Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb68650
DOI: 10.22146/jtbb.68650

Research Article

Growth of Kaffir Lime (*Citrus hystrix* DC) Cell Line Derived from Seed Explant After Yeast Elicitation Using Pure and Technical Grade Yeast

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Keywords:
cell line
elicitation
kaffir lime
S. cerevisiae
technical grade yeast
Submitted:
24 August 2021
Accepted:
11 December 2021
Published:
18 March 2022
Editor:
Miftahul Ilmi

ABSTRACT

The addition of elicitors in kaffir lime (*Citrus hystrix* DC.) culture is one of strategies for obtaining and increasing the production of secondary metabolites. *Saccharomyces cerevisiae* is one of the elicitors that can be used to increase secondary metabolites such as terpenoids. However, in its use, the pure cultures of *S. cerevisiae* are expensive. Therefore, the first objective of this study was to analyze the ability of technical grade (commercial baker's yeast) to be used as an elicitor and measure the growth of kaffir lime cell line after being elicited by pure and technical grade (commercial baker's yeast). The second objective is to determine the best time to subculture kaffir lime cell line after elicitation. We observed the morphology and measured the growth curve of pure and technical grade yeast until the 4th subculture generation. Furthermore, we used both grades of yeast for elicitation. Kaffir lime cell suspension was treated with 10 ppm pure grade or 5 ppm and 10 ppm technical grade yeast for 4 days. After elicitation, kaffir lime cell lines were subcultured and their growth was analyzed. The result showed that the morphology and growth curve of technical grade until 4th subculture generations was similar to the pure grade. On the other hand, after elicitation using pure and technical grade yeast and being subcultured, the growth of the elicitated kaffir lime cell line had the same pattern as the control group, but the cell density of the control group was higher than the elicitated group. The initial stationary phase of kaffir lime cell line was on the 17th day which is the best time to subculture. The subculturing process is important to maintain the viability of the kaffir lime cell line.

INTRODUCTION

Kaffir lime (*Citrus hystrix* DC.) leaf extract has the potential as an anticancer (Tunjung et al. 2015). However, the use of the extract from nature has several obstacles such as overexploitation of kaffir lime leaf and the production of

bioactive compounds that are strongly influenced by environmental conditions. One method for producing bioactive compounds can be done by using tissue culture techniques (Bourgaud et al. 2001). Unfortunately, the type and level of bioactive compounds in kaffir lime callus extract were less than in the leaf extract. Therefore, kaffir lime cell culture requires an elicitor to increase the production of bioactive compounds.

Elicitor is a biotic or abiotic compound that induces the synthesis of other specific compounds that are used for defense mechanisms in plants (Murthy et al. 2014; Ramirez-Estrada et al. 2016). Biotic elicitors consist of living organisms such as fungi, bacteria, and herbivores. Inorganic compounds such as heavy metals, pesticides, detergents, or physical factors (e.g. cold shock, UV light, and high pressure) are examples of abiotic elicitors. These biotic and abiotic elicitors trigger the enzymatic activities in plant stress responses (Gueven 2003; Ramirez-Estrada et al. 2016).

S. cerevisiae is a yeast that is able to increase the number of terpenoid compounds in some plants. Treatment by *S. cerevisiae* with a concentration of 1.5% for 72 h was able to increase the number of gymnemic acid by 9.3 fold - in the suspension culture of *Gymnema sylvestre* (Chodisetti et al. 2013). Furthermore, *S. cerevisiae* increase ajmalicine content in the cell aggregate culture of *Catharanthus roseus*. The number of ajmalicine was increased to 25.288 ± 0.102 jig/g DW after being treated with *S. cerevisiae* with a concentration of 0.5% for 24 h (Ratnasari et al. 2001). Moreover, according to Pereira et al. (2007), the production of triterpenes was increased after the addition of *S. cerevisiae* to the cell suspension culture of *Tabernaemontana catharinensis*.

Furthermore, the use of *S. cerevisiae* as an elicitor has several advantages such as it is easy to grow, has a short life cycle, can grow at low pH, and is safe for health because it is non-toxic (Sitinjak et al. 2000). On the other hand, in its use, the pure culture of *S. cerevisiae* has several disadvantages including not being sold freely, impractical, difficult to carry from one place to another because it must be in aseptic conditions, and expensive. Hence, we need another type of yeast elicitor that has the same ability as *S. cerevisiae*. Commercial baker's yeast consists of *S. cerevisiae* and has several advantages, including easy to find, easy to use, easy to carry because it is in powder form, and has an affordable price. However, no scientific report about commercial baker's yeast can be used as an elicitor.

