bibliometric analysis provides a comprehensive overview of the research trends on *C. ternatea* studies and can facilitate its future studies.

ACKNOWLEDGEMENT

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REPURPOSING NATURAL PRODUCTS AGAINST *PLASMODIUM KNOWLESI* LACTATE DEHYDROGENASE VIA *IN SILICO* APPROACH FOR ANTIMALARIAL DRUG DEVELOPMENT

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ABSTRACT: Malaria cases have increased globally, which is due to the emergence of zoonotic malaria parasites that infect human, along with the existence of artemisinin-resistant parasites. Hence, there is an urgent need to find new therapeutics to counter these issues. As a vital enzyme in the glycolytic pathway that serves as the parasite's primary energy source during its intraerythrocytic stage in human, lactate dehydrogenase from *Plasmodium knowlesi* (*Pk*-LDH) can be a potential drug target. This project aims to screen for existing natural products that have progressed to preclinical or advanced drug development phases against *Pk*-LDH via ligand-based virtual screening (LBVS) and to evaluate the potentials of these bioactives as repurposed drugs by binding energy estimation through structure-based virtual screening (SBVS). The LBVS method, which was conducted *via* LiSiCA and ChemMine, are based on shape-based molecular similarity calculations to screen for analogues of the query molecules, which are lactate and pyruvate. Subsequently, PyRx simulation software were utilised for docking studies with the aid of PyMOL and PLIP software. This study discovered that Compound 7, α-hydroxyisovaleric acid, and Compound 2, α-ketoisovalerate are structurally similar to compounds that directly involved in the metabolic pathways of *P. knowlesi*, lactate and pyruvate, with a similarity score of 0.75. Both compounds also formed strong interactions with *Pk*-LDH, resulting in strong binding affinities of -4.6 kcal mol^{-1} and -4.3 kcal mol^{-1}, respectively. These findings open possibilities for using natural products in drug repurposing as anti-malarial agents.

KEY WORDS: *Malaria, Plasmodium knowlesi, Lactate dehydrogenase, Glycolysis, and Drug repurposing*

1. INTRODUCTION

Malaria, a persistent global health challenge, continues to exact a significant toll, particularly in regions such as Asia and Africa. The disease is predominantly transmitted through the bites of *Plasmodium*-infected *Anopheles* mosquitoes [1]. The emergence of drug-resistant parasites, notably in Southeast Asia, has magnified the complexities of malaria treatment, which subsequently jeopardising the efficacy of widely used antimalarial

drugs. This includes Artemisinin (ART) and its derivatives, which is one of the most effective antimalarial treatments [2]. Hence, addressing the resistance issue is of paramount importance, compelling the exploration of novel antimalarials.

In the pursuit of innovative solutions, repurposing natural products stands out as a promising strategy. This approach involves identifying FDA-approved drugs or natural compounds and assessing their potential as antimalarial agents. Natural products, characterised by their diverse chemical structures, have garnered attention for their capacity to modulate biological functions across therapeutic sectors [3].

This study is driven by the urgent necessity to combat malaria by targeting *Plasmodium knowlesi* lactate dehydrogenase (*Pk*-LDH) by using repurposed natural products through *in silico* approaches. *Pk*-LDH, a crucial enzyme in the parasite's glycolytic pathway, plays a vital role in energy generation, facilitating *Plasmodium* replication within the host's blood cells [4]. Hence, targeting *Pk*-LDH presents a promising strategy to disrupt the parasite's metabolic processes and hinder its proliferation.

To identify potential *Pk*-LDH inhibitors, this research employs virtual screening strategies, by integrating ligand-based and structure-based approaches. These methods enable screening of databases for compounds that are similar to known ligands, followed by docking studies to evaluate binding energies of the compounds interacting with *Pk*-LDH, thereby assessing their potential as antimalarials. Notably, two specific natural products were explored in this study: α-hydroxyisovaleric acid (Compound 7) and α-ketoisovalerate (Compound 2). The first compound, α-hydroxyisovaleric acid is a derivative of valine, featuring a substitution of the amino group with a hydroxyl group and commonly used as a biomarker [5]. The second compound, α-ketoisovalerate serves as both a human and *Saccharomyces cerevisiae* metabolite, playing significant roles in various biological contexts, while also serving as an adjuvant and biomarker [6].

