PRODUCTION OF MALAYSIAN KENAF *Hibiscus cannabinus* CALLUS BIOMASS AND EXOPOLYSACCHARIDE IN A CELL SUSPENSION CULTURE

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FACULTY OF SCIENCE

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PRODUCTION OF MALAYSIAN KENAF *Hibiscus cannabinus* CALLUS BIOMASS & EXOPOLYSACCHARIDE IN A CELL SUSPENSION CULTURE

ABSTRACT

A local fibrous crop, kenaf (*Hibiscus cannabinus*) is commonly utilised within traditional fields such as paper and pulp production, textiles and bio-composites. Many often overlook its vast potential due to its well-established fibre usage where its future applications can eventually penetrate diverse industries such as medical, pharmaceutical, cosmeceutical and food, owing to its extracts' therapeutic activities. However, our heavy reliance on traditional farming to ensure a consistent supply of kenaf raw materials is deemed to be unsustainable. This is because concerns about land availability, crop quality and time efficiency will arise if we were to upscale its extract production for industrial purposes. Thus, plant cell suspension culture is opted to solve the aforementioned issues as this method can produce a large amount of kenaf callus biomass (KCB), the source of the extracts. But an optimization study involving its working parameters; sucrose concentration (g/L), agitation (rpm), 2,4-dichlorophenoxy acetic acid (2,4-D) concentration (mg/L) and naphthalene acetic acid (NAA) concentrations (mg/L) is a must. This is to ensure an efficient suspension culture of kenaf in terms of the cost, resources, time, and potential future upscaling activities using bioreactors. Furthermore, it is to maximise the production of the KCB and a potential therapeutic compound, kenaf exopolysaccharide (EPS) from the suspension culture. So, in this dissertation, a preliminary study of kenaf cell suspension culture was conducted using callus that is formed on the stem of 14-days old kenaf seedlings for the establishment of the kenaf callus growth curve. At the end of the suspension culture, KCB and EPS were harvested as the cultivation's products. It was found that the most suitable time to harvest the cultivated kenaf callus is on the 22nd day of the culture, with a KCB of 9.09 g/L and 1.10

g/L EPS. Then, the optimization of kenaf callus in suspension culture was conducted using response surface methodology (RSM) to study the interaction and optimize the working parameters (sucrose, agitation, NAA and 2,4-D) for the production of KCB and EPS. A central composite design (CCD) was utilised and the polynomial model was fitted to the experimental data. The models generated were significant (p<0.05) with sucrose, agitation and NAA having a strong effect on KCB and EPS production. The maximum predicted outcome of KCB (13.34 g/L) and EPS (1.62 g/L) can be achieved using the optimized parameters; 50 g/L of sucrose, 147.02 rpm and 2 mg/L of NAA. Then a molecular characterization of EPS was performed using Fourier-transform infrared spectroscopy (FTIR). The absorbance at 3299 cm^{-1}, 2933 cm^{-1}, 1628 cm^{-1}, 1081 cm^{-1} and 865 cm^{-1} in the spectra suggested that EPS from kenaf is of α-configuration (α-glucan). To conclude, this research provides a blueprint for kenaf callus cultivation in cell suspension culture for mass-production purposes to produce its extracts' source, callus. This study also determined the type of the excreted polysaccharide during the suspension culture of kenaf which is found to be α-glucan that has been previously proven to have useful properties such as anti-inflammatory, antibacterial and antioxidant.

Keywords: *Hibiscus cannabinus*; kenaf; optimization; callus; biomass; exopolysaccharide

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ABSTRAK

Tumbuhan tempatan, kenaf (*Hibiscus cannabinus*) biasa digunakan dalam sektor tradisional seperti pembuatan kertas, tekstil dan bio-komposit. Ramai yang terlepas pandang potensi besar kenaf oleh kerana penggunaan fibernya yang meluas, di mana kegunaan kenaf pada masa hadapan boleh menembusi sektor perubatan, farmasi, kosmetik dan makanan oleh sebab aktiviti terapeutik ekstraknya. Tetapi, kebergantungan kita terhadap penanaman secara tradisional untuk memastikan keberlangsungan bekalan kenaf adalah tidak mampan, sekiranya kita ingin memperbanyakkan penghasilan ekstrak untuk kegunaan industri kerana isu seperti ketiadaan tanah, kualiti tumbuhan dan kecekapan masa akan timbul. Jadi, kultur sel tumbuhan secara penggantungan dipilih untuk menyelesaikan masalah tersebut kerana cara ini boleh menghasilkan biojisim kalus kenaf (KCB) yang banyak, iaitu sumber ekstrak. Namun, proses mengoptimum yang melibatkan keadaan kultur; kepekatan sukrosa (g/L), pergolakan (rpm), kepekatan 'naphthalene acetic acid' (NAA, mg/L) dan kepekatan '2,4-dichlorophenoxy acetic acid' (2,4-D, mg/L) adalah diperlukan untuk memastikan kultur sel tumbuhan kalus kenaf yang efisyen dari segi kos, sumber, masa dan kerja peningkatan hasil pada masa hadapan menggunakan bioreaktor. Selain itu, ia untuk memaksimakan hasil pengeluaran KCB dan kompoun berpotensi untuk terapi, kenaf eksopolisakarida (EPS) dari kultur penggantungan. Jadi, dalam disertasi ini, satu percubaan awal untuk menyelidik kultur sel tumbuhan secara penggantungan kenaf dengan menggunakan kalus yang terbentuk daripada batang kenaf yang 14 hari untuk menghasilkan graf pertumbuhan kenaf. Hasil dari penyelidikan awal, ia didapati bahawa masa yang sesuai untuk mengambil hasil KCB dan EPS adalah pada hari ke-22 kultur dengan KCB sebanyak 9.09 g/L dan EPS sebanyak

1.10 g/L. Kemudian, proses mengoptimum kalus kenaf menerusi kultur penggantungan telah dijalankan dengan menggunakan perisian 'response surface methodology' (RSM), untuk menyelidik interaksi dan optimumkan keadaan kultur (sukrosa, pergolakan, NAA dan 2,4-D) untuk penghasilan KCB dan EPS. Reka bentuk 'central composite design' (CCD) telah digunakan dan model polinomial telah disuaikan dengan data eksperimen. Model yang terhasil adalah ketara (p<0.05), di mana sukrosa, pergolakan dan NAA mempunyai efek yang kuat bagi penghasilan KCB dan EPS. Hasil maksimum KCB (13.34 g/L) dan EPS (1.62 g/L) boleh dicapai dengan penggunaan keadaan kultur optimum; 50 g/L sukrosa, pergolakan pada kelajuan 147.02 rpm dan 2 mg/L NAA. Seterusnya, pencirian molekular EPS telah dilakukan dengan menggunakan 'Fourier-transform infrared spectroscopy (FTIR). Penyerapan pada 3299 cm^{-1}, 2933 cm^{-1}, 1628 cm^{-1}, 1081 cm^{-1} dan 865 cm^{-1} dalam spektra mencadangkan EPS dari kenaf adalah dalam konfigurasi α (α-glucan). Kesimpulannya, penyelidikan ini telah menghasilkan satu pelan tindakan untuk kalus kenaf menerusi kultur penggantungan untuk meningkatkan penghasilan sumber ekstraknya, iaitu KCB. Penyelidikan ini juga berhasil dalam mencirikan polisakarida yang terkeluar semasa kultur penggantungan kalus kenaf, di mana ianya adalah α-glucan yang telah direkodkan untuk mempunyai pelbagai manfaat seperti anti-radang, anti-bakteria dan antioksidan.

Kata kunci: *Hibiscus cannabinus*; kenaf; pengoptimuman; kalus; biojisim; eksopolisakarida

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
%	:	Percentage
°C	:	Degree Celsius
µg	:	Microgram
µL	:	Microlitre
2,4-D	:	2,4-dichlorophenoxy acetic acid
3D	:	Three-dimensional
ANOVA	:	Analysis of variance
BA	:	6-benzyladenine
BAP	:	6-benzyl amino purine
cm^{-1}	:	Wavelength per distance
DW	:	Dry weight
EPS	:	Exopolysaccharide
FTIR	:	Fourier-transform infrared spectroscopy
FW	:	Fresh weight
g	:	Gram
h	:	Hour
ha	:	Hectare
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
KCB	:	Kenaf callus biomass
L	:	Litre
LKTN	:	National Kenaf and Tobacco Board
m	:	Metre

mg	:	Milligram
MHz	:	Megahertz
min	:	Minute
mL	:	Millilitre
mm	:	Millimetre
mmol	:	Micromolar
MS	:	Murashige and Skoog
NAA	:	Naphthalene acetic acid
NMR	:	Nuclear magnetic resonance
PGR	:	Plant growth regulators
pH	:	Potential hydrogen
rpm	:	Rotation per minute
RSM	:	Response surface methodology
s	:	Second
t	:	Tonne
v/v	:	Volume per volume

CHAPTER 1: INTRODUTION

1.1 Research background

Kenaf or *Hibiscus cannabinus* is a herbaceous dicotyledonous annual plant that came from the same family as cotton and okra, the *Malvaceae* family (Meints & Smith, 2003). This plant which is believed to have originated from Africa is being progressively planted in various countries such as Thailand, China, India and even in Malaysia due to their suitable climatic conditions (Monti & Alexopoulou, 2013). Kenaf thrives with abundant solar radiation accompanied by high rainfall and it typically grows naturally within the tropical and temperate regions (Ayadi et al., 2017). At its best, kenaf can grow up to 5 m in height within 6 months and it can produce approximately 30 t/ha of dry stem material, a reliable part of the plant for a good natural fibre source (Coetzee et al., 2008). Conventionally, this plant is highly valued for its natural fibre which is found to be useful for numerous industries such as paper and pulp production, textiles, and including the manufacturing of bio-composites and absorption materials. But recently, kenaf has been regarded as a multipurpose plant as numerous discoveries have been made regarding its potential which exceeds the traditional uses including medicinal and therapeutic applications (Ayadi et al., 2017).

Traditional applications of kenaf include the production of cordage, rope and fishnet. It is also highly used in the paper manufacturing industry due to its superior quality compared to the usage of wood (Tahir et al., 2011). Other than that, kenaf can also be used for food production which is derived from its seed oil, which happens to be naturally healthy due to its high amount of mono-unsaturated and poly-unsaturated fatty acids (Coetzee et al., 2008). Even the leaves of kenaf are extensively used especially for animal forage due to their high crude protein content (Webber & Bledsoe, 2002). But with recent discoveries, many have noted its potential that can be further expanded beyond the aforementioned traditional uses (Norhisham et al., 2023). Kenaf was found to be a good

and dependable source of phytochemicals such as flavonoids, phenolics and polysaccharides that provide antioxidant and antimicrobial properties (Birhanie et al., 2021). Besides, other worth mentioning therapeutic properties of kenaf that have been pharmacologically tested include anti-tumour, anti-inflammatory, cardio-protective, anti-hypertensive, anti-oxidant, anticoagulant and antiplatelet (Adnan et al., 2020; Chew et al., 2021; Ghafar et al., 2012; Goh et al., 2021; Kai et al., 2015). With the ever-increasing concern to attain a sustainably healthy community, kenaf has piqued the interest of many as the alternative natural source for plant-based foods and drugs.

However, the majority of the reported therapeutics and pharmacological activities from kenaf have come directly from the extracts of the physical plant itself such as its leaves, stems and roots (Chew et al., 2021; Goh et al., 2021; Hanumegowda et al., 2022; Kai et al., 2015). Eventually, this generates a continuous request for kenaf raw materials through traditional and conventional farming to produce useful phytochemical compounds. However, our prolonged dependency on traditional farming and cultivation methods are not highly suggested as the quality of the intended product may vary from time to time due to climatic changes, plant varieties and farming practices. Even the issue of land and resource availability could be an obstacle in the future which will further hamper the true potential of kenaf. Alternatively, phytochemical compounds of any plant species can be retrieved from the unorganised mass of cells (callus) of the plant (Mamdouh & Smetanska, 2022; Murtazina et al., 2022; Shaikh et al., 2018). When the callus is allowed to be cultured in an agitated liquid medium, this increases the quantity of the callus biomass from which a phytochemical of interest can be retrieved in higher quantities (Shaikh et al., 2018). But, to fully reap the benefits of its therapeutic components that are retrieved from the plant callus biomass, its final yield must be high enough for it to be economically feasible for the industry (Ghafar et al., 2013). So, the main question arises, how can we ensure a sustainable production of any kenaf therapeutic phytochemicals that

guarantees a consistent quality with a high yield return that is enough to cater for industrial needs?

To solve the aforementioned issues, the cultivation of kenaf can be done in vitro using the tissue culture technique. Through this technique, the plant can be mass-produced aseptically in a shorter period due to the optimised culture conditions. The callus derived from the in vitro-grown kenaf can then be harvested and subjected to cell suspension culture using bioreactors to mass-produce any of the targeted biologically active phytochemicals (Vayabari et al., 2023). Since this technique does not depend on raising and maintaining crops, the production of the targeted phytochemicals can be done regardless of season, land or plant availability (Winson et al., 2020). Natively, kenaf is grown on a solid medium, so the alteration of its growing habitat from solid to liquid must be justified and optimised in terms of its cultivating parameters to bring about the best results. Thus, an optimisation study to determine the right parameters to cultivate kenaf callus in suspension culture is crucial, and must be executed. This is important to set a blueprint for any future industrial-scale cultivation through suspension culture, so the utilization of the bioreactor can be done effectively in terms of time, cost and resources.

Therefore, in this dissertation, the interaction between the parameters influencing cell suspension culture of kenaf; sucrose concentration, agitation and plant growth hormones concentrations along with their impact on the outcome of the responses was studied using the response surface methodology (RSM) method, which subsequently provided the optimised parameters for the culture.

1.2 Problem statement

Our total reliance on traditional farming for kenaf raw materials; leaves, seeds and flowers for the production of its phytochemicals is not sustainable, as several issues will unravel from time to time such as product quality, land and resource availability. Thus, other alternatives such as cell suspension culture seem to be very attractive (Murtazina et al., 2022; Winson et al., 2020). However, reports on kenaf callus cell suspension culture are also of low frequency. This further ponders questions about its optimum cultivating parameters in cell suspension culture. So, its best culturing parameters through suspension culture such as sucrose concentration (g/L), agitation (rpm) and plant growth hormones concentration (mg/L) remain unknown. Therefore, the aforementioned cell suspension culture working parameters must be optimised experimentally to result in the best outcome in terms of kenaf callus biomass (KCB) and kenaf exopolysaccharide (EPS).

1.3 Objectives

- To optimise the working parameters; sucrose concentration (g/L), agitation (rpm) and plant growth hormone concentration (mg/L) for the production of kenaf callus biomass (KCB) and kenaf exopolysaccharide (EPS) through cell suspension culture.

- To analyse the composition of the extracted kenaf exopolysaccharide (EPS) from the cell suspension culture of kenaf callus using Fourier-transform infrared (FTIR).

1.4 Scope of work

This study focused on the optimization of the culturing parameters of kenaf cell suspension culture; sucrose concentration, agitation and plant growth hormone concentration. First, a preliminary study to establish the growth profiling curve of kenaf

cell suspension culture was performed. Then, the working parameters of the suspension culture of kenaf were optimised using the response surface methodology (RSM) method which revealed the significant effects and interdependency of the tested parameters on the products of the culture (KCB and EPS). Then, the produced EPS were subjected to compositional analysis using Fourier-transform infrared spectroscopy (FTIR) to elucidate its structural conformation.

1.5 Dissertation outline

This dissertation is comprised of five chapters. Chapter 1 explained about the background and the necessity of the study. Chapter 2 provided an in-depth literature review about the plant of interest (kenaf) along with the cell suspension culture technique. Chapter 3 discussed about the methodological approach used in this study. Chapter 4 highlighted the results and discussion of the study. Chapter 5 provided the impact and insights of the study's outcome while Chapter 6 concluded the entire research.