Kaffir lime (*C. hystryx*) shows the potential to be used as a traditional medicine for several diseases such as cancer. In our previous study, we found that terpenoids are detected in the first subculture 35 days (control group) and callus preserves in 4°C of kaffir lime callus from seed explants. The type of terpenoids were squalene and geranyl acetate, whereas geranyl linalool was found in kaffir lime callus preserve in 4°C with alginate encapsulation. Furthermore, some compounds that act as anti-cancer were also detected in preserved callus such as lauric acid, palmitic acid, stearic acid, 1-decanol, undecylenic acid, oleic acid, 2H pyran-2-one, octadecane, 1-hexcosanol, hexane, methane, dodecane, tetracosane, 2 decenoid acid, and 3-dodecane

(Fajarina et al. 2021). Because of its capability to synthesize secondary metabolites especially terpenoids, kaffir lime callus, or callus cell suspension can be used as raw materials for traditional medicine. The number of terpenoid compounds that have the potential of anti-cancer needs to be increased. One method that can be used to increase the production of secondary metabolites is using elicitation in cell suspension culture.

Cell suspension culture contains a population of cells with a fast growth rate. Good culture conditions make the reproduction of cell suspension cultures suitable for increasing secondary metabolites production (Moscatiello et al. 2013). During incubation, the cell suspension culture was shaken using a shaker to make the single cells could divide and increase the aeration. Agitation or shaking in cell suspension cultures can increase the aeration to maintain cell viability during the incubation period (Dwimahyani 2007). Our previous study Damayanti et al. (2020) succeeded in optimizing the growth of kaffir lime suspension cells in MS liquid media with a concentration of 2 ppm 2,4-D addition. This condition was suitable for yeast elicitation. On the other hand, the synthesis of compounds that have the potential as an anti-cancer by callus or cell suspension needs to be preserved for long-term and large-scale use. So that, we need a method to maintain these compounds. A previous study reported that the cell suspension subculture method can stabilize alkaloids. Alkaloid production from two cell lines of *Tabernaemontana divaricata* cell suspension culture showed a maximum amount in the 4th subculture after changing it with the same medium and stabilized on a higher level than found in the original cell lines (Sierra et al. 1992).

The first objective of this study was to analyze the ability of technical grade (commercial baker's yeast) to be used as an elicitor and measure the growth of kaffir lime cell line after being elicited by pure grade (pure culture of *S. cerevisiae)* and technical grade (commercial baker's yeast). The second objective is to determine the best time to subculture kaffir lime cell line after elicitation.

MATERIALS AND METHODS

Materials

Kaffir lime fruit was obtained from kaffir lime orchards in Kaliduren Village, Candirejo, Borobudur, Magelang Regency, Central Java. We used fresh fruit, and the diameter of the fruit was approximately 5-6 cm, the length and width of the seed were approximately 0.7 - 1 cm and 0.3 – 0.5 cm, respectively. The pure cultures of *S. cerevisiae* were taken from the Center Studies for Food and Nutrition of Universitas Gadjah Mada whereas commercial baker's yeast powder was bought from a supermarket.

Yeast Observation

Culture medium

Pure cultures of *S. cerevisiae* and technical grade were grown and subcultured once a week on Peptone Glucose Yeast (PGY) extract solid media. Pure and technical grade subculture was carried out four times to obtain the fourth subculture (G4).

Morphological observation

Pure grade and technical grade culture were transferred into PGY liquid media for 24 hours. After 24 hours, 1 ml of yeast was taken from the liquid medium for morphological analysis using a binocular microscope at 100x magnification.

Growth measurement

One use of one week old pure cultures of *S. Cerevisiae* and technical grade / commercial baker yeast were inoculated into 10 ml of PGY liquid medium then they were incubated at room temperature without being shaken. Cells were harvested at 2 h to 24 h time intervals to determine growth curves. The cell number was calculated using a spectrophotometer at a wavelength of 660 nm, with 3 replicates.

Growth of Kaffir Lime Cell Suspension

Medium Preparation and sterilization of explant

The tissue culture method was conducted according to Damayanti et al. (2020). The basal medium is Murashige and Skoog (MS) (Damayanti et al. 2020) containing myo-inositol, it is added with sucrose (30 g/l), agar (8 g/l), and 2 ppm concentration of 2,4-D and distilled water. The medium pH was adjusted to 5,8 using 1 N HCl or 1 N KOH. Then, the medium was sterilized using the autoclave at 121^{o}C with pressure 1 atm for 15 min.

Kaffir lime seeds were removed from the fruit then sterilized using sodium hypochlorite 5.25% for 5 min. The seeds were washed with distilled water 2 times for 5 min each. After sterilization, the seed explants were transferred to sterile petri dishes lined with filter paper.