Given the escalating malaria cases, which signifies the threats of drug-resistant *Plasmodium* strains, the urgency to explore alternative antimalarials has never been more critical [7]. Artemisinin, known as the most potent antimalarial drug, has served as the primary treatment for *P. falciparum*, with Artemisinin-based Combination Therapies (ACTs) developed and recommended as the first-line treatments in endemic areas. Nevertheless, the emergence of drug resistance, particularly along the Thai-Cambodian border, has raised global concerns due to the lack of equally effective alternatives [8]. The conventional drug discovery process is intricate, time-consuming, and costly [9]. However, the application of *in silico* techniques offers a rapid and cost-effective alternative, particularly in repurposing existing natural products for antimalarial properties.

By focusing on repurposing natural products against *Pk*-LDH, this study aligns with Sustainable Development Goal (SDG) 3, which aims to ensure good health and well-being of the nations. Identifying potential inhibitors against *Plasmodium* holds promises for advancing malaria elimination efforts *via* virtual screening approaches.

2. MATERIALS AND METHODS

2.1. Ligand-Based Screening

Ligand-Based Virtual Screening (LBVS) was performed to identify repurposable natural products that resemble the query molecules; pyruvate and lactate, which are associated with *Pk*-LDH enzyme in the glycolytic pathway. LiSiCA and ChemMine programmes were utilised to analyse multiple databases, including PubChem and other

databases, to identify potential natural product-derived compounds for further screening. Both LiSiCA and ChemMine use the Tanimoto technique as a scoring function to measure the structural similarity between compounds. A higher Tanimoto coefficient indicates a greater degree of similarity between the compounds.

Initially, OpenBabel, a chemical toolbox, was employed to convert the SDF file from the databases to the MOL2 format. The screening process starts with the initial screening of unknown compounds in LiSiCA, where compounds with Tanimoto scores of 0.4 or above were identified. To further enhance the similarity scores, the MCS Tanimoto function provided in ChemMine was utilised. The results were narrowed down to natural product-derived compounds that have reached the pre-clinical or advanced drug development phases. In this analysis, chemical databases such as PubChem, CheMBL, Drugbank, and Clinicaltrials were used to identify these compounds. Finally, natural products with high similarity scores, determined by setting a cutoff of 0.75 using the MCS Tanimoto score, were selected as ligand candidates for further evaluation.

2.2. Structure-Based Screening

Structure-Based Virtual Screening (SBVS) was employed to identify potential ligands from LBVS that are capable of binding to *Pk*-LDH proteins through molecular docking analysis *via* PyRx software, with subsequent analysis of their interactions using PyMOL and PLIP programmes. The 3D structure model of the *Pk*-LDH protein, obtained from a prior work [10], was used for this screening.

Compound candidates from LVBS that exhibit significant similarities to one of the query molecules with a high MCS Tanimoto score derived from ChemMine, were chosen for further assessment in the structure-based screening. Initially, the protein structure of *Pk*-LDH underwent modifications to ensure proper preparation before the docking process in PyRx. This preparation encompassed necessary adjustments such as the removal of water molecules from the cavity, charge stabilization, completion of absent residues, and construction of side chains according to defined parameters [11].

For the preparation of the ligand candidates, conversion from SDF to PDBQT format was carried out using OpenBabel, an integrated tool within PyRx. Subsequent steps involved energy minimisation of the ligands, facilitated by PyRx functionalities. The compounds were then categorised as protein or ligand, and the grid box was set to encompass all protein residues.

In the docking process facilitated by PyRx, flexibility was allowed for the compound candidates, while the protein structure remained rigid. Docking results were visualised using the software, displaying poses along with their respective binding affinities and additional parameters such as RMSD. The most favorable poses with the lowest binding energies were selected among the nine poses generated for further analysis. These chosen compounds, along with *Pk*-LDH protein, were subjected to detailed binding affinity assessments using PyMOL and PLIP software to explore atomic binding interactions within the ligand-enzyme complexes. Furthermore, for additional validation, ADME analyses were conducted to evaluate the drug-likeness of the selected ligand candidates.