CHAPTER 2: LITERATURE REVIEW

2.1 Kenaf's taxonomy

Kenaf is a fibrous plant that is scientifically known as *Hibiscus cannabinus*. This plant is closely related to cotton (*Gossypium hirsutum*), okra (*H. esculentum*), hollyhock (*Althaea rosea*) and hibiscus (*H. hibiscum*) as kenaf originated from the *Malvales* order, and *Malvaceae* family, and situated under the *Hibiscus* genus (Izran et al., 2014; Sim & Nyam, 2021). Looking deeper, *Hibiscus* genus can be further broken down into several sections; *Furcaria*, *Alyogen*, *Abelmoschus*, *Ketmia*, *Calyphillia* and *Azanza*. Taxonomically, kenaf has been classified within the *Furcaria* section, a section that is famously known for its food, fibre and medicinal uses (Sim & Nyam, 2021). Kenaf can also be recognised by other names based on its locations and regions such as Ambari (Taiwan), Gambo (West Africa), Stokroos (South Africa), Til (North Africa), Mesta (India), and Java Jute (Indonesia) (Ayadi et al., 2017; Izran et al., 2014). Interestingly, kenaf has always been associated with hemp or marijuana, *Cannabis sativa* due to their similarities in terms of their leaves' shape and nomenclature origin; taken from a Persian word, *kanab*. However, both plants are not associated with one another as hemp originated from the *Moraceae* family (Izran et al., 2014).

2.2 Kenaf's botanical description

Natively, kenaf is renowned for its good quality fibre source that can be retrieved from its stem. Kenaf stem can be distinguished and categorised according to the three types of fibre it makes; bast, core and pith (Ayadi et al., 2017). Kenaf bast is the stem's outer region which makes up approximately 30% of the total stem's dry weight. The bast is claimed to produce a superior fibre quality compared to other parts of the stem (Khalil et al., 2010). Meanwhile, the kenaf core can be observed as the inner white part of the stem which constitutes the remaining of the stem's dry weight. For kenaf pith, it can be

recognised by its polygonal parenchymal cells (Tahir et al., 2011). This prickly, unbranched and green-reddish stems of kenaf can grow to up to 4 - 5 m with sufficient solar radiation and abundant rainfall due to their high growth rate capacity (Figure 2. 1A). This feature permits the plant to be harvested for industrial usage within 6-8 months (LKTN, 2021).

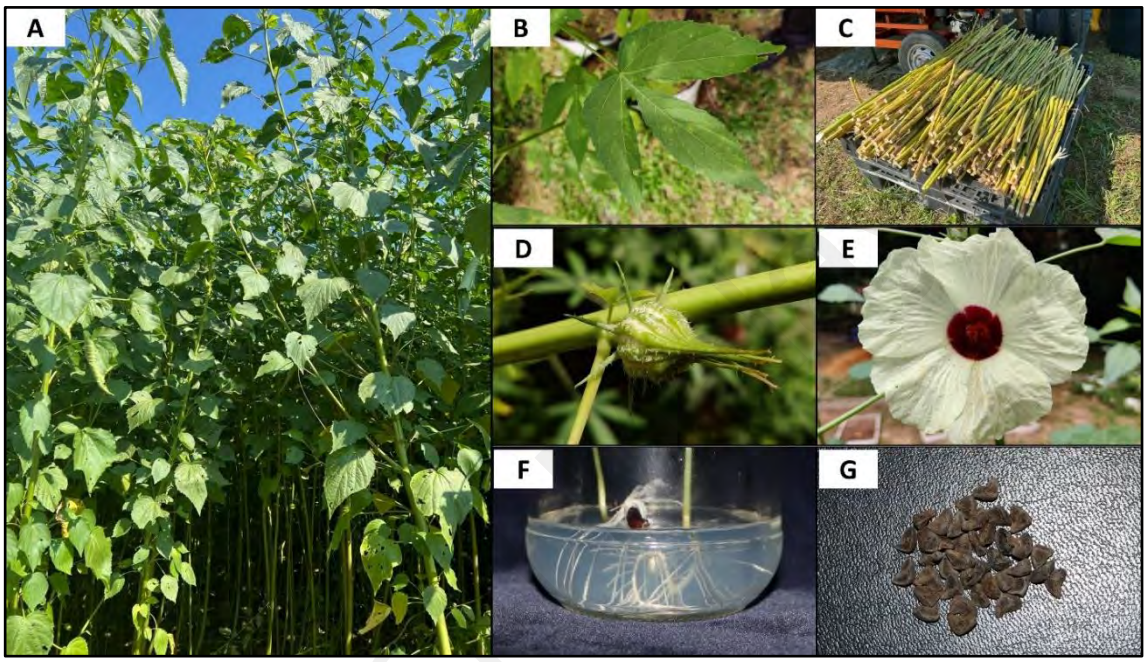

Figure 2. 1: Kenaf plant structure. (A) Whole plant. (B) Leaf. (C) Stem. (D) Fruit. (E) Flower. (F) Roots. (G) Seeds.

Botanically, depending on its variety and age, kenaf can produce either simple (entire) or compound (divided) green leaves, that are arranged alternately along the kenaf stem (Figure 2. 1B). Typically, young leaves that are found on kenaf seedlings are simple, entire and cordate. But, as the plant matures, new palmately compound leaves begin to appear whereby they resemble the palm of a hand, with leaflets ranging from 3 to 7 radiating outwards from a single point. Besides, kenaf produces creamy to light yellow-coloured flowers with a reddish-maroon coloured centre that is bell-shaped and widely open, which makes the flowers hugely conspicuous (Figure 2. 1E). The flowers which open and close in a single day are solitary with short-stalked that consist of 5 petals, 5 sepals and

numerous stamens. Nevertheless, the flower is said to have a superior ovary, whereby other floral organs such as sepal, petal and stamen are arranged below or around it. Then in terms of pollination, kenaf flowers can conduct both self-pollination and cross-pollination which is characterised by the twisting-closing movement of the petals, and with the help of domesticated honey bees, respectively (Webber & Bledsoe, 2002). Moreover, the kenaf fruit is distinguished by its hairy, rounded and coarse structure which is also segmented (Figure 2. 1D). Most segments are seeded, each containing 20-26 seeds. Kenaf seeds are hairless with the shape of kidney beans-like triangular (wedged-shaped) along with sharp corners that are brown with bright yellow dots (Figure 2. 1G) (Arumingtyas, 2015; Webber & Bledsoe, 2002). Other than that, for a better absorption rate, kenaf plant has a long tap root with extensive lateral roots (Figure 2. 1F) that consequently make it more sensitive to changes in soil moisture and a better soil penetrator for deep soil water absorption (Danalatos & Archontoulis, 2004).

2.3 Kenaf's growth

Kenaf is a type of plant that has a wider range of adaptation to soils and climatic conditions compared to other fibrous plants that are cultivated for commercial purposes. The plant thrives very well under abundant solar radiation and high rainfall which can be usually found within the tropical and temperate regions of the globe (Ayadi et al., 2017). The presence of high temperature and moisture are favoured for kenaf's growth. The plant is also equipped with an extensive root system; a long taproot and lateral roots which allow kenaf to adapt and respond appropriately to soil water availability and moisture variations (Danalatos & Archontoulis, 2004). Due to its wide ecological adaptability, the composition of the soil and its origin do not have a major impact on kenaf growth which in theory, it can grow on any type of soil. Unlike other commercial crops which are poorly adapted to unfertile soil. Interestingly, it can also be planted on marginal land provided that the soil does have a good drainage system (Ayadi et al., 2017). Besides, even though

kenaf is regarded as a high water-demanding plant, it adjusts and reacts relatively well according to water availability through its effective stomatal conductance and transpiration rate. Under a stressed condition where the water availability is very limited, both stomatal conductance and transpiration rate will be hugely reduced (Ayadi et al., 2017). However, under a prolonged period of water scarcity, especially during the seedling stage, its growth will be severely hampered. It is also important to note that the temperature for kenaf cultivation should remain above 10 °C throughout its growing period to ensure its fibre and seed yields are maximised (Ayadi et al., 2017). For kenaf floral induction, it depends on temperature and photoperiod. Kenaf's flowering will occur once the daylight hour decreases below 12.5 h, lest it will remain vegetative (Meints & Smith, 2003).

2.4 Kenaf in Malaysia

2.4.1 History

In 1999, the National Economic Action Council (NEAC) of Malaysia initiated the idea of kenaf plantation to investigate its potential and feasibility to become another commercialised crop besides rubber and oil palm. Since 2000, multiple agencies including the Malaysian Agricultural Research and Development Institute (MARDI), Malaysian Rubber Board (LGM), Malaysian Palm Oil Board (MPOB) and Universiti Putra Malaysia (UPM) formed a committee that drove the research and development of kenaf which subsequently fast-tracked its progress and adoption in the country. Since then, many types of research that covered the aspects of upstream and downstream processing including variety screening, agronomic practices for cultivation, harvesting and mechanization, retting and fibre processing for industrial applications such as bio-composite and animal feed were undertaken (Basri et al., 2014). This scenario is illustrated by the recent citation and publication trends associated with kenaf in Malaysia (Figure 2. 2).

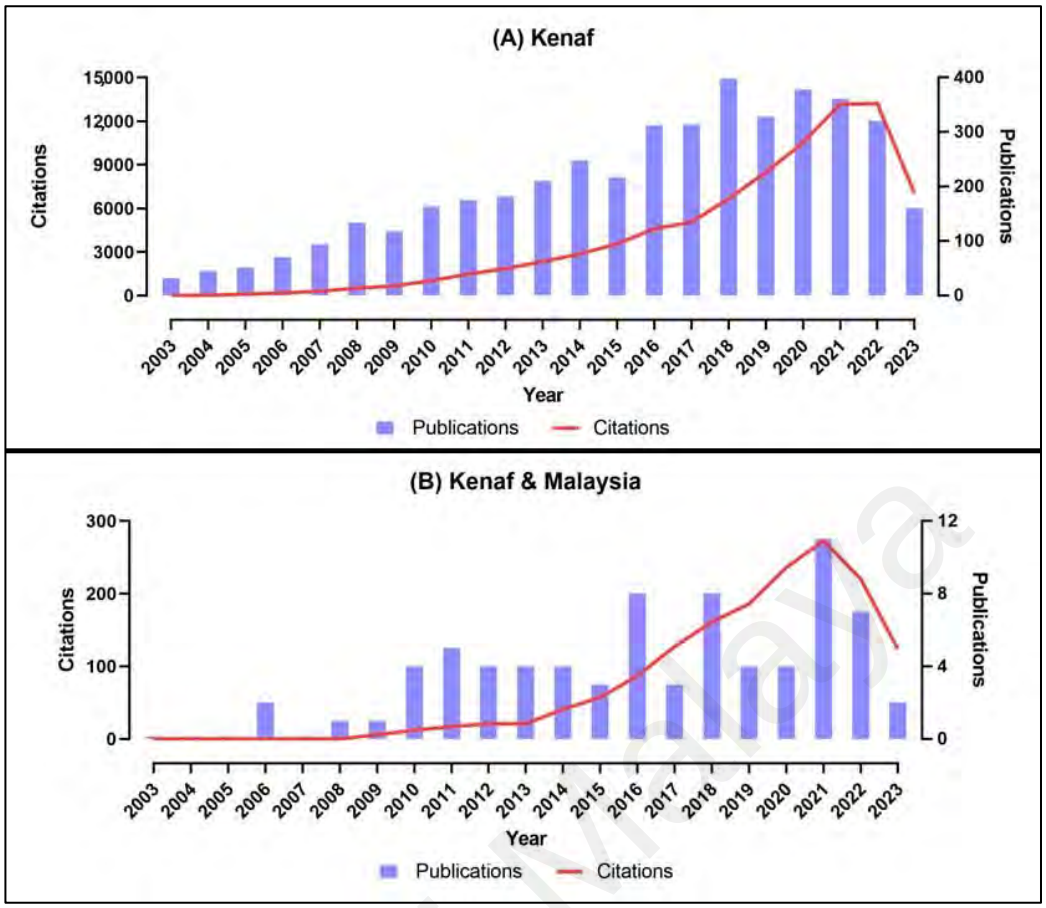

Figure 2. 2: Publication and citation trends using the keywords; (A) Kenaf (B) Kenaf and Malaysia. Data retrieved from Web of Science.

At first, kenaf plantation began in 2004 as a familiarisation crop mostly in the East side of Malaysia, Kelantan and Terengganu on BRIS (Beach Ridges Interspersed with Swales) soil before being cultivated nationwide for commercial purposes in 2010. The introduction of kenaf into the country concurrently served as an alternative to the tobacco plant which was also a part of the government's strategies to counter the price and import duties reduction of tobacco (Tan et al., 2017). The initiation of the ASEAN Free Trade Area (AFTA) in 2010 had negatively affected the marketability of Malaysian tobacco farmers against the neighbouring countries. It was revealed that the cost of production in Malaysia was nearly doubled compared to other tobacco producers such as Thailand and Indonesia (Edeerozey et al., 2007; Mossello et al., 2010). Thus, kenaf has been viewed as the potential plant to replace tobacco.

2.4.2 Current status

As of 2021, kenaf is being planted in Malaysia for two reasons; seed production, and fibre and crop production (LKTN, 2021). Throughout the country, kenaf cultivar V36 is selected for commercial cultivation because of its excellent growth rate and yield, where the plant needs approximately 90 to 150 days, depending on the purpose of cultivation to mature for harvesting. For seed production, kenaf cultivation happens in the Malaysian Northern region; Kedah and Perlis which amount to almost 500 ha (Figure 2. 3).

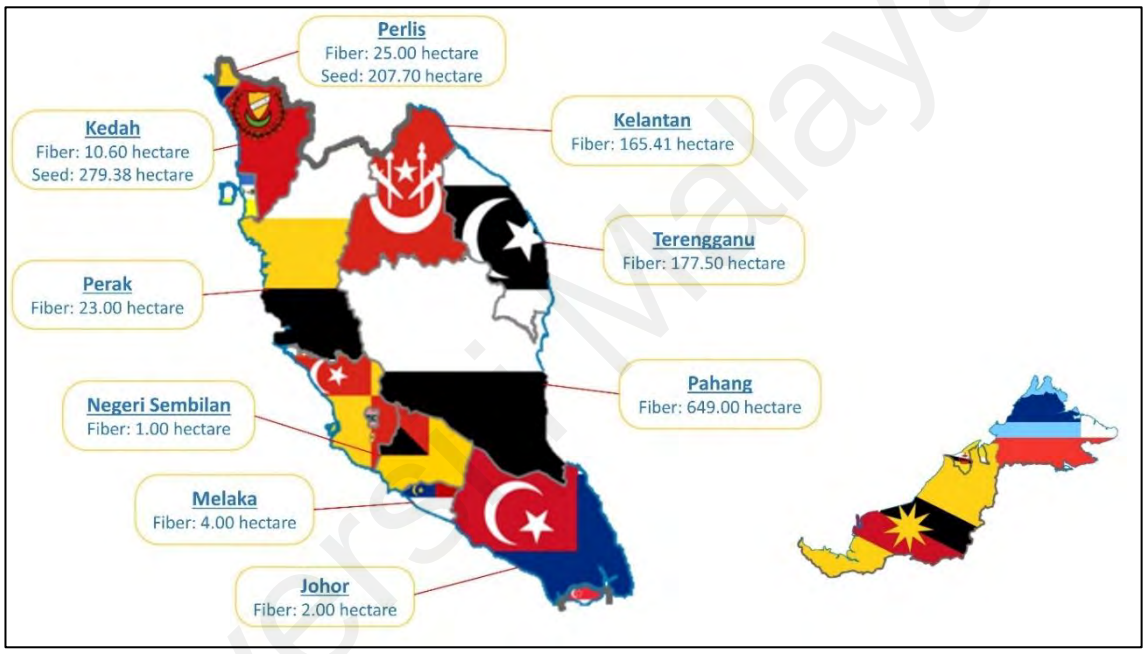

Figure 2. 3: Kenaf and seed hectarage by state (hectare).

Meantime, the total crop hectarage for fibre and core production was about 1000 ha, located in Kelantan, Terengganu, Pahang, Johor, Melaka, Perak, Negeri Sembilan and Kedah (Figure 2. 3). Because of the suitable tropical climate in Malaysia along with the adaptability of the kenaf accession V36 that is classified as a photoperiod-insensitive and late flowering cultivar, the kenaf seeds' germination rate per hectare for both of the aforementioned purposes was above 80%. This in return produced a yield/ha of 0.25 t and 10 t for seed production, and fibre and core production, respectively. For seed production,

it is sold at RM 15 (3.15 U$D) per kg, generating a gross income of RM 3750 (788.63 U$D)/ha. Meanwhile, RM 5000 (1051 U$D)/ha can be raised from the fibre and core production as the kenaf dried stems were sold at RM 500 (105 U$D)/t (LKTN, 2021).

2.5 Uses and applications

2.5.1 Traditional uses

Traditionally, kenaf is often used in the production of rope, cordage and fishnet. Besides, it is also used within the paper-manufacturing industry as it was suggested that kenaf-based paper is superior compared to wood-based paper (Ayadi et al., 2017). Furthermore, kenaf can also be a new source of healthy food whereby its extracted oil from the seeds happens to have a high amount of mono-unsaturated and poly-unsaturated fatty acids (Coetzee et al., 2008). Interestingly, even the leaves of kenaf can be used for animal forage due to their high crude protein content (Ayadi et al., 2017).