Callus Induction

This protocol was referred to Damayanti et al. (2020). The seed explants were grown on an MS solid medium containing 2 ppm of 2,4-D. Seeds were maintained and stored in the incubation room under dark conditions (25^{o}C) until they reached the stationary phase (G0), and then 1st subcultures (G1) were done every 25-30 days.

Cell Suspension Establishment

This method is according to a previous study (Damayanti et al. 2020). Kaffir lime callus G1 at 25-30 days (early stationary phase) was transferred to 50 ml MS containing 2 ppm 2,4-D for 21 days and shaken at 100 rpm. After 21

days, homogenates were separated from the medium and used as an inoculum for the establishment of suspension culture. Homogenates contain a single cell population, explant debris, and dead cells. To distinguish single cells and dead, we analyze the homogenates under the microscope. Only single cells were subcultured into the fresh medium for 16 days, shaken at 100 rpm. The incubation was carried out at room temperature in dark conditions.

Elicitation of Cell suspension

Pure and technical grades yeast were inoculated into PGY liquid medium, then incubated at room temperature without shaking. After incubation, yeast cells were autoclaved for 15 min at 121 ^{0}C with 1 atm. The yeast cells were centrifuged and the resulting pellets were rinsed and subjected to be used as an elicitor. Elicitation was carried out by adding technical grade (5 ppm, 10 ppm) and *pure grade* (10 ppm) yeast into 50 ml of kaffir lime cell suspension culture. Kaffir lime cell suspension was treated with yeast for 4 days with 3 replicates.

After harvested, cell lines were filtered using a 100 µm nylon filter and washed using MS liquid medium to ensure that there was no yeast contamination inside cell suspension. The filtered cells were subcultured into 40 ml of MS containing 2 ppm 2,4-D. The growth of cell lines and the control group were measured using a Neubauer hemacytometer for 27 days.

RESULTS AND DISCUSSION

The growth of yeast

In this study, we optimized technical grade yeast as an elicitor. Commercial baker's yeast is a technical grade yeast that is very easy to obtain, widely traded, and has an affordable price (one tube pure culture's price of *S. cerevisiae* is Rp 500.000,00 while one sachet of commercial baker / technical grade yeast is Rp 5.000,00). Therefore, in this study, we compared these two types of yeasts to see their ability as an elicitor.

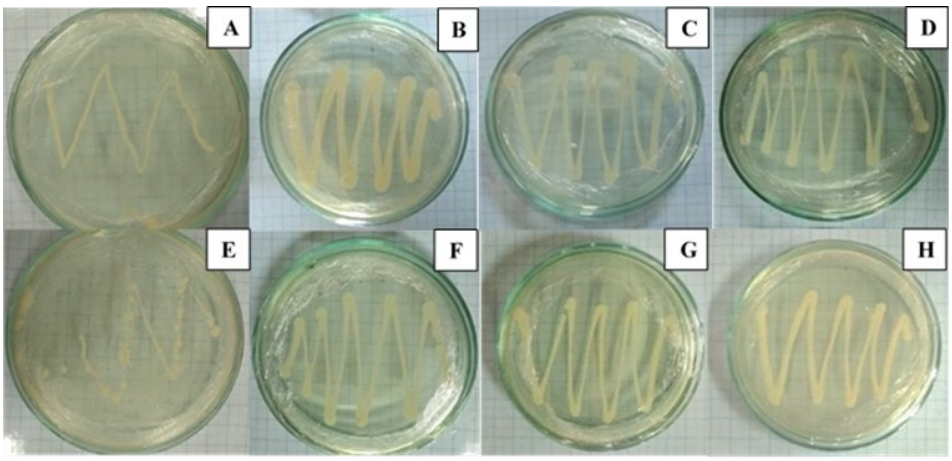

Figure 1. The growth of yeast on PGY solid media.
Pure grade G0(A), G1(B), G2 (C),G4 (D); technical grade G0(E), G1 (F), G2 (G) -G4 (H). (G0: before subculture; G1: 1th subculture; G2: 2nd subculture; G4: 4th subculture).