3. RESULTS

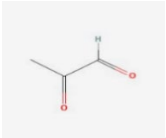
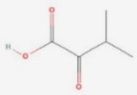
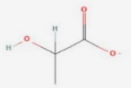
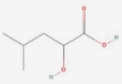
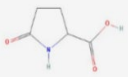
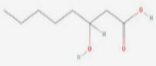
3.1. Ligand-based Virtual Screening

In the Ligand-Based Virtual Screening (LBVS), the performance of both ChemMine and LiSiCA was compared for similarity screening, aiming to evaluate their effectiveness. Initially, LiSiCA software was utilised to rank compounds based on their 2D structural resemblances to pyruvate, oxamate, and lactate. LiSiCA was chosen as the primary screening tool due to its capability to screen diverse compound databases from various sources, a feature lacking in ChemMine. Among various databases that have been screened, only databases namely PubChem, NP ATLAS, and AnalytiCon Discovery possess compounds with a similarity score of 0.4 or above with at least one of the three reference molecules. Notably, ChemMine displayed a more promising similarity score compared to LiSiCA, attributed to its employment of the Maximum Common Substructure (MCS) Tanimoto coefficient for assessing structural similarity. In this screening, 24 unknown compounds that have MCS Tanimoto scores of 0.4 and above with at least one of the three reference molecules have been screened using LiSiCA.

The subsequent phase of screening targeted the identification of natural products with potential as anti-malarial agents. Compounds sourced from natural origins and those in advanced drug development stages were referenced from chemical databases such as PubChem, CheMBL, Drugbank, and Clinicaltrials. In this phase, compounds with lower structural similarity scores were eliminated, setting a cutoff value of 0.75 to identify natural products potentially repurposable as antimalarials. While previous research recommended a cutoff of 0.85 [12], the initial scores obtained did not consistently meet this threshold. Lowering the cutoff to 0.75 enabled the inclusion of compounds exhibiting reasonable similarity, enhancing the chances of identifying potential candidates for repurposing. This adjustment balanced capturing significant similarity while expanding the pool of potential candidates. These natural products with high similarity scores, presented in the Table 1, are indicative of ligands that may possess similar biological activity to the reference molecules.

Subsequently, three ligand candidates meeting the predefined criteria were identified after the rigorous screening process. Ligand candidates with PubChem CIDs 49 and 880 exhibited notable resemblances to pyruvate, while ligand with PubChem CID 99823 displayed high similarity to lactate. These compounds bear substantial structural likeness to pyruvate and lactate, essential metabolites involved as the substrate and product, respectively, in the LDH enzyme's activity in *P. knowlesi*. Targeting these metabolites or interfering with *Pk*-LDH enzymatic activity presents a potential avenue to disrupt the parasite's energy metabolism and inhibit its growth.

Table 1: The analogues of the query molecules (Pyruvate and Lactate)

Compound	Ligand Structure	PubChem CID	Drug Development Phase	Natural Source	MCS Tanimoto Score
			Pyruvate		
3		880	Preclinical	*Arabidopsis thaliana, Sesamum indicum*	0.833
2		49	Preclinical	*Aloe africana, Euglena gracilis*	0.75
1		1662	Clinical but unknown phase	*Lemna perpusilla,Lotus creticus*	0.417
			Lactate		
7		99823	Preclinical	*Vitis vinifera, Saccharomyces cerevisiae*	0.75
8		92779	Preclinical	*Annona muricata, S. cerevisiae*	0.667
5		743	Preclinical	*Escherichia coli* (strain K12, MG1655)	0.5
6		499	Preclinical	*Stellaria palustris, Synechocystis*	0.5
4		26613	Investigative	*Streptomyces, Fragaria*	0.417

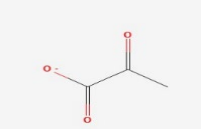
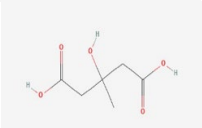
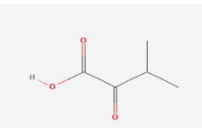
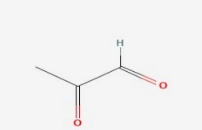
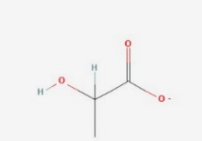
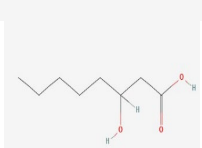
3.2. Structure-Based Virtual Screening

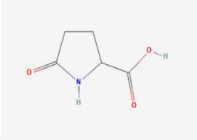
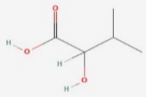
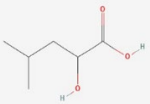
To further assess the potential of these compounds, Structure-Based Virtual Screening (SBVS) methods were subsequently employed for in-depth analysis and exploration of their interactions with *Pk*-LDH enzymes.