2.5.2 Old folk medicine

For generations, kenaf has long been used in India and Africa as an effective folk medicine to treat bilious conditions, puerperium and fever, thanks to its natural phytochemicals such as tannins, phenolics, alkaloids, saponins and essential oils (Ayadi et al., 2017; Kobaisy et al., 2001). For example, treatment of anaemia and fatigue can be done using kenaf's stem peelings which act as a haematic agent. Meanwhile, its leaves can be used to treat dysentery, and throat and blood disorders. Besides, its fattening seeds can also treat conditions like aches and bruises. Other than that, kenaf is regarded to be an aphrodisiac, anodyne stomachic, purgative and aperitif as well (Sim & Nyam, 2021).

2.5.3 Pharmacological uses

Kenaf can be safely considered as a multipurpose crop. With recent discoveries, its potential applications can now be expanded more than its native uses which even include various industries such as medicinal, pharmaceutical and food. Many of the reported pharmacological activities of kenaf came directly from its own extracts either from its seeds, leaves or flowers. Due to this, other than functioning as a multipurpose crop, kenaf also fits the zero-waste concept whereby every part of the crop is useful. Some of kenaf's pharmacological activities are further elaborated in Table 2. 1.

Table 2. 1: Pharmacological activities of kenaf.

Pharmacological activities	Description
Anticancer	The chemopreventive capacity of kenaf was reported when kenaf seed oil that is extracted from the supercritical carbon dioxide fluid extraction demonstrated a strong cytotoxicity (IC_{50} 200 μg/mL) against human colorectal cancer cell lines (HT29). Cells of HT29 showed characteristics of apoptosis such as plasma membrane blebbing (Ghafar et al., 2013). Besides, Adnan et al. (2020) also proved kenaf's anticancer properties when ethanolic kenaf seed extract and n-hexane kenaf seed extract documented 37% and 34% cell death of A549 (lung cancer cells), respectively.
Antibacterial	Extracts of kenaf seeds in ethyl acetate, ethanol and water also managed to show antibacterial activities through the zone of inhibition assay against *Bacillus cereus*, *Bacillus subtilis* and *Escherichia coli* (Adnan et al., 2020). Meanwhile, extracts from kenaf leaves showed its potent antibacterial activity against *Staphylococcus aureus*, *Salmonella enterica*, *Staphylococcus epidermis* and *Pseudomonas aeruginosa* (Birhanie et al., 2021; Chew et al., 2021).
Antifungal	Plant pathogenic fungi such as *Colletotrichum fragariae*, *Colletotrichum gloeosporioides* and *Colletotrichum accutatum* can also be addressed using oil that is extracted from kenaf leaves as it demonstrated fungicidal activity against the fungi (Kobaisy et al., 2001). Other than that, kenaf seed peptides produced using fermentation process showed antifungal properties against *Fusarium sp.* and *Aspergillus niger* with a minimum inhibitory concentration of 0.18 mg/mL and 1.41 mg/mL, respectively (Arulrajah et al., 2021).

Table 2.1: Continued

Pharmacological activities	Description
Antithrombotic	Cardiovascular complications can also be treated using kenaf where Hanumegowda et al. (2022) proved that the kenaf seed protein extract can serve as an anticoagulant and antiplatelet agent which ultimately will hamper the progression of thrombotic disorders. The study showed that the protein extract inhibited platelet aggregation by 56% and 34% for adenosine diphosphate and epinephrine-induced platelet aggregation, respectively. The efficacy of the protein extract was also shown in vivo whereby it managed to delay the bleeding time of mice.
Anti-hypercholesterolemic	Moreover, various types of extract from kenaf seeds also managed to reduce the amount of serum total cholesterol and malondialdehyde levels of hypercholesterolemic rats. The outcome of the study using kenaf seed extract was comparable with hypercholesterolemic rats that are treated with a commercial drug, simvastatin. Even the indicators of the deposition of cholesterols into tissues such as atherogenic index and coronary risk index were lowered using the kenaf seed extracts (Kai et al., 2015).
Anti-hyperpigmentation	Furthermore, kenaf can also tackle the beauty industry as Sim et al. (2022) tested a kenaf based-lotion, made from its seed oil and leaves extract for its anti-tyrosinase activity and anti-melanogenesis activity, which are important to prevent skin hyperpigmentation or more commonly known as the formation of dark spots. The lotion managed to reduce the level of intracellular melanin content in normal human epidermal melanocytes (NHEM). The study proposed that both seed oil and leaves extract of kenaf work together to disrupt the formation of skin-darkening pigment, melanin at both transcriptional and translational levels.
Antioxidant	Kenaf seed extracts also have antioxidant ability as they demonstrated scavenging activities in the DPPH free radical assay (73%) and hydroxyl free radical assay (67%) (Adnan et al., 2020). This antioxidant activity was also demonstrated using both leaves and flower extracts of kenaf (Birhanie et al., 2021; Ryu et al., 2017).

Table 2.1: Continued

Pharmacological activities	Description
Antihypertensive	A study done by Zaharuddin et al. (2020) showed that protein hydrolysates of kenaf that are produced through enzymatic hydrolysis managed to inhibit angiotensin I-converting enzyme (ACE) activity. ACE is responsible for activating a vasoconstrictor while inactivating a vasodilator which will subsequently raise blood pressure and encourage the development of hypertension. The protein hydrolysates also managed to reduce the systolic blood pressure of spontaneously-induced hypertensive rats. These findings suggested that kenaf can be another source of hypertensive cure.
Phytotoxic	Interestingly, oil from kenaf leaves can also manifest phytotoxic activity. This was demonstrated by Kobaisy et al. (2001) where the oil started to show its phytotoxic behaviour towards the growth of lettuce and bentgrass seedlings at 0.1 mg/mL.
Antidiabetic	Kenaf leaves can also be used to make tea which has antidiabetic properties. Referring to a study by Goh et al. (2021), kenaf leaves tea that is infused with goji berries and red dates tea for flavour enhancement showed a hypoglycaemic effect by disrupting the activity of α-amylase enzyme. The enzyme has a crucial function in starch and glycogen conversion in the system.

2.6 Plant cell suspension culture

2.6.1 Introduction to plant cell suspension culture

Cell suspension culture is one of the plant tissue culture disciplines that implies the concept of in vitro dedifferentiated cultures. Taking advantage of the totipotency of dedifferentiated plant cells, which is the capability of each cell to produce one whole new plant, the desired plant's phytochemicals can be produced through cell cultures (Mustafa et al., 2011). A plant cell suspension culture is a sterile system that consists of dispersed cell aggregates or friable unorganised mass of cells (callus) in an agitating liquid medium. The culture is initiated by introducing friable callus into a sterile liquid medium of the right nutritional composition (Taghizadeh et al., 2020). This technique is suitable especially for the mass-production of phytochemicals using bioreactors as well as for the

study of plant molecular and cellular processes (Mustafa et al., 2011). However, several requirements for this method must be highlighted. First, a must-have equipment for the commencement of this technique is an orbital shaker that provides a uniform agitation for the suspension cultures. It is highly encouraged to place the shaker in an air-conditioned room to regulate the temperature accordingly (Erkoyuncu & Yorgancilar, 2021). Moreover, Erlenmeyer flasks with wider-mouth are preferred as the culture vessels for the ease of cultivation with a working volume ranging from 20 mL to 100 mL, depending on the volume of the flasks used. The flasks should also be sealed properly with aluminium foil to ensure sterility while allowing gas exchange along with minimizing evaporation. It is also worth noting that the usage of cotton wool plugs is not advisable during the cultivation as they can cause contamination in flasks that are left on a shaker for a prolonged period (Chawla, 2002). One of the notable advantages of this technique is that it maintains a homogenous plant cell population which enables the cultured cells or callus to have direct contact with the supplied nutrients, plant growth hormones, carbon sources and vitamins. Since the cultures are continuously agitated, the friable callus or cell aggregates will be broken up into smaller clumps or single cells by the gentle shear force or pressure which will increase its surface area for better nutrient absorption activity (Büyükdinç et al., 2023). The agitation also helps with the aeration of the cells as it provides a better gaseous exchange between the liquid medium and air (Chawla, 2002). Owing to these benefits, callus grown using cell suspension culture can be regarded as superior compared to callus cultivated on a solid medium.

2.6.2 Types of plant cell suspension cultures

2.6.2.1 Batch culture

A batch culture can be defined as a suspension culture that is grown in a constant volume of the nutrient-packed medium. In this type of culture, the increment of the cell biomass through cell division and cell growth will be halted once one of the components

in the culture environment becomes a limiting factor such as nutrient or oxygen depletion. Generally, this culture will portray four phases of a growth cycle; lag phase, log or exponential phase, stationary phase and death phase (Erkoyuncu & Yorgancilar, 2021). First, when the desired cells or callus are placed or subcultured into a fresh medium, no cell division occurs where the cells are still preparing to divide. The lag phase may also be used to synthesise any metabolite of interest and also to regulate the rate of its physiological activity. The inoculum or the starting culture used will also affect the period of the lag phase. Then the culture will enter a stage where the highest rate of cell division occurs, the exponential phase. This can be defined as the rate of increase of biomass per unit of biomass concentration that is constant and can be measured. After some period of cultivation, the culture will enter the stationary phase which can also be defined as plateauing phase (Khanpour-Ardestani et al., 2015). Due to the depletion of nutrients along with the accumulation of waste products to toxic levels, the number of cell divisions equals the number of cell death which eventually makes the culture's population constant. Lastly, once the cell death exceeds the cell division activity, the population of the culture will suffer a decrease which is usually associated with detrimental nutrient availability and excessive waste accumulation. In this state, cell dry weight decreases and no net synthesis of biomass or increase in cell number happens in the culture (Chawla, 2002).

2.6.2.2 Continuous culture

In a continuous cell suspension culture, the culture will be continuously fed with a fresh medium containing nutrients. Despite this, the volume of the culture remains constant and this type of culture can be further divided into two; open continuous culture (OCC) and closed continuous culture (CCC). For OCC, the inflow of the fresh medium is balanced out by the outflow of the culture which includes the cultured callus or cells. Typically, the rate of the inflowing medium and outflow culture will be controlled, to ensure that the culture remains permanently at the sub-maximal growth rate. Once the

steady-state is achieved, the rate of new cell formation is equivalent to the rate of the outgoing cells (Khanpour-Ardestani et al., 2015). This condition can also be defined as a balanced growth whereby most of the cells in the culture are metabolically similar. However, for CCC, the inflow of the new medium is offset by the outflow of the old cultured medium only, without involving any cells or callus. The cells in the outflow medium will be separated mechanically and added back into the culture. So, the callus biomass will continue to increase throughout the entire culture period. CCC is highly useful in the case of the production of secondary metabolites that are excreted into the liquid medium such as coumarins, lignin, polysaccharides and monoterpene derivatives (Chawla, 2002).

2.6.3 Initiation of plant cell suspension culture

A typical plant cell suspension culture begins with the initiation of callus directly from the cut pieces of surface-sterilised plant or plantlets, called the explants. The explants or these differentiated plant parts can be in the form of buds, stems, leaves, roots, flowers and petioles. Usually, buds, young leaves or seedlings are chosen as the explants as they possess the actively dividing tissues called meristematic tissues, which are highly favourable for callus initiation (Zuraida et al., 2014). However, aerial plant parts (stems, leaves, seeds, bud, flowers) are preferred because they are less contaminated compared to explants retrieved from below the ground (roots). Due to this, the selection of explants will greatly influence the sterilization procedure to remove contaminants while ensuring the explants' viability. Once they are sterilised, the explants are placed on a solid medium supplemented with the right hormone combination to initiate the production of callus, a process called callusing. It may take up to 6 weeks for the callus to appear. To obtain an axenic callus, the grown callus will be removed from the explants and transferred to a new medium of similar composition to enhance callus growth to a reasonable amount (Erkoyuncu & Yorgancilar, 2021). Then, the callus will be inoculated into the liquid

medium and agitated to allow the establishment of suspension cultures for the next few days or weeks, depending on the species and the medium composition used. Interestingly, cell suspension culture can also be initiated from sterile seedlings or leaves through a mechanical method where the soft tissues will be gently grounded using a glass homogenizer. But it is crucial to note that the success of plant cell suspension culture largely depends on the initial callus, especially during callus initiation. Even the growth performance and friability of the callus produced can differ because of the type of explants used (Chawla, 2002; Mustafa et al., 2011).

2.6.4 Growth measurement of plant cell suspension cultures

To ensure the reproducibility of plant cell suspension culture growth either for experiment or industrial purposes, the measurement of growth parameters is crucial. Generally, the growth of a plant cell suspension culture can be done in various ways according to the desired parameters such as the number of cells, the cell mass, the volume of cells and many more (Taghizadeh et al., 2020). Some of the simplest and most practical growth measurement methods will be described briefly. First, it can be done by measuring the fresh weight (FW) or the dry weight (DW) of the cultured cells. Implying this technique involves harvesting the cultured cells to measure the FW or DW of cells per volume of cell suspension culture. Due to its simplicity, this method is often chosen to be used as the growth parameters for plant cell suspension culture. Besides, the growth of the culture can also be monitored based on the number of cells. Since the process of cell division causes the number of cells to increase, this can be the parameter to be used to oversee the culture's growth over some time (Pan et al., 2020). This can be done with the help of a haemocytometer whereby the number of cells will be counted per volume of suspension. Next is through a method called packed cell volume (PCV). Throughout the cultivation, when the number of cells increases, the volume occupied by cells per mL of suspension culture also increases which can be another parameter for growth

measurement. PCV is determined after the cells are allowed to be sedimented which can be assisted by centrifugation. So the PCV is the resulting volume of pellet per volume of the culture which is normally expressed as a percentage (Mustafa et al., 2011).

2.6.5 Growth media

The composition of a growth medium is crucial in affecting the outcome of the plant cell suspension culture. Depending on the purpose of the culture along with the species or genotype used, the medium may be altered according to their needs as different in vitro cultures such as for plants, plant cells, tissues, callus and protoplasts require specific medium compositions. Generally, a typical plant cell suspension culture medium will consist of carbon sources, inorganic nutrients (macroelements, microelements) vitamins, plant growth regulators (PGR) and water. This section justifies the selection of carbon source (sucrose concentration) and plant growth hormones as one of the cultivating parameters chosen for the optimization study (Table 2. 2).

Table 2. 2: Summary of growth media optimization

Growth media composition	Recommendation	Need for optimization (Yes/No)	References
Carbon source	Sucrose usage is 2-5% of the working volume	Yes	(Chawla, 2002)
Inorganic nutrients	Included in the MS medium	No	(Mustafa et al., 2011)
Plant growth hormones	NAA and 2,4-D are chosen as they can induce the formation of kenaf callus within the range of 0 – 2 mg/L	Yes	(Sultana et al., 2016a)
Vitamins	Included in the MS medium	No	(Chawla, 2002; Mustafa et al., 2011)
pH	Best working pH between 5.75 – 5.85	No	(Chawla, 2002; Mustafa et al., 2011)

2.6.5.1 Carbon source (s)

Carbon source or energy source typically comes in the form of glucose, sucrose and fructose. The standard carbon source that is widely used in plant tissue culture is either sucrose or glucose. This is because fructose is less efficient. The sucrose within the medium will be converted into glucose and fructose, where the former will be utilised first, followed by the latter. The general concentration of sucrose that is used in the medium ranged between 2% and 5% of the total working volume. Other carbon sources such as lactose, maltose, galactose and starch were found to be inferior compared to sucrose and glucose. Other than that, the media can also be included with myo-inositol which theoretically can improve cell growth (Chawla, 2002). Therefore, the right amount of carbon source (sucrose) must be determined experimentally for the kenaf callus cell suspension culture to achieve an optimum process. In addition, since the carbon source is the major component that can be found in the culture medium, its concentration needs to be optimized which in return will save cost and resources for any future cultivation of the intended species (Mamdouh & Smetanska, 2022).