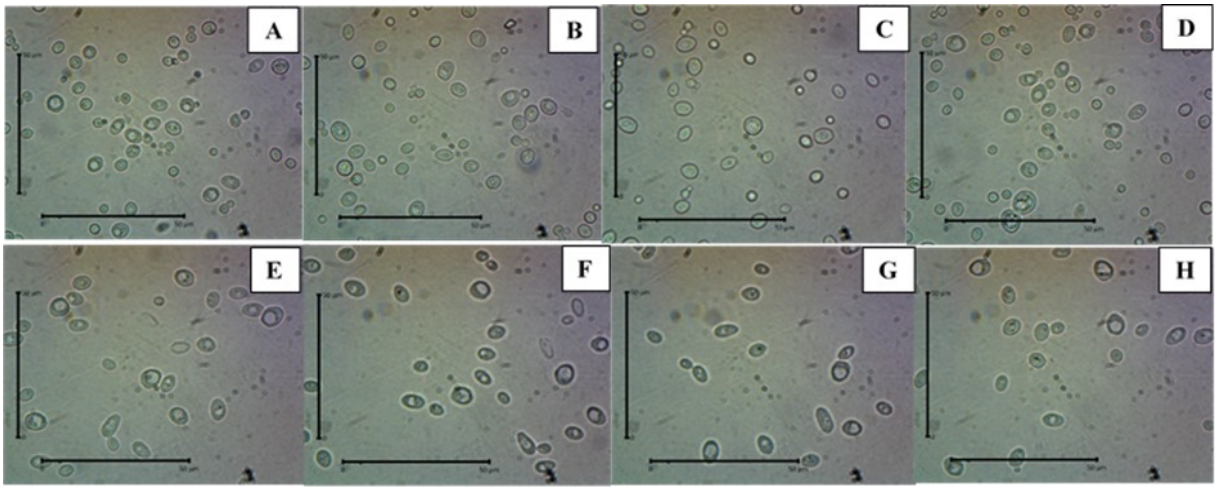

Figure 2. Morphology cell of pure and technical grade yeast after subculture every 7 days.Pure grade at G0 (A), G1 (B), G2 (C)-G4 (D); technical grade yeast at G0 (E), G1 (F), G2 (G)-G4 (H). (G0: before subculture; G1: 1st subculture; G2: 2nd subculture; G4: 4th subculture) At 100× magnification.

Figure 1 showed that technical grade yeast is stable during subculture until G4. This study is the first scientific using technical grade yeast as an elicitor in tissue culture. We subcultured the yeast until the 4th generation because we wanted to ensure its viability and growth stability. Technical grade yeast contains *S. Cerevisiae* and an emulsifier (sorbitan monostearate E491), affecting yeast growth. If the growth of yeast could be stabilized until the 4th subculture, we assumed that technical yeast grade is able to grow well. Thus it can be used as an elicitor. The result of morphological observations of two types of yeast (pure and technical gradeculture) can be seen in Figure 2.

The cells of *S. cerevisiae* pure culture and technical grade yeast cultures exhibited similar shapes and size (Figure 2 and Table 1). They have spherical, oval, and elongated shapes. The size of pure and technical grade before culture and after 4th sub culture was similar. The results are consistent with the description of *S. cerevisiae* by Montes de Oca (2016). This data showed that technical grade yeast is referred to as an elicitor candidate for kaffir lime cell suspension culture. However, the morphological characters cannot be used to identify and compare the yeast because the Saccharomycetaceae family consists of members with similar cell morphology. Therefore, further research with more accurate methods such as Biochemical and DNA sequencing methods is needed.

Table 1. Size of pure and technical grade yeast cell before subculture and after 4th subculture.

Grade of yeast	Subculture phase	Diameter of cells (μm)
Pure culture	Before subculture	6,36 ± 0,12
Pure culture	after 4th sub-culture	6,01 ± 0,21
Technical grade	Before subculture	6,55 ± 0,47
Technical grade	after 4th sub-culture	6,21 ± 0,46

Furthermore, we measured the yeast growth curve. This curve was to determine the best time to apply yeast on the kaffir lime cell suspension as elicitation treatment begin. The results can be seen in Figure 3.