Through this analysis, compounds shortlisted from the earlier LBVS underwent docking simulations to assess their binding affinity. These compounds were docked specifically to the active site of the *Pk*-LDH protein. Through this process, compounds exhibiting the most negative binding energy indicating stronger binding affinity were listed as top candidates, which is a practice corroborated previously [13] [14].

To ascertain compounds with acceptable binding affinity *via* the docking technique, a comparative analysis was conducted between the docking scores of the selected compounds and the binding affinity values of their closest reference molecules. This comparative approach aimed to prioritise compounds demonstrating similar or superior binding affinity to the reference molecules, suggesting their potential as regulators of *Pk*-LDH enzyme. The PyRx docking approach highlighted pyruvate with the highest docking score of -4.1 kcal mol^{-1} and lactate with a score of -3.8 kcal mol^{-1}. Based on these scores, eight ligands exhibiting acceptable binding affinities were identified. These ligands, detailed in Table 2, showcased binding affinities ranging from -5.5 to -3.6 kcal mol^{-1}, indicating their potentials to interact effectively with the *Pk*-LDH protein and potentially evoke similar biological responses as the query molecules.

Table 2: The binding affinities of selected compounds and the query molecules

Compound	Structure	PubChem CID	Binding Affinity [kcal/mol]
Pyruvate		107735	-4.1
1		1662	-5.5
2		49	-4.3
3		880	-3.6
Lactate		91435	-3.8
4		26613	-4.8
5		743	-4.8

6		499	-4.7
7		99823	-4.6
8		92779	-4

4. DISCUSSION

Through both LBVS and SBVS analyses, Compound 2 (CID 49, α-ketoisovalerate) and Compound 7 (CID 99823, α-hydroxyisovaleric acid) emerged as the top candidates following a thorough analysis encompassing both screening approaches. The combination of screening strategies yields a comprehensive insight into the candidates' structural resemblance to known active molecules and their favorable interactions with the target protein. The integration of data acquired from both screening approaches enhances the overall reliability of the findings. As tabulated in Table 3 and Table 4, both compounds 2 and 7 exhibit noteworthy similarity scores with their respective reference molecules, which are pyruvate and lactate, while also demonstrated favorable binding affinities of -4.3 kcal mol^{-1} and -4.6 kcal mol^{-1}, respectively. These compounds have been identified as potential inhibitors that specifically target the *Pk*-LDH enzyme.

Table 3: Comparison of Compound 2 binding affinities with its respective reference compound (Pyruvate)

Compound	**PubChem CID**	**Binding Affinity [kcal/mol]**
Pyruvate	107735	-4.1
Compound 2	49	-4.3

Table 4: Comparison of Compound 7 binding affinities with its respective reference compound (Lactate)

Compound	**PubChem CID**	**Binding Affinity [kcal/mol]**
Lactate	91435	-3.8
Compound 7	99823	-4.6

Subsequently, docked complexes interaction analysis was performed, which centers on the identification of precise amino acid residues involved in the interactions between the selected compounds and *Pk*-LDH, to comprehend the pivotal binding sites and molecular interactions essential for inhibiting the enzyme's activity. Diverse interaction types, including hydrophobic contacts, hydrogen bonds, π-stacking, salt bridges, amide stacking, and cation-π interactions, collectively contribute to the stability and binding affinity of the ligand-protein complex. Notably, highly efficient ligands often exhibit a higher prevalence

of hydrophobic interactions, while fragment inhibitors commonly used in drug development demonstrate increased occurrences of hydrogen bonds [15, 16].

The first phase of the analysis involved visualising the lactate-*Pk*-LDH and Compound 7-*Pk*-LDH complexes. A comparative assessment between these complexes, depicted in Fig. 1, highlights that Compound 7-*Pk*-LDH complex did not involve salt bridges, which are not considered crucial for overall binding stability [17]. Despite forming fewer interactions, Compound 7, structurally akin to lactate, displayed a higher binding affinity (-4.6 kcal mol^{-1}) to *Pk*-LDH, compared to *Pk*-LDH-lactate interactions (-3.8 kcal mol^{-1}). Compound 7 interacted notably with ALA-80A and PHE-82A *via* hydrophobic interactions, underscoring the significance of these residues in the binding process.