2.6.5.2 Inorganic nutrients

Inorganic nutrients which are also known as mineral elements are crucial for plants. For instance, nitrogen is an important building unit of amino acids, nucleic acids and proteins, whereas calcium and magnesium are vital for plant cell walls and chlorophyll, respectively. Similarly, certain enzymes require elements like iron, zinc and molybdenum to properly function. Other than the well-known elements; carbon, hydrogen, nitrogen and oxygen, there are another 12 more elements that are deemed to be essential for plant growth (Zuraida et al., 2014). These inorganic nutrients can be divided into two categories. Those that are required in a concentration of more than 0.5 mmol/L are considered macroelements (nitrogen, potassium, phosphorus, calcium, sulphur, magnesium). Meanwhile, anything less than the aforementioned concentration such as

iron, manganese, boron, copper, zinc, iodine, molybdenum and cobalt are known as microelements. Fortunately, all the inorganic nutrients can be supplied into the medium using the commercially available Murashige and Skoog (MS) medium as it not only contains the essential nutrients, but also the actual and right concentrations of the inorganic nutrients (Chawla, 2002; Mustafa et al., 2011).

2.6.5.3 Plant growth regulators (PGR)

Plant hormones are organic compounds that are naturally produced in plants that play a crucial role in growth and development. These hormones usually work in a minute quantity and are active at their site of production. Apart from these naturally derived compounds, synthetic compounds that can mimic the actions of plant hormones are called plant growth regulators (PGR), which are heavily used in plant tissue culture. However, the optimum concentration of PGR depends on plant species and genotype, and must be determined experimentally (Khanpour-Ardestani et al., 2015). Generally, there are two classes of PGR and each class will bring out different effects. They are known as auxins and cytokinin. Auxins such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), indole acetic acid (IAA) and indole butyric acid (IBA) are important for the induction of cell division, cell elongation, formation of callus and adventitious roots. Auxins usually inhibit the formation of the shoot. Meanwhile, for cytokinin, it is often used to stimulate growth and development along with promoting shoot growth. Some examples of cytokinin are kinetin, 6-benzyl amino purine (BAP) and 6-benzyladenine (BA) (Chawla, 2002; Mustafa et al., 2011). In this study, the combination of NAA and 2,4-D is chosen as the PGR that will be used. This is because the aforementioned hormones can induce callus formation of kenaf. Since both of the hormones are auxins, the range of concentration that will be used is between 0 mg/L and 2 mg/L (Sultana et al., 2016b).

2.6.5.4 Vitamins

Naturally, plants can produce vitamins on their own for growth and development. However, in-vitro-cultured plant cells need to be supplied with vitamins. Some examples of vitamins that are important for plants include vitamin B1 (thiamine), nicotinic acid, vitamin B6 (pyridoxine), inositol, biotin, folic acid, ascorbic acid and many more. The vitamins can be supplemented into the medium in various forms and concentrations. Thankfully, the widely commercially available MS medium is now included with vitamins (Chawla, 2002; Mustafa et al., 2011).

2.6.5.5 pH

The pH of the prepared media will determine various vital aspects of the culture that includes the structure and the activity of the biological macromolecules. It is stated that any pH higher than 7.0 or lower than 5.0 will generally stop the growth and development of the culture (Pan et al., 2020). After the process of autoclaving, it is also expected that the pH of the media will vary and can fall by 0.3 to 0.5 units from the original pH. In general, it is recommended to adjust the pH of the media to be in the range between 5.75 and 5.85 before sterilization because the uptake of nutrients by the plants is good at that particular pH range (Chawla, 2002; Mustafa et al., 2011).

2.6.6 Growth conditions

Other than the medium composition for the establishment of plant cell suspension cultures, the growth conditions which can also be termed physical factors shall not be neglected. This is because controllable factors such as agitation rate, temperature and light will have effects on the outcome of the cultures. This section also highlights the need for optimizing the agitation rate for kenaf cell suspension culture (Table 2. 3).

Table 2. 3: Summary of growth conditions optimization

Growth conditions	Recommendation	Need for optimization (Yes/No)	References
Agitation	Inconsistent agitation rate depending on the species	Yes	(Chawla, 2002)
Light	Total darkness to induce callus formation	No	(Odahara et al., 2020)
Temperature	Culture at 25 °C for optimum growth	No	(Linh et al., 2021)

2.6.6.1 Agitation

Agitation can be regarded as one of the most important factors in conducting plant cell suspension culture. Its purpose is to supply a mild and gentle pressure on the cell aggregates, that breaks them into smaller pieces which eventually increases their surface area for better nutrient absorption. The agitation also helps in maintaining a uniform distribution of cells, callus clumps and nutrients to achieve homogeneity. Besides, the agitated and circulated medium will provide a good gas exchange for the cultured cells (Taghizadeh et al., 2020). However, since the cultured plant cells may be sensitive to shear and due to this, a higher shaker speed may result in negative effects on their viability. So it is crucial to set the agitation rate just enough for the culture to reach homogeneity without causing any unnecessary damage to the plant cells (Chawla, 2002; Mustafa et al., 2011). Since there is no report regarding kenaf cell suspension culture, it is crucial to determine its optimum agitation in this study.

2.6.6.2 Light

Cell suspension culture could also be influenced by the light whether the culture is conducted in total darkness, periodic dark, light intervals or continuous light. It all depends on the purpose of the cultivation because even a short exposure of the culture to the light may trigger specific biosynthetic pathways. So, early precautions should be taken if the cultures were to be done in total darkness. Moreover, special lamps that only emit

light at a particular wavelength that shall not distress the plant cells are also available and can be considered when conducting plant cell suspension culture (Chawla, 2002; Mustafa et al., 2011). For inducing and maintaining kenaf callus, Odahara et al. (2020) suggested to incubate the culture in total darkness, to prevent the process of organogenesis.

2.6.6.3 Temperature

The temperature of the culture will affect its growth performance. Any temperature above the recommended temperature will halt the culture's cell division which will negatively impact its resulting callus biomass. If the temperature is below the optimum, it may prolong the cell division cycles which eventually will lengthen the log phase period, an undesirable condition, especially for the industry (Linh et al., 2021). Linh et al. (2021) found that the best temperature for plant cell suspension culture is 25 °C, which is in accordance with the suggestion (24-25 °C) given by Mustafa et al. (2011).

2.6.7 Previous studies on plant cell suspension cultures

Some of the previous studies on plant cell suspension culture for the production of a variety of phytochemicals are listed in Table 2. 4. But, up to now, no studies have documented kenaf callus cultivation through cell suspension culture. This proves to be the gap that is yet to be filled in the kenaf field.

Table 2. 4: Previous studies on plant cell suspension culture

Plant	Sucrose (g/L)	Growth hormones	Agitation (rpm)	Phytochemicals	Reference
Tuberose	-	2,4-D	-	EPS	(Honda et al., 1997)
Timothy Proso millet Harding grass Rice Barley	-	2,4-D Cytokinins	-	EPS	(Sims et al., 2000)
Black bamboo	30	2,4-D BA	110	Free amino acids	(Ogita, 2005)
Lemon balm	30	2,4-D NAA BAP	110	-	(Meftahizade et al., 2010)
Costa Rican pitahaya	0-60	2,4-D Picloram	120	Betalain	(Winson et al., 2020)
Boxthorn	5-30	NAA	120	Phenolics Flavonoids	(Mamdouh & Smetanska, 2022)
Wheat	-	2,4-D Abscisic acid	140	EPS	(Murtazina et al., 2022)

2.7 Plant polysaccharides

2.7.1 Introduction to polysaccharides

Polysaccharides are a class of naturally occurring macromolecules, alongside proteins, nucleic acids and lipids. These abundant natural biopolymers are made from individual monosaccharide units that are linked together through glycosidic bonds. Polysaccharides can be retrieved from natural sources such as plants, algae, bacteria and fungi (Mohammed et al., 2021; Shi, 2016). Interestingly, due to their unique and various structural features that are formed from the interconnection at several points of attachment, leading to either branched or linear structures, polysaccharides have the highest capacity for carrying biological information. Unlike nucleic acids and amino acids which can be interconnected in linear chains only. Despite their structural complexity, polysaccharides backbone chains are often glucan, mannan, xylan, fructan, galactan, pectin and galactomannan (Shi, 2016). At the cellular level, polysaccharides function as

either an energy reserve (starch) or structural components (cellulose) of the membrane or cell wall of organisms (Shi, 2016). Besides, polysaccharides which are also the third major class of biopolymers, have important roles in various physiological processes and tumour metastasis (Zhang & Wang, 2015). Other than that, they also provide protection, adhesion, and stimuli responsiveness along with having responsibilities in the immune system, pathogenesis prevention, blood clotting and therapeutic effect (Ngwuluka, 2018). In fact, with more attention given to the study of polysaccharides, polysaccharides also have many beneficial biological activities such as antioxidant, antitumour, anti-hyperglycaemic, and immune regulation activities (Shi, 2016). Even the polysaccharides extracted from kenaf leaves were proven to have antibacterial and antioxidant activities (Birhanie et al., 2021).

2.7.2 Classification of polysaccharides

Commonly, polysaccharides can be distinguished according to their chemical structures which are made of monosaccharide residues, bonded through glycosidic linkages. The glycosidic bond is formed through the anomeric carbon atom between the glycosidic bond donor and the acceptor, forming either branched or linear chains. Polysaccharides can also be covalently bonded to other structures such as amino acids, peptides and lipids. Polysaccharides that consist of the same monosaccharides are known as homoglycans or homopolysaccharides. Meanwhile, if they are made of different monosaccharides, they are known as heteroglycans or heteropolysaccharides (Mohammed et al., 2021). For instance, glucans are glucose homopolysaccharides whereas mannose homopolysaccharides are known as mannans (D'Ayala et al., 2008). Some of the most common components of polysaccharides include glucose, galactose, arabinose, mannose, xylose and arabinose. There are also some monosaccharide derivatives found in polysaccharides that are bonded with simple sugar acids (glucuronic and iduronic acid) and amino sugars (glucosamine and galactosamine) along with their

derivatives (N-acetylneuraminic acid and N-acetylmuramic acid). Moreover, polysaccharides can also be classified based on their monosaccharide composition, the length of their chains and the pattern of the chains either branched or linear (Mohammed et al., 2021). They can also be differentiated based on their charges (polyelectrolytes). For example, chitosan is deemed to be a positively charged polysaccharide while alginate, hyaluronic acid, pectin and alginate are negatively charged polysaccharides (Liu et al., 2008). Furthermore, polysaccharides can also exist either in the cell wall of plants and animals or outside of them. If they are within the cell wall, they are known as intracellular polysaccharides. But if they can be found outside of the cell wall, they can be called extracellular polysaccharides or exopolysaccharides (Shi, 2016).

2.7.3 Plant exopolysaccharides

The secretion of exopolysaccharides (EPS) into the growth medium is common in plant cell suspension cultures. These secreted polysaccharides can be classified based on their major molecular structures such as glucans, xyloglucan, galacturonan, arabinogalactan, rhamnogalacturonan, glucomannan, fructan and many more (Honda et al., 1997). Interestingly, EPS that are found in the medium is representative of the polysaccharides that are still intact in the cell walls of cultured plants (Honda et al., 1997; Sims et al., 2000; Webster et al., 2008). Even if the EPS collected differs from those in the primary cell walls, it is because the cultured cells are not fully dedifferentiated (Webster et al., 2008). The production of EPS happens during cell wall expansion. During this process, native enzymes within the cell wall of plant cells participate in the hydrolysis of glycosidic linkages of the polysaccharide matrix. Eventually, this will loosen the cell wall and cause expansion while the products of the hydrolysis, the EPS, are released into the medium (Honda et al., 1997). Many studies have been conducted on the production of EPS through cell suspension culture of different plant species such as aloe vera, pear, rice and grain (Kim et al., 2012; Sims et al., 2000; Webster et al., 2008). For instance, cell

suspension culture of aloe vera using growth regulators like 2,4-D, kinetin and NAA managed to produce EPS that is similar to the structure of β-1,4-glucomannan (Kim et al., 2012). Meanwhile, xyloglucan was found to be the major component of the secreted polysaccharide of *Pyrus communis*, pear during suspension cultures (Webster et al., 2008). Even the EPS from the broth of suspension culture of mentha and rice was predominated by xyloglucan (Maruyama et al., 1996; Sims et al., 2000). In addition, EPS from the culture medium of wheat callus that is supplemented with abscisic acid was found to contain glucose as its major monosaccharide composition, indicating the EPS is glucan (Bishimbayeva et al., 2017). On top of that, Murtazina et al. (2022) proved that the extracted EPS from wheat cell suspension culture can suppress the proliferation of colon cancer cells. Interestingly, *Gossypium hirsutum L.* (cotton), a family of kenaf was found to have pectin and xyloglucan as the main composition of its excreted polysaccharides (Buchala et al., 1989). However, to date, there is no report regarding the EPS produced from kenaf cell suspension culture, which is why there is still a knowledge gap yet to be filled. However, an early indication of kenaf's polysaccharides potency was demonstrated by Birhanie et al. (2021) where they recorded antimicrobial and antioxidant activities of the extracted polysaccharides from kenaf's leaves.

2.8 Optimization using response surface methodology (RSM)

In this study, the optimization of the selected culturing parameters of the kenaf callus cell suspension culture was conducted using the response surface methodology (RSM) method that follows the Box-Behnken experimental design. When it comes to optimizing the chosen parameters, RSM is a great tool that offers model building, designing experiments and statistical analysis (Kalil et al., 2000). RSM offers the ability to investigate the individual and combined effect of independent parameters on the dependent parameter of an experiment. It also suggests what treatment combinations should be avoided and where the model borders should lay for the study (Boey et al.,

2013; Chellamboli & Perumalsamy, 2014). RSM is also an iterative investigational method that focuses on unravelling the role of the individual cultivating parameter along with the effect of their interaction with one another in determining the final output of the experiment (Hasni et al., 2017). For this dissertation, RSM uses a mathematical model that stimulates the optimization of the chosen cultivating parameters (sucrose concentration, plant growth hormone concentration and agitation) for maximizing the production of KCB and EPS. Other benefits of employing this technique include cost-saving, and lowering the number of experiments needed to be performed which eventually helps in saving much time, resources and effort.

2.9 Summary of literature review

In short, the plant of interest for this study, kenaf, has tremendous potential for further development in Malaysia. This is evident from the increase in its production, adoption, and research trends. Additionally, its previously reported pharmacological activities underscore its significant utility in various industries, including pharmaceuticals, cosmeceuticals, and food. Therefore, cultivating kenaf through cell suspension culture to produce kenaf callus biomass (KCB) and its exopolysaccharide (EPS) as a new source of bioactive compounds is essential. However, optimizing its culturing conditions is crucial to maximize KCB and EPS production. Based on the compiled literature of previous works, crucial factors such as sucrose concentration (g/L), agitation rate (rpm), NAA and 2,4-D concentrations (mg/L) were selected to be tested further in this study. Meanwhile, for the EPS compositional analysis, the type of the polysaccharide excreted during the kenaf cell suspension culture can be determined based on the major component that makes up the polysaccharide backbone itself.

CHAPTER 3: METHODOLOGY

The overall workflow of the methodology used in this study is briefly described in Figure 3. 1.

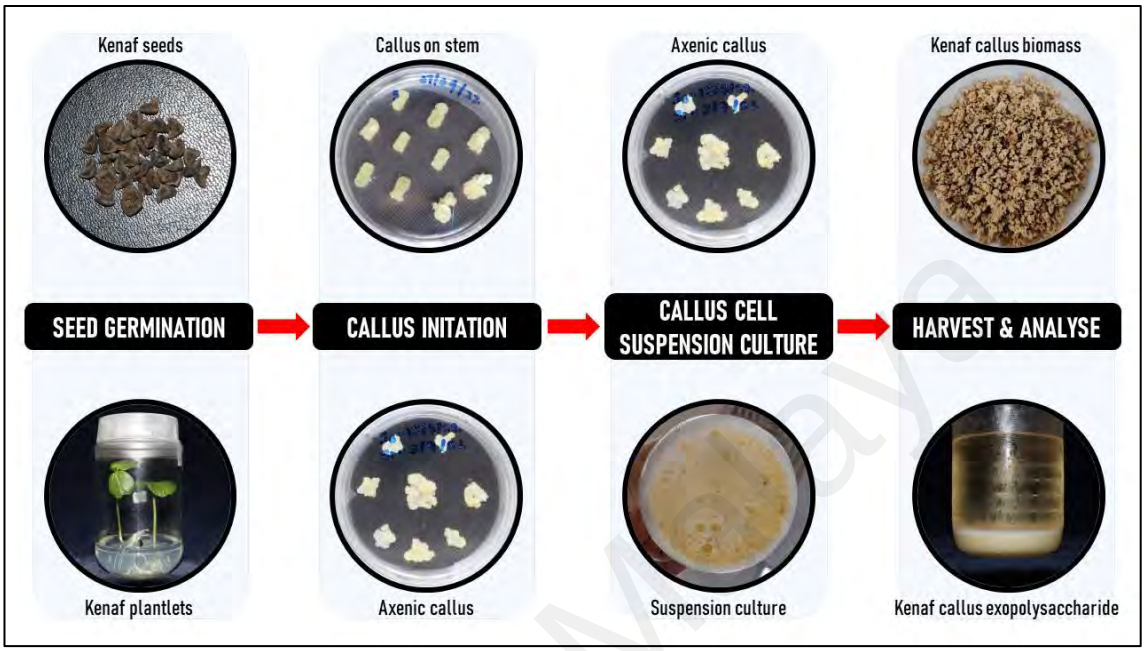

Figure 3. 1: General overview of the methodology workflow of this study.