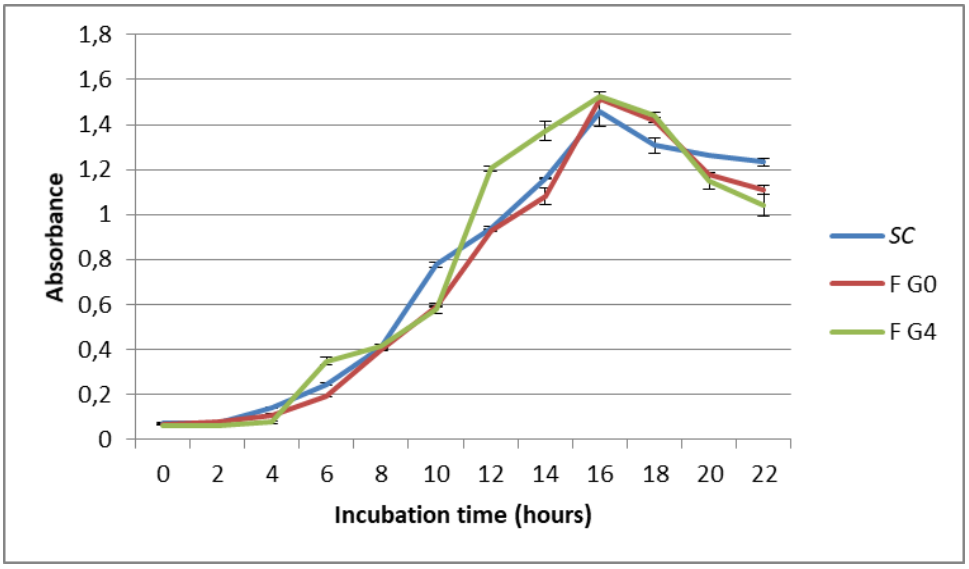

Figure 3. Growth curves of pure and technical grade yeasts.
Note: SC = Pure culture of *S. cerevisiae*; F = Technical grade yeast; G0 = before culture; G4 after 4th subcultur.

Sigmoid curves were achieved when both yeasts reached their maximum growth (Figure 3). The peak period of both pure and technical grade of yeast was at the 16th hour. After peak growth, cells reached their stationary phase. This peak period is the best time to harvest the yeast cells for the elicitation process. According to Klis et al. (2002), In this phase, yeast cells are at the highest growth, and the yeast cell wall, the elicitor component of the yeast is well-formed and thicker and considerably has higher turgor pressure compared to exponentially growing cells. Furthermore, at the stationary phase, the rate of yeast cells divisions is declining thus extra energy is allocated to form a compact cell wall structure (Aleu et al. 1999). According to Chen and Chen (2000), the response of plant cells to elicitors is directly related to the composition of the yeast cell wall, especially glucan, which can be recognized by plant cells as stress, so that plant cells will respond by producing secondary metabolites.

The Comparison Growth of Cell Line After Yeast Elicitation

A Friable callus is needed as a raw material for cell suspension. The friable texture of callus facilitates the separation between cells into a single cell in cell suspension culture (Damayanti et al. 2020). The addition of 2,4-D to the culture medium caused the middle lamellae of the plant cell wall to break, thereby promoting the bonding between cells to break off and form a crumbly callus (Leksonowati et al. 2017). Therefore, 2 ppm of 2,4-D was added into the medium for callus induction and cell suspension. Callus was suspended in a sterile liquid medium containing various nutrients and growth

factor compounds were needed by cells for their growth (Damayanti et al. 2020).

Figure 4 showed the kaffir lime seeds that were used as explants in this study. Kaffir lime seeds that were used for explants were part of the endosperm. Endosperm as a food reserve for embryos contained nutrients in the form of carbohydrates and proteins. These nutrients are needed at the beginning of callus growth (Sukmara et al. 2014). Friable endosperm callus can be used as a raw material for cell suspension culture. According to Pasitvilaiturm and Pankasemsuk (2012), cell suspension cultures of *Jatropha curcas* L. Are able to grow and produce oil (Figure 5). Callus on the 25th day has a crumb texture with a moderate-yellow color (Figure 5A). G1 callus has a crumb texture with a moderate-yellow color (Figure 5B). The friable callus is needed as a raw material for making cell suspension (Damayanti et al. 2020). Therefore, G1 callus is a raw material that was subcultured into a liquid medium to form a single cell aggregate (Figure 5C).