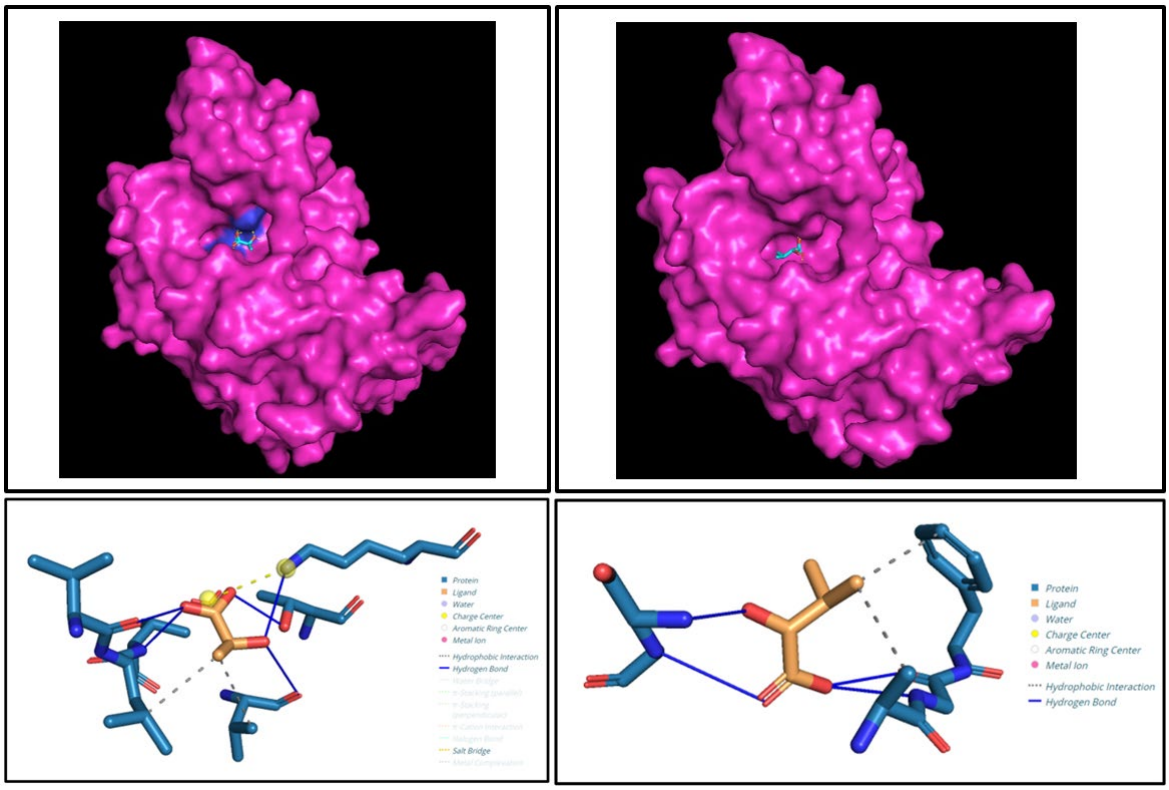


Fig 1. Visualisation of lactate-*Pk*-LDH (left) and Compound 7-*Pk*-LDH (right) complexes using PyMOL sofware. The specific interactions are also shown for both complexes.

Subsequently, visualisation of pyruvate-*Pk*-LDH and Compound 2-*Pk*-LDH complexes were employed. As illustrated in Fig. 2, Compound 2, resembling pyruvate structurally, formed a greater number of interactions compared to pyruvate. These included five hydrophobic interactions and one salt bridge. These findings corroborate the higher binding affinity exhibited by Compound 2 (-4.3 kcal mol^{-1}) in contrast to pyruvate (-4.1 kcal mol^{-1}), indicating a more robust binding with *Pk*-LDH. Further evaluation of hydrogen bonds unveiled the interaction of both Compound 2 and pyruvate with the specific amino acid residue HIS-182A, suggesting a common binding site within the *Pk*-LDH enzyme. The shared interaction with HIS-182A in both Compound 2 and pyruvate points toward a conserved binding region for these ligands within the *Pk*-LDH enzyme.