3.1 Plant material and seed germination

Kenaf seeds were purchased from Lembaga Kenaf and Tembakau Negara (LKTN), Kuala Lumpur, Malaysia. The seeds were sterilised and germinated according to (Odahara et al., 2020) with slight modifications. Kenaf seeds were rinsed with tap water for 2 h followed by a 30-second wash using a commercial detergent. Then the seeds were sterilised with 70% (v/v) ethanol for 20 s and then with 50% sodium hypochlorite (v/v, Clorox®) for 15 to 20 min before being rinsed with 2 L sterilised distilled water. After the seeds were air dried, they were placed on the seed germinating medium (pH 5.7 – pH 5.8) containing Murashige-Skoog (MS, Duchefa Biochemie) medium with 3% sucrose (w/v, Duchefa Biochemie) and 0.3% gelrite (Duchefa Biochemie). The seeds were

cultivated at 25 °C under dark condition in an incubation room (Agro-Biotechnology Institute, Serdang).

3.2 Callus induction and proliferation

14 days old kenaf seedlings were used to initiate the growth of callus (Figure 3. 2A). The upper stem of the kenaf seedlings was cut into 3 mm – 5 mm individual fragments before being placed on callus induction medium containing MS medium with 3% sucrose, 0.3% gelrite along with the addition of plant growth regulators (PGR); 0.2 mg/L 1-Naphthaleneacetic acid (NAA) and 0.2 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) which then were grown under the same condition as seed germination. The two weeks old grown callus on the kenaf stem (Figure 3. 2B) was subcultured onto new media by scrapping the callus from the explants gently to obtain an axenic callus culture. The axenic callus was allowed to grow for another 4 weeks (Figure 3. 2C).

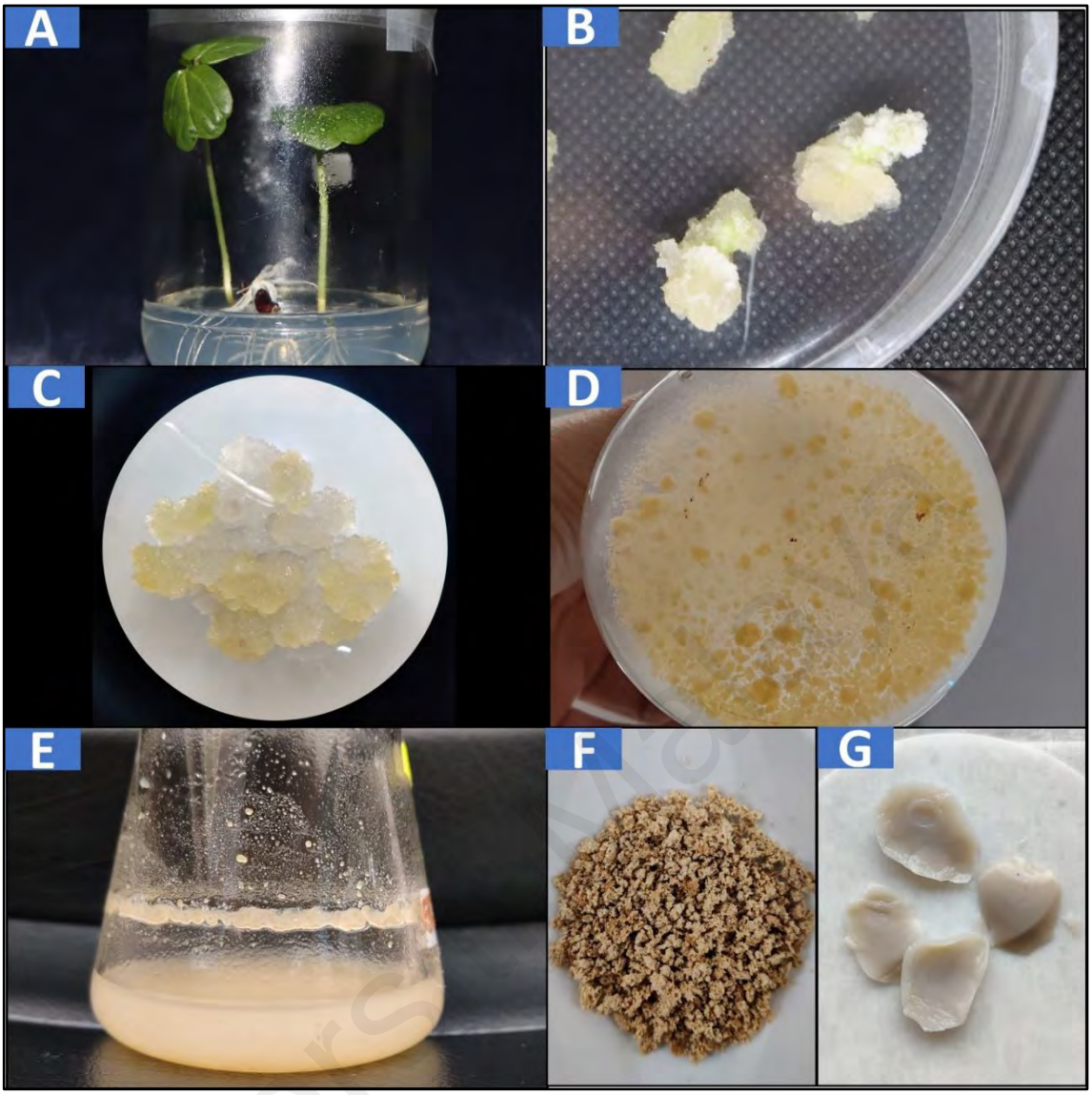

Figure 3. 2: (A) Germinated kenaf seedlings. (B) Callus grown on kenaf stem. (C) Axenic kenaf callus. (D) & (E) Kenaf callus in cell suspension culture. (F) KCB. (G) EPS.

3.3 Cell suspension culture

3.3.1 Preliminary study: Kenaf's cell suspension culture growth curve

The 6 weeks old grown callus (Figure 3. 2C) was subjected to the cell suspension culture according to (Kumar et al., 2015; Mustafa et al., 2011) with slight alterations. 0.1g of the grown callus was homogenised gently before being transferred into 100 mL shake flasks containing liquid medium (10 mL) that contains the same medium composition as the callus induction medium except for gelrite. The kenaf callus was cultivated for 30

days at 25°C under dark conditions (Linh et al., 2021). KCB and EPS of the cultures were harvested every two days and this was done in triplicates. The highest output of KCB and EPS was found to be on the 22nd day of the cultivation.

3.3.2 Main study: Optimisation of kenaf cell suspension culture parameters

The 6 weeks old grown callus (Figure 3. 2C) was subjected to the cell suspension culture according to (Kumar et al., 2015; Mustafa et al., 2011) with slight alterations. 0.5 g of the grown callus were homogenised gently before being transferred into 250 mL shake flasks containing liquid medium (50 mL) that contains the same medium composition as the callus induction medium except for gelrite (Figure 3. 2D, E). The kenaf callus were cultivated at 25°C under dark conditions. KCB and EPS of the cultures were harvested on the 22nd day of the cultivation based on kenaf cell suspension culture's growth curve that was established in the preliminary study (Methodology 3.3.1).

3.4 Kenaf callus biomass (KCB) separation and dry weight estimation

KCB (Figure 3. 2F) was separated from the cultured filtrate using the Buchner filter system and an air pump (Wan-Mohtar et al., 2021). The filtered sample was dried at 30 °C where its dry weight was determined once the weight of KCB is constant using a pre-dried filter paper that is based on Equation 3.1.

$$\text{KCB(g/L)} = \frac{\left(\text{Dried filter paper (g)} + \text{ dried sample (g)}\right) - \text{ Dried filter paper (g)}}{\text{Working volume (mL)}} \times 1000 \quad (3.1)$$

3.5 Kenaf exopolysaccharide (EPS) separation, extraction and dry weight estimation

EPS (Figure 3. 2G) was separated using an improvised method (Balamurugan et al., 2021; Usuldin et al., 2021) where it was extracted and precipitated from the obtained filtrate (cultured broth) with the help of a Buchner filter system. The EPS in the filtrate

was precipitated by adding 95% (v/v) ethanol at a 1:4 ratio before being incubated for three days at 4 °C. Then the precipitated EPS was isolated by centrifugation (10,000 rpm, 10 min), where the formed pellet was be collected, dried and weighed accordingly (Günter et al., 2004). The dry weight of the EPS was determined based on Equation 3.2.

$$\text{EPS(g/L)} = \frac{\left(\text{Dried falcon tube (g)} + \text{ dried sample (g)}\right) - \text{ Dried falcon tube (g)}}{\text{Working volume (mL)}} \times 1000 \quad (3.2)$$

3.6 Optimisation of kenaf cell suspension culture parameters using response surface methodology (RSM)

The MS medium concentration, initial pH and temperature were kept constant at 4.41 g/L, pH 5.7-5.8 and 25°C respectively throughout the entire study. Kenaf callus cultivation in cell suspension culture requires both physical and chemical adjustments to be a success. The selected parameters are chosen based on the justification given by previous studies which are briefly explained in Table 3. 1.

Table 3. 1: Brief justification of the chosen parameters for kenaf callus optimization study through cell suspension culture

Factors	Importance	References
Sucrose concentration	Carbon source (source of food for the kenaf cells to grow and multiply)	(Chawla, 2002)
Agitation	Provide uniform culture distribution of the supplied nutrients along with a good aeration and nutrient transfer	(Chawla, 2002; Mustafa et al., 2011)
NAA concentration 2,4-D concentration	Combination of both hormones are important for inducing and maintaining the formation of kenaf callus	(Chawla, 2002; Mustafa et al., 2011; Sultana et al., 2016b)

To optimise the production of KCB and EPS (g/L) along with the selected culturing parameters in cell suspension culture; sucrose concentration (10 – 50 g/L), agitation (50 – 150 rpm), and NAA and 2,4-D concentrations (0 – 2 mg/L), a complete factorial central

composite design (CCD) for response surface methodology (RSM) was utilised using Design Expert 13.0 software. In Table 3. 2, the levels and the range of the parameters are shown for this study based on the suggestions and recommendations stated in the literature review (Table 2. 2 and Table 2. 3).

Table 3. 2: Experimental range and levels of independent parameters

Independent parameters	Range and levels		
	-1	0	1
Sucrose (g/L)	10.0	30.0	50.0
Agitation (rpm)	50.0	100.0	150.0
NAA (mg/L)	0.0	1.0	2.0
2,4-D (mg/L)	0.0	1.0	2.0

Thirty experiments were generated using CCD in Design Expert 13.0 software as listed in Table 3. 3. All experiments were carried out in triplicates to guarantee the maximum yield of responses whereby it was analysed using ANOVA (analysis of variance) and plotted as (3D) three-dimensional models. The influence of the parameters upon the responses were done through an empirical model that is according to the second quadratic-order equation as follows:

$$Y=b'_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} {x_i}^2 + \sum_{i=1}^{n} \sum_{j=i}^{n} b_{ij} x_i x_j \quad (3.3)$$

Where; Y = predicted response, b'_0 = the constant coefficient, b_i = the linear coefficient, b_{ii}= the quadratic coefficient, b_{ij} = the interaction coefficient, and $x_i x_j$ = the coded values (Balamurugan et al., 2021; Usuldin et al., 2023).

Table 3. 3: Illustrations of the levels and range used in this study

Run	Factor 1	Factor 2	Factor 3	Factor 4
	A: Sucrose (g/L)	B: Agitation (rpm)	C: NAA (mg/L)	D:2,4-D (mg/L)
1	50	150	2	2
2	30	150	1	1
3	50	150	0	0
4	30	100	1	1
5	30	100	0	1
6	50	50	2	0
7	10	50	0	2
8	30	100	1	0
9	50	50	2	2
10	50	150	2	0
11	50	50	0	0
12	30	100	1	1
13	10	50	2	2
14	50	50	0	2
15	50	150	0	2
16	30	100	1	2
17	30	100	1	1
18	30	100	1	1
19	10	50	0	0
20	50	100	1	1
21	30	100	2	1
22	30	100	1	1
23	10	150	0	2
24	30	50	1	1
25	10	100	1	1
26	30	100	1	1
27	10	50	2	0
28	10	150	0	0
29	10	150	2	0
30	10	150	2	2

A: Sucrose concentration (g/L), B: Agitation (rpm), C: 1-Naphthaleneacetic acid (NAA, mg/L) and D: 2,4-Dichlorophenoxy acetic acid (2,4-D, mg/L).

3.7 Statistical analysis

All experiments were conducted in triplicates where the corresponding mean and standard deviation were measured using the GraphPad Prism 9 software (version 9.0.0). If the error bar is absent, it indicates that it is shorter than the size of the symbol. Meanwhile, the analysis of variance (ANOVA) for the CCD of the RSM was done using

the Design Expert 13.0 software (StatEase®). The p-values (p<0.05) were used to show the statistical significance levels for the tested coefficient models (Usuldin et al., 2023).

3.8 Characterisation of EPS using Fourier-transform infrared spectroscopy (FTIR)

FTIR analysis for EPS was conducted using Agilent Cary 630 which is equipped with diamond ATR (Attenuated Total Reflectance). The data generated was collected using MicroLab software (Usuldin et al., 2020) and analysed according to Pavia et al. (2001) and Hong et al. (2021).

CHAPTER 4: RESULTS & DISCUSSION

4.1 Kenaf callus suspension culture growth curve

The kenaf callus suspension culture was conducted for 30 days whereby the produced KCB and EPS were collected every two days in triplicates. Typically, the harvesting time of a suspension culture occurs during the end of the log phase right before the culture enters the stationary phase (Mustafa et al., 2011). Based on Figure 4. 1, the cultivation of kenaf callus through cell suspension culture was slow during the first 18 days which indicated the occurrence of the extended lag phase.

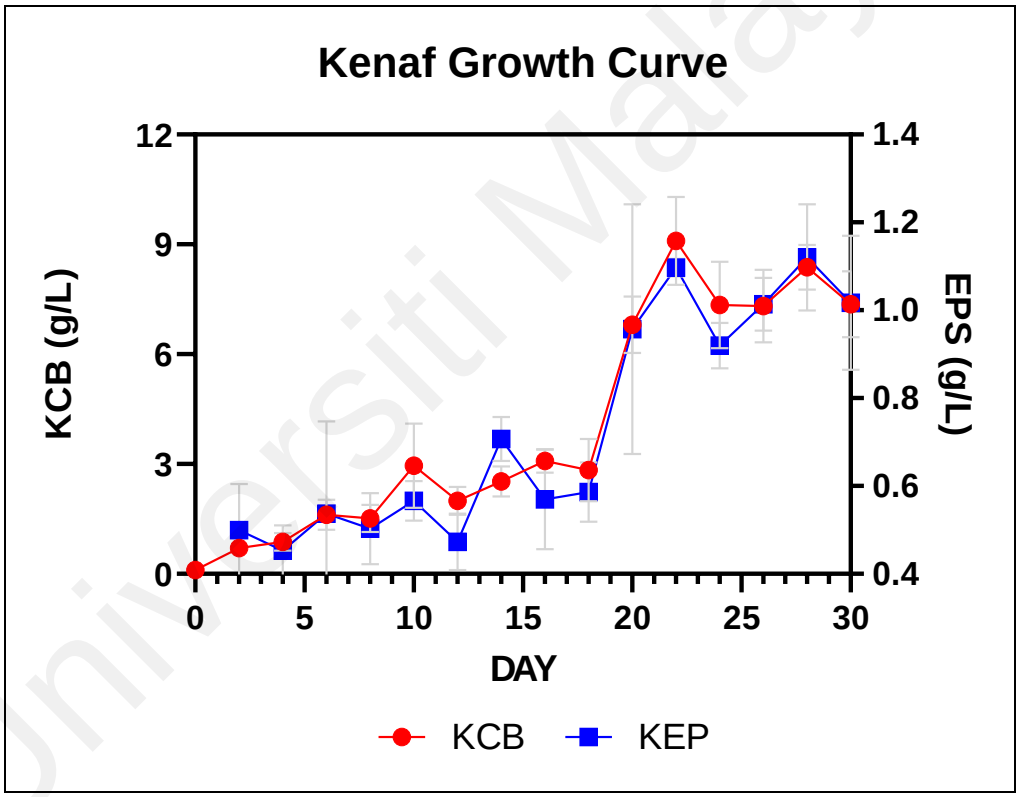

Figure 4. 1: Growth curve of kenaf callus in cell suspension culture

This can be seen by the low and stagnant production of the KCB and EPS. Then, the kenaf callus experienced exponential growth from Day 18 to Day 22 whereby both of the KCB and EPS showed an increasing production trend and this can be categorised as the log phase. However, after Day 22, the growth of kenaf callus and the production of its

EPS decreased, an indication of the transition phase which is then followed by a stationary phase that can be depicted by the plateauing of the plot in Figure 4. 1. In terms of the highest amount of KCB produced, it occurred during Day 22 (9.09 g/L ± 3.19) of the cultivation. Meanwhile, for EPS, the amount secreted on Day 22 of the cultivation was 1.10 g/L ± 0.02. Usually, the maximum quantity of KCB and EPS can be observed during the end of the log phase or initial stationary phase (Wan-Mohtar et al., 2016). This suggested that Day 22 of the kenaf callus cell suspension culture manifested the criteria of the end of log phase (Figure 4. 1), which should be taken as the harvesting time of the cultivation.