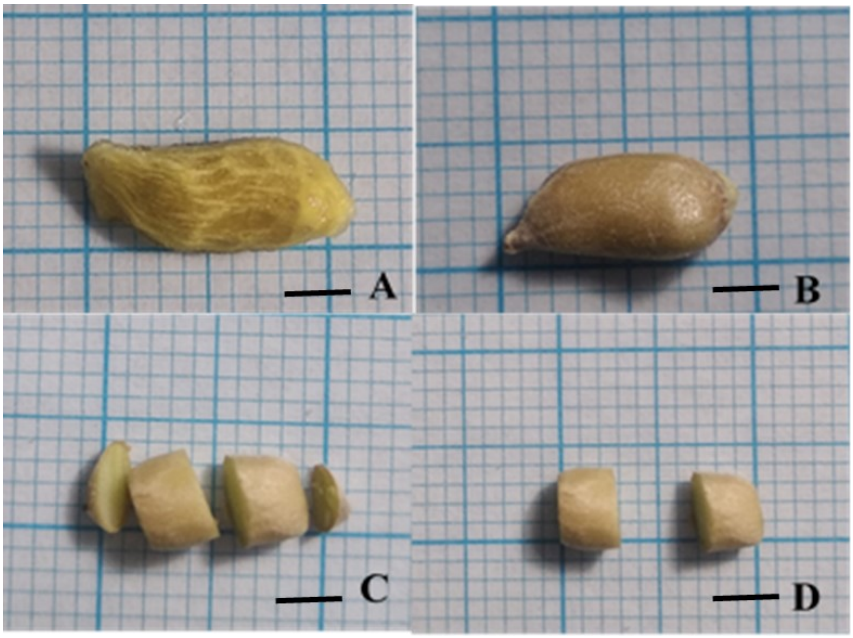

Figure 4. Kaffir lime seeds. The seed coat (A); the seed coat is removed (B) The seed is sliced (C); The seed is ready to culture (D). Bars: 0,3 cm.

G1Callus aged 25-30 days (early stationary phase) was transferred into the liquid medium and placed in a shaker for 21 days. In the liquid medium

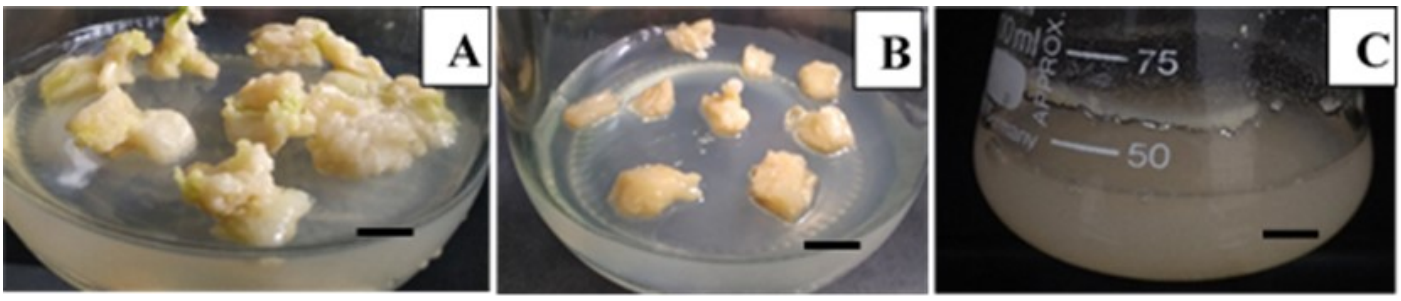

Figure 5. Kaffir lime callus and cell suspension. Callus on 25th day (G0) (A); Callus on 25th day (G1) (B); Suspension culture in the flask (C). Bars: 1 cm.

will be found a single cell population, explant debris, and dead cells. The suspensions aged 21 days were subcultured into the new medium for 16 days. On the 16th day, the new cell growth entered the stationary phase. Hence, this is the best time for elicitation (Damayanti et al. 2020).

Cell suspension culture consists of cells population with fast growth rate and good culture conditions make the reproduction of cells suspension culture suitable to increase the production of secondary metabolites (Moscatiello et al. 2013). It was found in mangosteen (*Garcinia mangostana* L.) culture that the production of secondary metabolites in callus treated with 100 µM methyl jasmonate or 0.5 g/l casein hydrolysate as an elicitor for 5 days and 7 days respectively was lower (21 metabolites) than in cell suspension (34 metabolites). These differences in secondary metabolites production may be due to agitation (Jamil et al. 2018). During incubation, the suspension culture was shaken using a shaker so the single cells could divide and increase the aeration. Agitation or shaking in *Jatropa Curcas* Cell suspension cultures can increase the aeration to maintain cell viability during the incubation period (Dwimahyani 2007).

Damayanti et al. (2020) showed that the beginning of a stationary phase in kaffir lime cell suspension was on the 16th day. Cell lines are the cells that are able to survive biotic stress and are expected to be able to produce high levels of metabolites. Cell line suspension subculture is one of the methods that could stabilize the growth of the cell line.