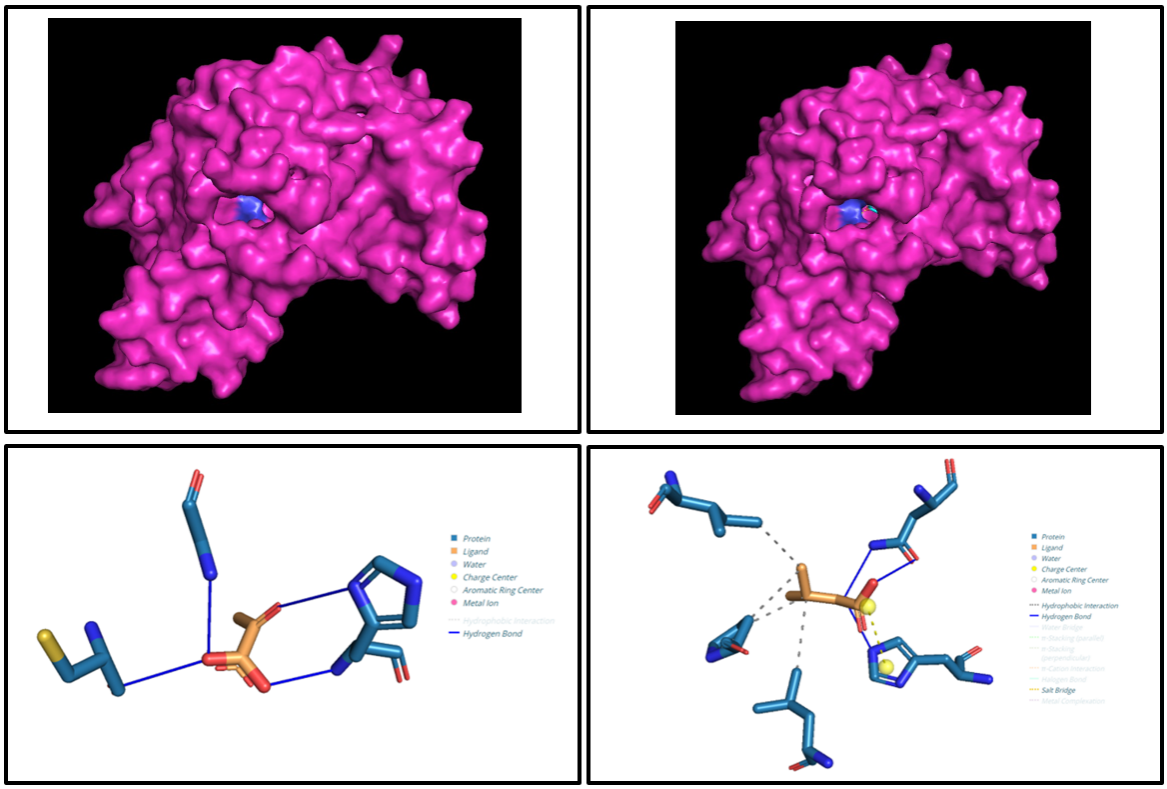


Fig 2. Visualisation of pyruvate-*Pk*-LDH (left) and Compound 2-*Pk*-LDH (right) complexes using PyMOL software. The specific interactions are also shown for both complexes.

5. CONCLUSION

The implementation of drug repurposing approaches presents promising avenues in identifying potential treatments for artemisinin-resistant malaria parasites. This approach not only expedites research endeavors but also significantly reduces the associated costs in the drug design pipeline. Through employing virtual screening and molecular docking techniques, this study has pinpointed α-hydroxyisovaleric acid (Compound 7) and α-ketoisovalerate (Compound 2) as potential inhibitors of lactate dehydrogenase from *P. knowlesi* (*Pk*-LDH). Compound 7 demonstrated stronger binding affinity despite fewer interactions, while Compound 2 displayed enhanced binding affinity over pyruvate due to specific interactions with interacting residues in *Pk*-LDH. These findings highlight the potential of repurposing Compound 7 and Compound 2 as alternative treatment options for artemisinin-resistant malaria. In the future, further research avenues should encompass rigorous validation, structural optimisation, potential combination therapies, mechanistic elucidation, and pathways for effective clinical translation, aiming to pave the way for the development of viable treatments against artemisinin-resistant malaria.

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