4.2 Kenaf callus biomass (KCB) production optimization

For the optimization of KCB and EPS production, 30 distinct sets of cultivating conditions were tested using RSM (Table 4. 1). The influence of sucrose concentration, agitation speed, NAA and 2,4-D concentrations on the production of KCB and EPS of kenaf was assessed over 22 days for each experimental setup, where each pre-determined condition was conducted in triplicates.

Table 4. 1: RSM with CCD matrix and the outcomes of the KCB and EPS production

Run	Experimental variables				Responses			
	Sucrose (g/L)	Agitation (rpm)	NAA (mg/L)	2,4-D (mg/L)	KCB (g/L)		EPS (g/L)	
					Actual	Predicted	Actual	Predicted
1	50	150	2	2	9.87	9.32	1.70	1.77
2	30	150	1	1	7.15	6.87	0.95	0.76
3	50	150	0	0	4.22	4.65	0.75	0.85
4	30	100	1	1	9.07	8.10	0.56	0.65
5	30	100	0	1	7.26	7.43	0.18	0.00
6	50	50	2	0	4.80	4.92	0.38	0.41
7	10	50	0	2	4.80	4.90	0.11	0.04
8	30	100	1	0	10.95	10.68	1.06	0.70
9	50	50	2	2	2.24	2.35	0.28	0.26
10	50	150	2	0	13.41	13.26	1.59	1.63
11	50	50	0	0	0.79	0.19	0.34	0.47
12	30	100	1	1	8.06	8.10	0.66	0.65
13	10	50	2	2	1.58	1.08	0.05	0.03
14	50	50	0	2	1.97	1.65	0.22	0.21
15	50	150	0	2	4.68	4.75	0.88	0.88
16	30	100	1	2	9.60	10.31	0.59	0.74
17	30	100	1	1	8.76	8.10	0.45	0.65
18	30	100	1	1	8.43	8.10	0.68	0.65
19	10	50	0	0	1.23	1.71	0.08	0.09
20	50	100	1	1	4.15	5.04	1.86	1.50
21	30	100	2	1	9.56	9.83	0.33	0.34
22	30	100	1	1	7.78	8.10	0.55	0.65
23	10	150	0	2	3.1	2.92	0.19	0.24
24	30	50	1	1	2.97	3.69	0.08	0.05
25	10	100	1	1	3.07	2.62	0.82	0.96
26	30	100	1	1	7.82	8.10	0.39	0.65
27	10	50	2	0	2.04	1.92	0.02	0.00
28	10	150	0	0	1.24	1.09	0.01	0.00
29	10	150	2	0	4.91	5.17	0.64	0.73
30	10	150	2	2	2.41	2.97	1.23	1.07

Table 4. 2 presents the ANOVA for the KCB optimization (Figure 4. 2). The results showed that the model was significant as the value of "Prob>F" was <0.0001 (p<0.05). The coefficient of determination (R^2 = 0.9828) indicates that the model can describe 98.28% of the response or the outcome variations. The result of the adjusted coefficient of determination (Adj. R^2 = 0.9667) implies the significance of the model that is in reasonable agreement with the predicted coefficient of determination (Predict. R^2 =

0.9201) with a difference of less than 0.2. The model can be expressed in terms of the actual experimental variables for KCB production using Equation 4.1.

$$\begin{aligned} KCB = {} & -1076246 + 0.539019 \times A + 0.206755 \times B - 2.48409\,C - 2.63263\,D \\ & + 0.001273 \times A \times B + 0.056531 \times A \times C - 0.021656 \times A \times D \\ & + 0.019363 \times B \times C - 0.006812 \times B \times D - 1.00812 \times C \times D \\ & - 0.010680\,A^2 - 0.001129\,B^2 + 0.528070\,C^2 + 2.39307\,D^2 \end{aligned} \quad (4.1)$$

Where; A = sucrose, B = agitation, C = 1-naphthalene acetic acid (NAA), D = 2,4-dichlorophenoxy acetic acid (2,4-D).

Table 4. 2: The results of analysis of variance (ANOVA) generated from the CCD quadratic model in RSM for KCB

Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Model	335.48	14	23.96	61.17	< 0.0001*
A: Sucrose	26.28	1	26.28	67.09	< 0.0001*
B: Agitation	45.35	1	45.35	115.75	< 0.0001*
C: NAA	25.75	1	25.75	65.74	< 0.0001*
D: 2,4-D	0.6198	1	0.6198	1.58	0.2277
AB	25.93	1	25.93	66.20	< 0.0001*
AC	20.45	1	20.45	52.21	< 0.0001*
AD	3.00	1	3.00	7.66	0.0144
BC	15.00	1	15.00	38.28	< 0.0001*
BD	1.86	1	1.86	4.74	0.0459
CD	16.26	1	16.26	41.51	< 0.0001*
A^2	47.28	1	47.28	120.70	< 0.0001*
B^2	20.63	1	20.63	52.67	< 0.0001*
C^2	0.7225	1	0.7225	1.84	0.1945
D^2	14.84	1	14.84	37.88	< 0.0001*
Residual	5.88	15	0.3918		
Lack of fit	4.50	10	0.4499	1.63	0.3065
Pure error	1.38	5	0.2755		
Cor total	341.36	29			
Std. Dev	= 0.6259		R^2	= 0.9828	
Mean	= 5.60		Adjusted R^2	= 0.9667	
			Predicted R^2	= 0.9201	

*significant

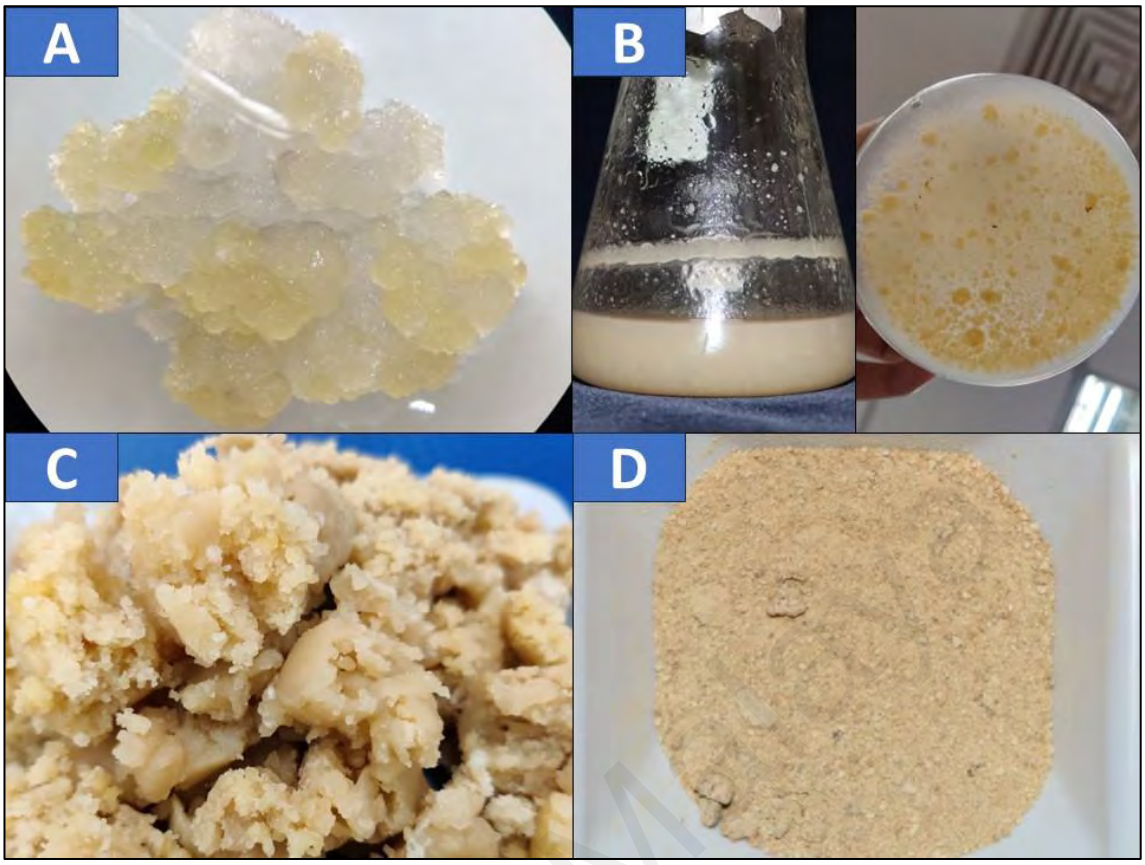

Figure 4. 2: Optimization of KCB. (A) Axenic kenaf callus. (B) KCB in cell suspension culture. (C) Filtered and harvested KCB. (D) Dried KCB.

Based on the results obtained (Table 4. 2), sucrose concentration (A), agitation (B) and NAA (C) can greatly influence the outcome of KCB production (p<0.05). Besides, the interactions between sucrose and agitation (AB), sucrose and NAA (AC), sucrose and 2,4-D (AD), agitation and NAA (BC), agitation and 2,4-D (BD), NAA and 2,4-D (CD) along with the quadratic terms of sucrose (A^2), agitation (B^2) and 2,4-D (D^2) also do have a significant (p<0.05) impact on the production of KCB through this model. However, 2,4-D concentration (D) and the quadratic term of NAA (C^2) had negative effects on the model. Based on the model, maximising the KCB production will require the usage of 40.03 g/L of sucrose, 1.87 mg/L of NAA and 0.04 mg /L of 2,4-D with an agitation speed of 129.59 rpm.

Figure 4. 3 shows the combined effects and interactions between all the experimental parameters on the production of KCB. Based on Figure 4. 3 (A-F), it is safe to say that all variables are dependent upon each other when it comes to the production of KCB. For instance, the influence of 2,4-D on KCB production with sucrose (Figure 4. 3C), and with agitation (Figure 4. 3E) is minimal. But the contribution of 2,4-D becomes higher upon its interaction with NAA as the increase of both hormones will lead to an increase in the KCB production (Figure 4. 3F). The same phenomena can be observed for the KCB outcome in the interaction between NAA with sucrose, agitation, and 2,4-D (Figure 4. 3B, D, F). This indicates that the variables' interdependency among each other is important in maximizing the production of KCB through cell suspension culture.

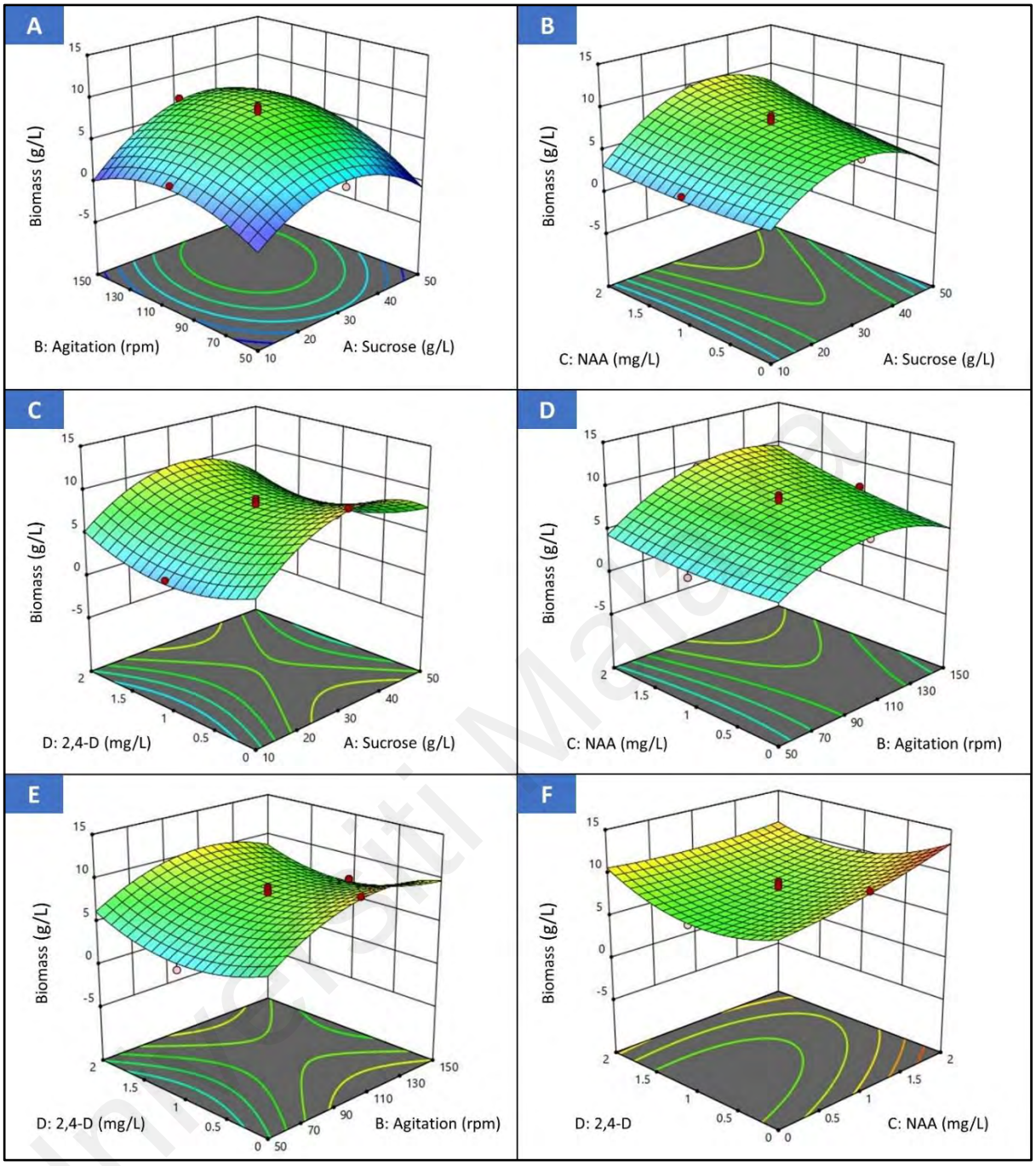

Figure 4. 3: Three-dimensional (3D) response surface plot of KCB production of *H. cannabinus* showing the interaction between; (A) sucrose and agitation, (B) sucrose and NAA, (C) sucrose and 2,4-D, (D) agitation and NAA, (E) agitation and 2,4-D and (F) NAA and 2,4-D.

In KCB optimization, sucrose concentration is crucial in maximizing the culture's output. Sucrose acts as the sole carbon source in the culture. This sugar acts as the energy and food source for the cultured kenaf callus to grow and multiply through cell division and is also used for other metabolic pathways such as cell enlargement, elongation and even in the production of secondary metabolites (Mustafa et al., 2011). Since the culture involved the usage of kenaf undifferentiated cells (callus), the presence of these carbon sources is crucial in ensuring its growth. When sucrose is used, it will be converted into glucose and fructose where the former will be utilised first for metabolic processes followed by the latter (Chawla, 2002). Therefore, the presence of sucrose can be considered crucial in determining the success of the kenaf callus cell suspension culture.