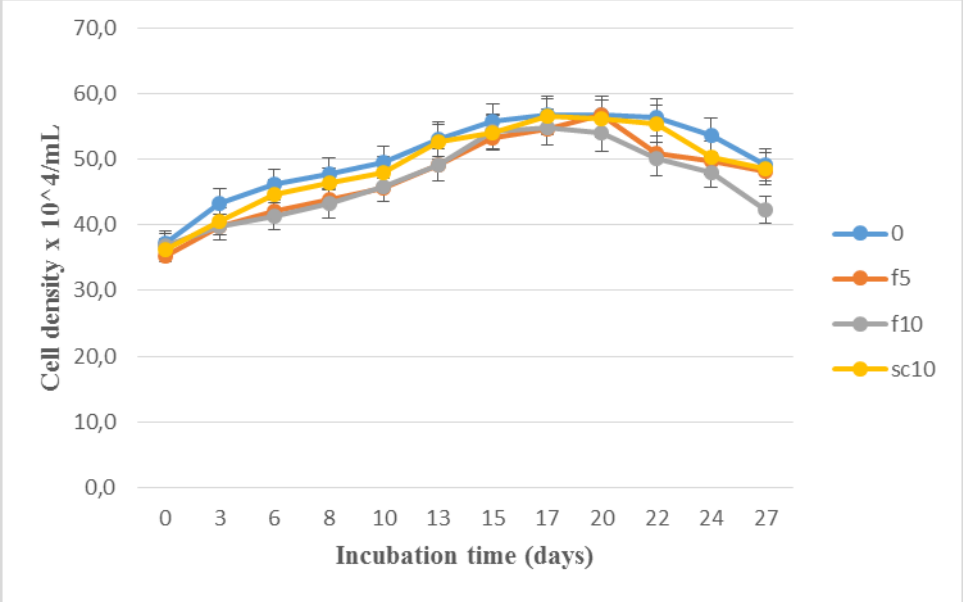

Figure 6. Growth curve of kaffir lime cell line after elicitation. F5 and F10 are cell lines that were elicited by technical grade yeast with 5 and 10 ppm respectively; Sc10 is cell line was elicited by pure grade yeast with 10 ppm.

Figure 6 showed the measurement of the cell growth after addition with pure and technical grade yeast. The cell density of the control group was higher than the elicitated group. This is because of screen cells' elicitation and cell lines' formation. High doses of elicitors can induce a hypersensitive

response that causes the cell to death (Namdeo 2007), so we assume that cells that can survive are cells that are resistant to biotic stress and probably be able to produce high levels of metabolites.

The growth phases of kaffir lime cell line after yeast elicitation consists of the lag phase, exponential phase, stationary phase, and death phase (Figure 6). The lag phase occurs on the 0 to 6th day. This phase occurs when the cell line adjusts to the new environment after previously being exposed to elicitors of pure and technical grade yeast. After experiencing the adjustment phase, the cell line starts to actively divide, called the exponential phase. The exponential phase occurs from the 8th day to the 17th day. In that day range, the maximum cellular division occurred. After carrying maximum cellular division, nutrients became limited in the culture medium and cell viability gradually decrease to reach the stationary phase. The graph above shows that the stationary phase starts on the 17th day until the 20th day. After the stationary phase, the nutrient content in the medium became exhausted and the toxic substances will be produced by the cells as a defense mechanism from stress (Bhojwani & Razdan 1983 in Khanpour-Ardestani et al. 2015). After that, decreasing amount of the cell density can be seen from the 24th day until the 27th day. This phase is called the death phase.

The morphology of kaffir lime cell line can be seen in Figure 7. There were three cell shapes were observed, namely spherical, comma, and elongated shapes. During the incubation period, cells change in shape due to