Moreover, the plant growth regulators (PGR) chosen for this study were both in the form of auxin; NAA and 2,4-D. PGR are synthetic compounds that can imitate the effects of naturally produced plant hormones, and they are typically required in a very minute quantity (Mustafa et al., 2011). Auxins are crucial for the initiation of cell division, cell elongation and the formation of callus. This class of PGR also tend to inhibit the production of shoots (Chawla, 2002). The usage of NAA and 2,4-D was found to be effective in the callus formation of a *Malvaceae*-family plant, *Helicteres isora L.* (Shaikh et al., 2018) which is in accordance with this study.

Besides, agitation is another crucial factor in plant cell suspension culture which is needed primarily for generating a gentle pressure to break the cultured kenaf callus aggregates into smaller pieces. This gives a larger total surface area of the kenaf callus for better nutrient exchange with the supplied medium. The agitation also helps the culture in achieving a homogenous mixture by maintaining a uniform distribution of the cells and nutrients, and provides good gas exchange activity in the cultivation system (Chawla, 2002; Mustafa et al., 2011). Taken together, a good agitation rate that will not cause negative effects on the cultured plant cells will promote the growth of the callus in

liquid. Taken together, regulating all the aforementioned parameters will result in high KCB production.

4.3 Kenaf exopolysaccharide (EPS) production optimization

Meanwhile, Table 4. 3 shows the ANOVA for the EPS production (Figure 4. 4). The results stated that the model was significant because the value of "Prob>F" was <0.0001 (p<0.05). The R^2 of 0.9176 shows that the model can explain 91.76% of the response or the outcome variations for EPS. The result of the Adj. R^2 (0.8406) implies the significance of the model that is in reasonable agreement with the Predict. R^2 (0.6833) with a difference of less than 0.2. The model can be expressed in terms of the actual experimental variables for EPS production using Equation 4.2.

$$\begin{aligned} \mathrm{EPS} = {} & 0.087251 - 0.083189 \times \mathrm{A} + 0.017643 \times \mathrm{B} + 0.741844\,\mathrm{C} - 0.201142\,\mathrm{D} \\ & + 0.000118 \times \mathrm{A} \times \mathrm{B} + 0.000656 \times \mathrm{A} \times \mathrm{C} - 0.002531 \times \mathrm{A} \times \mathrm{D} \\ & + 0.004187 \times \mathrm{B} \times \mathrm{C} + 0.001462 \times \mathrm{B} \times \mathrm{D} + 0.025625 \times \mathrm{C} \times \mathrm{D} \\ & + 0.001445\,\mathrm{A}^2 - 0.000099\,\mathrm{B}^2 - 0.506842\,\mathrm{C}^2 + 0.063158\,\mathrm{D}^2 \end{aligned} \quad (4.2)$$

Where; A = sucrose, B = agitation, C = 1-naphthalene acetic acid (NAA), D = 2,4-dichlorophenoxy acetic acid (2,4-D).

Table 4. 3: The results of analysis of variance (ANOVA) generated from the CCD quadratic model in RSM for EPS

Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Model	6.68	14	0.4774	11.93	< 0.0001*
A: Sucrose	1.31	1	1.31	32.65	< 0.0001*
B: Agitation	2.26	1	2.26	56.50	< 0.0001*
C: NAA	0.6651	1	0.6651	16.62	0.0010*
D: 2,4-D	0.0080	1	0.0080	0.2004	0.6608
AB	0.2233	1	0.2233	5.58	0.0321
AC	0.0028	1	0.0028	0.0689	0.7966
AD	0.0410	1	0.0410	1.02	0.3275
BC	0.7014	1	0.7014	17.52	0.0008*
BD	0.0856	1	0.0856	2.14	0.1644
CD	0.0105	1	0.0105	0.2625	0.6159
A^2	0.8661	1	0.8661	21.64	0.0003*
B^2	0.1579	1	0.1579	3.94	0.0656
C^2	0.6656	1	0.6656	16.63	0.0010*
D^2	0.0103	1	0.0103	0.2582	0.6187
Residual	0.6004	15	0.0400		
Lack of fit	0.5357	10	0.0536	4.14	0.0651
Pure error	0.0647	5	0.0129		
Cor total	7.28	29			
Std. Dev	= 0.2001		R^2	= 0.9176	
Mean	= 0.5877		Adjusted R^2	= 0.8406	
			Predicted R^2	= 0.6833	

*significant

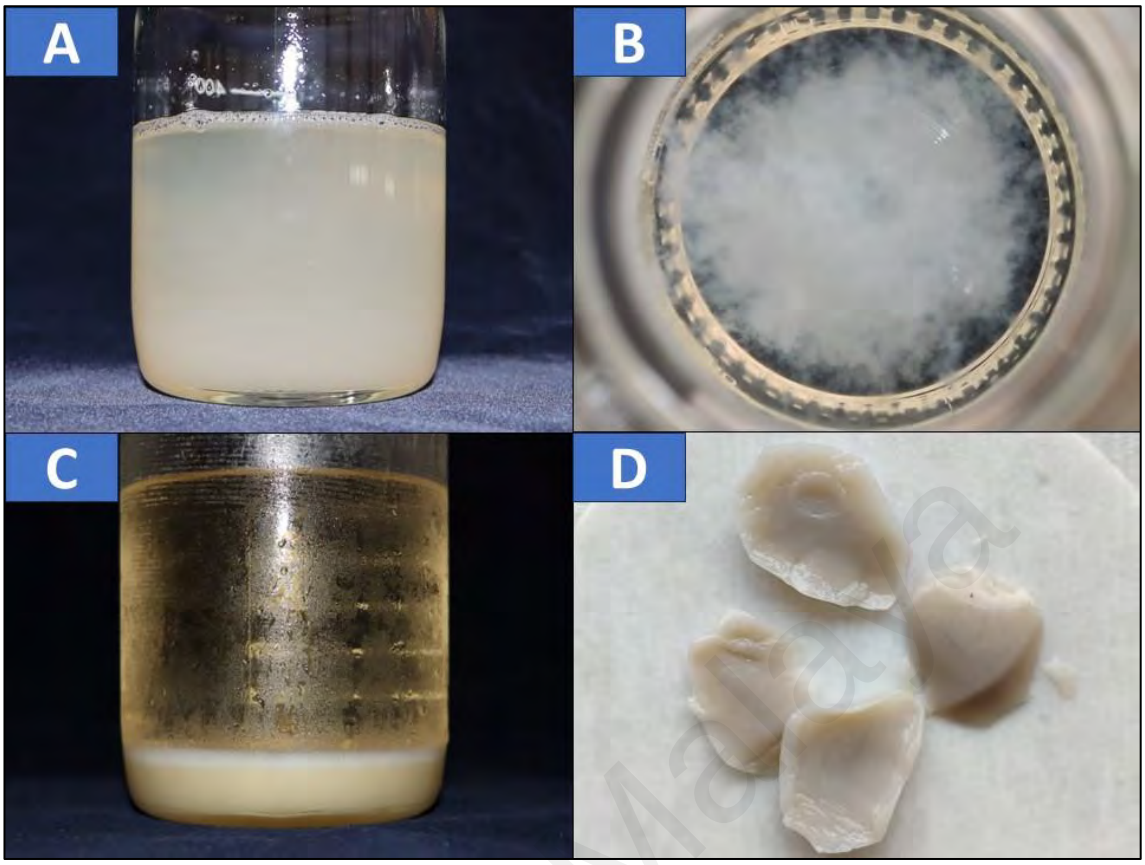

Figure 4. 4: Optimization of EPS. (A) Filtered filtrate of kenaf callus cell suspension culture. (B) Precipitation of kenaf EPS after the addition of alcohol. (C) Sedimented kenaf EPS. (D) Harvested and separated kenaf EPS.

Based on the results in Table 4. 3, sucrose concentration (A), agitation (B) and NAA concentration (C) can significantly affect the outcome of EPS production with *p* values of less than 0.05. Furthermore, the interaction between the variables especially between sucrose and agitation (AB), and agitation and NAA (BC) along with the quadratic terms of sucrose (A^2) and NAA (C^2) also turned out to be significant in producing EPS through this model ($p<0.05$). But, other interactions and quadratic terms; AC, AD, BD, CD, B^2 and D^2 with *p* values of above 0.05 will negatively affect the EPS production of kenaf callus cell suspension culture using this model. According to the model, the maximum amount of EPS can be generated by agitating the culture at 134.25 rpm with media supplemented with 49.99 g/L of sucrose, 1.34 mg/L of NAA and 1.983 mg/L of 2,4-D.

Figure 4. 5 shows the significant interactions (p<0.05) between experimental variables (sucrose, agitation and NAA) on influencing the amount of EPS produced. Based on Figure 4. 5A, the EPS produced will increase with the increase of both sucrose concentration and agitation. Meanwhile, the interaction between agitation rate and NAA concentration will also have a huge effect on the final EPS generated.

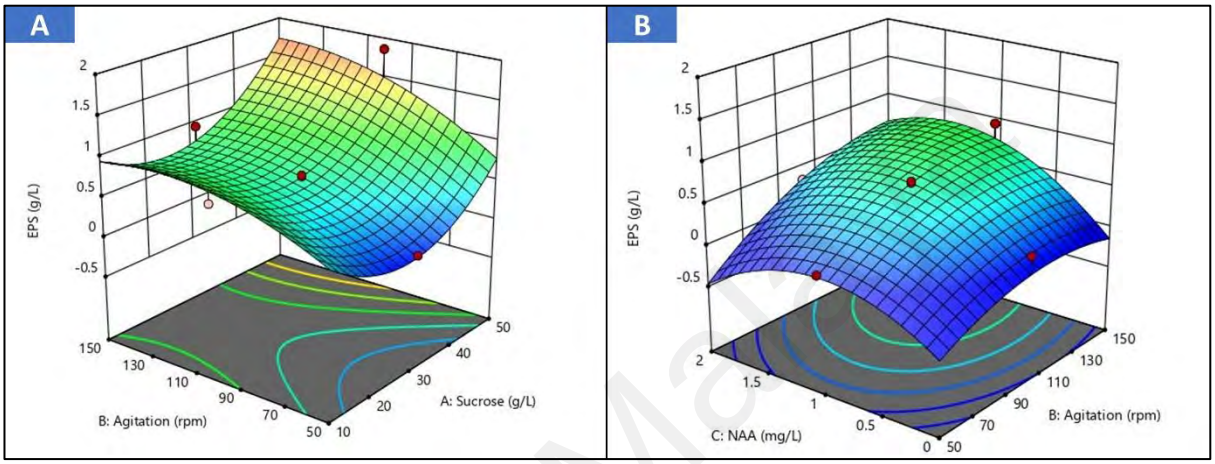

Figure 4. 5: Three-dimensional (3D) response surface plot of EPS production of *H. cannabinus* showing the interaction between; (A) sucrose and agitation, and (B) agitation and NAA.

The presence of sucrose in the culture will aid in the production of the secreted EPS. As the carbon source in the culture, it serves as the basic building block (monomer) of the backbone of the synthesised kenaf EPS which heavily influences the output of the EPS at the end of the culture. Then, with the help of the continuously agitating medium, this creates a gentle shear force that breaks down the cell wall of the kenaf callus which subsequently releases more EPS into the culture liquid medium, resulting in higher EPS output.

Additionally, the production of EPS through plant cell suspension culture that can be retrieved from the growth medium is common (Honda et al., 1997; Kim et al., 2012; Murtazina et al., 2022; Sims et al., 2000). The EPS produced can be categorised based on their major molecular structures such as glucans, galacturonan and xyloglucan. The EPS

secreted into the liquid medium is of the same nature as the type of polysaccharides that make up the cell wall of the cultured plant cells (Honda et al., 1997; Webster et al., 2008). The production of EPS occurs during cell wall expansion whereby the in-situ enzymes of the cells will initiate the process of hydrolysis that breaks down the glycosidic linkages of the polysaccharide matrix. This loosens the cell wall and causes it to expand where the product of the process which is the EPS is secreted into the cultivating medium concurrently (Honda et al., 1997).

4.4 Optimized conditions for kenaf callus suspension culture and its verification

Based on the optimized parameters for the production of KCB and EPS using kenaf callus in cell suspension cultures (Table 4. 4), the experimental parameters can be manipulated for a variety of purposes. Maximizing KCB output would require the usage of 30 g/L sucrose, 1.87 mg/L NAA, 0.04 mg/L 2,4-D and agitated at 129.59 rpm. To increase the EPS production, an agitation rate at 134.25 rpm and medium composition supplemented with 49.99 g/L sucrose, 1.34 mg/L NAA and 1.983 mg/L 2,4-D will be favoured. But if the cultivation requires the maximum outputs of both KCB and EPS, the usage of media with 50 g/L, 2 mg/L NAA and an agitation speed at 147.02 would be employed.

Table 4. 4: Optimized conditions for kenaf callus cell suspension culture

To maximise	Experimental variables				Responses	
	Sucrose (g/L)	Agitation (rpm)	NAA (mg/L)	2,4-D (mg/L)	KCB (g/L)	EPS (g/L)
KCB	40.03	129.59	1.87	0.04	11.97	-
EPS	49.99	134.25	1.34	1.983	-	0.73
KCB and EPS	50.00	147.02	2.00	0.00	11.87	0.91

Table 4. 4 also shows the cultivating parameters to optimise the production of KCB and EPS which were executed to verify the generated statistical model. 250 mL shake flasks were used to validate the model. Conditions for maximising KCB and EPS production produced 11.97 g/L of KCB and 0.73 g/L of EPS which are 87% and 40% of the predicted values (13.58 g/L KCB and 1.87 g/L EPS), respectively. Meanwhile, 89% and 56% of the predicted values (13.34 g/L KCB and 1.62 g/L EPS) were achieved with 11.87 g/L of KCB and 0.91 g/L of EPS produced using 50 g/L of sucrose and 2 mg/L of NAA at 147.02 rpm. The EPS produced deviates from the predicted responses. This could be due to the age and condition of the callus. Callus older than four weeks, exhibiting signs of browning, displayed suboptimal performance in EPS production within the liquid medium. This diminished productivity may stem from the kenaf callus cells' incapacity to engage in the crucial processes of cell division and elongation. Consequently, this inability leads to cell death and the accumulation of toxins in the culture. Such conditions contribute to the deterioration of the overall quality of the kenaf cell suspension culture.

4.5 Characterisation of kenaf EPS using fourier-transform infrared (FTIR)

The extracted kenaf callus EPS was subjected to FTIR (Figure 4. 6A) spectroscopy to study the interaction between its chemical bonds and infrared radiation which helps in characterising its functional groups within its carbon ring in comparison to the EPS retrieved from kenaf core powder (Figure 4. 6B).

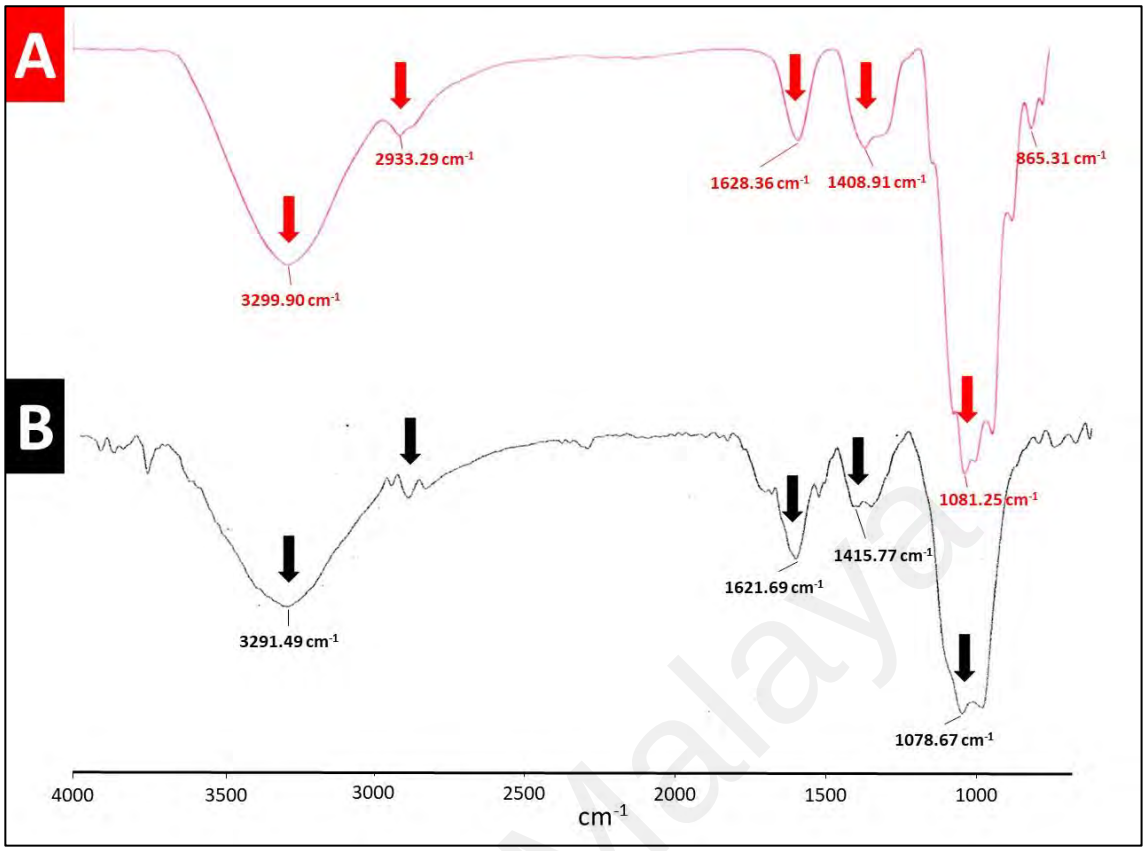

Figure 4. 6: FTIR spectra of (A) EPS from kenaf callus in suspension culture, and (B) EPS from kenaf core powder.

The single-broad absorption peak in the region between 3000 cm^{-1} – 3500 cm^{-1} suggested the presence of hydroxyl group (-OH) in the kenaf EPS structure. A low absorption peak is observed at 2933 cm^{-1} which indicated the stretching vibration of C-H in the sugar ring. Besides, the peak manifested (1628 cm^{-1}) between 1600 cm^{-1} – 1650 cm^{-1} confirmed the stretching vibration of the carbonyl group (C=O). The strong absorption peak at 1000 cm^{-1} – 1300 cm^{-1} designated the C-O-C vibration in the pyran structure of kenaf callus EPS. The resulting peak at 865 cm^{-1} indicated the presence of the α-linked glycosidic linkages in the sugar ring (Wang et al., 2022). These structural confirmations suggested that the extracted kenaf callus EPS are glucans in the form of α-configuration (α-glucan). Interestingly, several studies have reported the bioactivities of α-glucan such as anti-inflammatory, antibacterial and antioxidant (Buddana et al., 2015; Huang et al., 2018). This shows that the application of EPS extracted from the callus of

kenaf through cell suspension culture can be further explored in various industries such as pharmaceutical, cosmeceutical and even food (Norhisham et al., 2023). Additionally, the absorption peaks of kenaf callus EPS (Figure 4. 6A) and EPS retrieved from kenaf core powder (Figure 4. 6B) are considerably identical which is shown by the red and black arrows pointed in Figure 4. 6. This further proves that the EPS of kenaf produced from cell suspension culture is of the same nature as the one retrieved directly from the physical kenaf plant itself. Taken altogether, this negates and nullifies the concerns over any possible alteration in terms of the EPS formed.

4.6 Comparison with previous studies

A comparison of the total plant callus biomass produced along with its respective EPS through cell suspension culture is compiled in Table 4. 5. To the best of my knowledge, this is the first-ever study to ever attempt an optimization process in maximizing the amount of KCB and kenaf EPS through cell suspension culture method. While other study has attempted to optimize callus production through solid cultivation on a culture plate (Odahara et al., 2020), none has covered kenaf plant cell suspension culture. The callus biomass produced using the optimized parameters will generate a considerably high amount of KCB (13.34 g/L) compared to other plant species (1.25 – 19.20 g/L) despite initiating the culture with the lowest weight of callus used (1% of the total working volume) (Table 4. 5). This shows that by optimizing the cell suspension culture's working conditions, the requirement for initial callus to start the culture will be lower, a key in saving resources, time and cost. Besides, kenaf EPS is also expected to increase by 0.7-fold, from 1.10 g/L to 1.62 g/L (Table 4. 5) if the optimized parameters are employed during the cultivation. This is crucial especially if we were to maximize the total output of kenaf EPS for industrial usage and applications. However, studies on other plant species stated in the table did not report on their final EPS produced.

Table 4. 5: Total biomass and EPS produced by other plant species

Plant species	Weight of callus used (% of working volume)	Sucrose (g/L)	Agitation (rpm)	NAA (mg/L)	2,4-D (mg/L)	Biomass produced (g/L)	EPS produced (g/L)	References
Kenaf *(H. cannabinus)*	1	30	100	0.2	0.2	9.09	1.10	Current study[1]
Kenaf *(H. cannabinus)*	1	50	146	2	0	13.34	1.62	Current study[2]
Boxthron *(Lycium schweinfurthii)*	2	30	120	2	0	NA	NA	(Mamdouh & Smetanska, 2022)
Avartani *(Helicteres isora)*	7	30	50-60	0	0	19.20	NA	(Shaikh et al., 2018)
Silver birch *(Betula pendula* Roth)	1	30	100	0	2.5	1.25	NA	(Hajati et al., 2016)
Lemon balm *(Melissa officinalis*)	3	NA	110	NA	1	11.63	NA	(Meftahizade et al., 2010)

[1]Based on the KCB and EPS obtained in establishing kenaf growth curve. [2]Based on the estimated KCB and EPS produced using the optimized working parameters. NA = not available.

CHAPTER 5: RESEARCH'S CONTRIBUTIONS & INSIGHTS

5.1 Significance of the study

The execution of this research to optimize the parameters for kenaf callus in cell suspension culture is relevant to the current settings, which are briefly summarised in (Figure 5. 1).

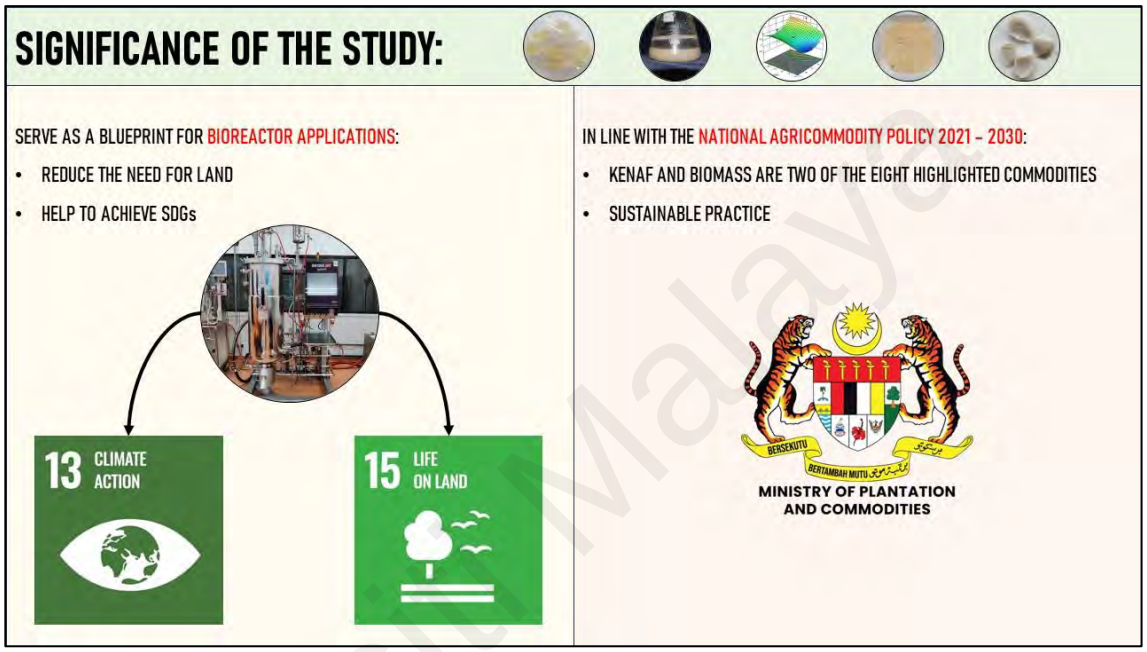

Figure 5. 1: Summary of the significance of the study

First, the study is in line with the newly announced Malaysia's National Agricommodity Policy 2021 – 2030 (DAKN 2030) which focuses on strengthening and enhancing eight nation's commodities; oil palm, rubber, timber, cocoa, pepper, kenaf, biomass and biofuel. The policy emphasizes five core principles when conducting any of the aforementioned commodities' activities which revolve around sustainability, productivity, value generation, market development and inclusivity (Bernama, 2022). The selection of the plant species in this study (kenaf) comes at the right time. However, since more farmers and kenaf stakeholders are focusing on expanding kenaf's plantation (Bernama, 2023), this will undoubtedly create the need for acres of land to be used for

the cultivation and farming of kenaf. This could eventually lead to severe deforestation activities and the destruction of flora and fauna.

This study paves the way for the usage of a sustainable biotechnological approach in addressing the issues mentioned. The optimized parameters gained from this study can serve as a blueprint for the upscaling purposes of the production of KCB and EPS using a more well-established and complex yet efficient device such as bioreactors, favourably a pilot-scale to cater for the demand for the industries. Kenaf biomass receives demand both domestically and internationally to be used in the building materials, pulp, biodegradable, livestock feed and bio-composites production sectors (Bernama, 2023). Additionally, its therapeutic and pharmaceutical properties that have been previously discussed (Norhisham et al., 2023) should not be neglected. These industrial and economic situations prove the necessity of the commencement of the study. But this biotechnological approach serves as an alternative way of culturing and cultivating kenaf and not as a way to diminish kenaf's plantation as a whole. This potential blueprint for kenaf application in bioreactors acts as a potential problem-solver in mitigating plantation concerns.

Besides, looking at a broader perspective, this study is also aligned with two of the Sustainable Development Goals (SDGs) which are Goal 13: Climate Action (G13) and Goal 15: Life on Land (G15). The ability to culture kenaf cells in cell suspension culture and potentially in a bioreactor to produce its biomass eliminates the requirement for land. Since bioreactors can be operated in small areas, the need to build more physical kenaf farms is no longer required, which ultimately will reduce any potential deforestation activities. According to the United Nations (2023b), the world's temperature will increase by 1.5 °C in 2035 and potentially 2.5 °C warming by 2100, which can lead to disastrous climate catastrophes events such as intensifying heatwaves, droughts, flooding, wildfires and sea-level rise. Thus, forest preservation is one of the efforts that can be done in

addressing this worrying event, as forests act as the natural carbon sink function, removing greenhouse gases that contribute towards climate change (Melillo, 2021). So, forest preservation through the usage of bioreactors for kenaf's biomass production can be considered as an initiative action to combat climate change which suits G13 well. Moreover, forest preservation is also in accordance with G15. Agricultural activities that require deforestation result in forest loss, land degradation and the extinction of species (United Nations, 2023a). Therefore, the privilege to opt for another method such as through cell suspension culture and bioreactors for agricultural activities should not be taken for granted to protect, restore and promote sustainable use of terrestrial ecosystems.

5.2 Challenges, limitations and potential future works

The in vitro cultivation of kenaf callus in cell suspension culture needs massive attention, and care and must be executed aseptically. During the suspension culture, concerns about the homogeneity of the culture will arise if the callus used to initiate the culture is not gently and aseptically homogenised. Differences in terms of the size of the callus used along with the presence of the clumped callus inside the culture environment are not preferred. This is because distribution and the nutrient exchange rate between the media and the growing callus are not uniform. Even the total surface area of the clumped callus used is lower compared to the well-homogenised and dispersed callus, which will deteriorate its performance and result in irreproducible results (Mustafa et al., 2011). This non-uniform production is not acceptable if kenaf callus were to be brought into the industry where a consistent quality and amount is upmost desired. Therefore, it is highly encouraged to homogenise the callus gently before initiating cell suspension culture for better cultivation performance.

Next, the precipitated EPS from the culture filtrate was found to be insoluble in water. This characteristic makes the EPS difficult to go for any bioassay activities, which can unearth its potential pharmaceutical benefits. When it comes to polysaccharides, some are

readily soluble in water such as pullulan and amylopectin, and some with poor water solubility such as xylan and mannan (Guo et al., 2017). The EPS from kenaf callus seems to belong to the latter group. Thus, to fully reap its pharmacological benefits, pre-treatment to turn it into a water-soluble compound is needed which can be done through either biological modifications (enzyme degradation), chemical modifications (sulfation, carboxymethylation, phosphorylation, methylation etc), or physical modifications (sonication, irradiation, autoclaving, heating etc) (Jayawardena et al., 2017).

In the future, further validation using a bioreactor is needed to fully realise its potential and feasibility for industrial applications. This study serves as the blueprint for KCB and EPS upscaling activities. The experimental parameters used in this study (sucrose concentration, agitation, NAA concentration and 2,4-D concentration) are applicable to the bioreactor system to maximize the output of KCB and EPS. The performance of the culture inside the bioreactor is expected to better the culture in shake flasks as more variables such as aeration (L/min), the concentration of dissolved oxygen and the pH of the media can be monitored and adjusted accordingly. However, a suitable type of impeller is needed to ensure minimum damage to the cells due to the shear force, and for plant callus, a marine-type impeller (Figure 5. 2) is recommended (Eibl & Eibl, 2008).

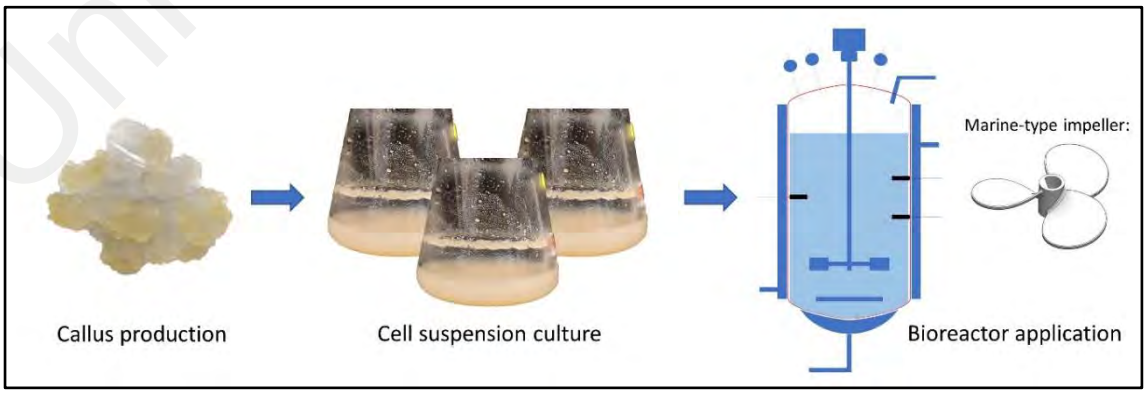

Figure 5. 2: Future validation and mass production workflow of KCB and EPS using a bioreactor

So, for industrial-scale production of KCB and EPS, it is highly suggested to apply the usage of bioreactors, with considerations of the optimized parameters and growth environment. Lastly, the produced KCB should undergo further screening to identify any useful extracts such as phenolic, flavonoid, polysaccharides (intracellular polysaccharides) and other secondary metabolites that are useful in various industries including pharmaceutical, food and cosmeceutical. According to Shaikh et al. (2018), the callus of plants can be the source for recovering high quantities and qualities of phytochemicals.

CHAPTER 6: CONCLUSION

The study performed in this thesis focused on the cultivation of kenaf callus in cell suspension culture along with the optimization of its working parameters to maximize the culture's output which are kenaf callus biomass (KCB) and exopolysaccharide (EPS). A specific optimization software, response surface methodology (RSM) was employed. The software gave conditions that needed to be tested and the results obtained were analyzed using analysis of variance (ANOVA), where the significant effects of each of the tested parameters (sucrose concentration, agitation, NAA and 2,4-D concentrations) on the culture's production of KCB and EPS were evaluated. Finally, the characteristics and the molecular structure of the extracted EPS from the kenaf callus cell suspension culture were examined using Fourier-transform infrared spectroscopy (FTIR).

To conclude, the optimization process managed to set cultivating conditions (50 g/L sucrose, 147.02 rpm and 2 mg/L NAA) that can increase the production of KCB and EPS simultaneously. This serves as a method to upscale the production of KCB and EPS for various applications such as in the pharmaceutical, cosmeceutical and food sectors. Then, the EPS produced was found to be of α-glucan nature, based on the peaks of the spectroscopy at 3299 cm^{-1}, 2933 cm^{-1}, 1628 cm^{-1}, 1081 cm^{-1} and 865 cm^{-1}. α-glucan has been reported previously to have pharmacological activities such as anti-inflammatory, antibacterial and antioxidant. This study also highlighted its relevance and importance, both domestically and globally in terms of achieving two of the Sustainable Development Goals (Goal 13: Climate Action and Goal 15: Life on Land), and is in accordance with Malaysia's Agricommodity Policy 2021 – 2030.

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