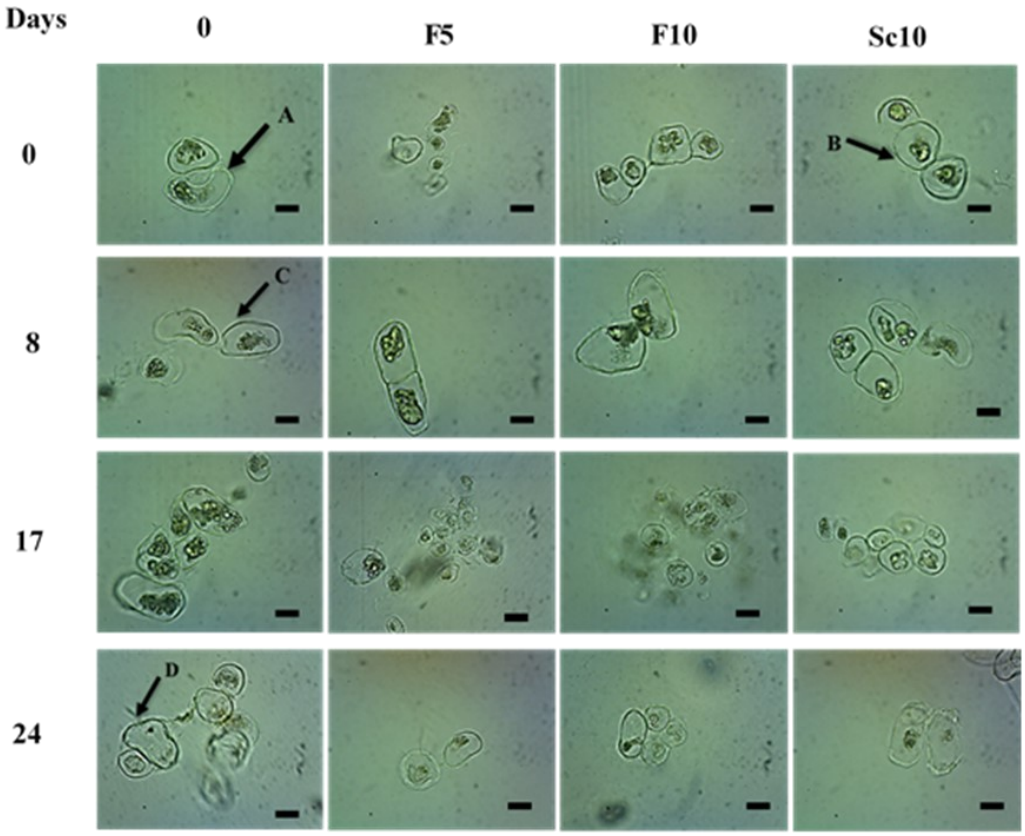

Figure 7. Morphology of kaffir lime cell line after elicitation from Day-0 to Day-24. (A) comma or sickle shape, (B) spherical shape, (C) elongated shape, (D) unviable cell, scale bars 100 μm. F5 and F10 are cell lines that were elicited by technical grade yeast with 5 and 10 ppm respectively; Sc10 is cell line was elicited by pure grade yeast with 10 ppm.

their response to the environment and nutrients. The spherical shape indicates that the cell is the result of previous cell division in *Stelechocarpus burahol* (BI.) Hook. F. And it is embryogenic cells with activated division in *Saccharum officinarum* L. Cells with a spherical shape will differentiate into elongated cells. In *S. burahol*, elongated cell indicated the non-viable condition and non-embryogenic cell showed in *S. officinarum* (Habibah et al. 2017; Thorat et al. 2017). According to Ogita et al. (1997) in dos Santos et. Al (2010), long binucleated cells undergo continuous cell division resulting in the development of adventitious somatic proembryos in *Larix leptolepis*. However, any species after passing through the exponential phase, the shape of the cell changes back to a spherical shape. The spherical shape of the cell persists until the cell death phase. This also occurs in *C. arizonica* cells which are spherical at the end of their growth (Sparapano & Bruno 2004).

In this study, we found that the best time for subculturing kaffir lime cell line suspension is on the 17th day, which is the final exponential phase (the initial stationary phase). The final exponential phase is better to be subcultured because when it enters the end of exponential and start the beginning of the stationary phase, the nutrient in the medium will decrease and the cells begin to produce bioactive compounds, especially secondary metabolite as a defense mechanism because of the limitation of nutrient in the medium. According to a study conducted by Khanpour-Ardestani et al. (2015), the content of acetonide compounds in *S. striata* is in the highest amount during the exponential phase and gradually decreased in the stationary phase. Subculturing the cell line with a certain period is one method to maintain cell viability to produce bioactive compounds. Sierra et al. (1992) succeeded in stabilizing alkaloid compounds in two cell line cultures of the *Tabernaemontana divaricata*. The highest production of alkaloids in the 4th day after the change of the medium and the growth remains stable during 30 subculture with a subculture interval every 9 days. The other study was found in the production of betaxanthins in callus culture of *Beta vulgaris* L. var 'Dark Detroit'. Production of betaxanthins in callus cells line of the *B. vulgaris* increased 1.8-fold after 48 subcultures with subculture intervals every 14 days (Trejo-Tapia et al. 2008). As well as the stability of synthesized verbascoside in cell line culture suspension of *Buddleja cordata* Kunth after being subcultured for 5 continuous years (Arano-Varela et al. 2020). This study provided an efficient way for further regulation of biosynthesis and production of bioactive compounds on scale-up in kaffir lime cell line culture. Analyzing post-subculture bioactive compounds and how appropriate the subculture cycle maximizes the production of secondary metabolites needs further investigation.

CONCLUSION

Technical grade yeast is an elicitor candidate for kaffir lime cell suspension culture based on morphology and growth pattern observation as pure grade yeast. Hence we could use either pure or technical grade (commercial baker)

yeast in kaffir lime cell elicitation. The elicited kaffir lime cell line has the same growth pattern as the control cell. The initial stationary phase was on the 17th day. Subculturing kaffir lime cell line at this phase is needed to maintain the viability of kaffir lime cell line.

AUTHORS CONTRIBUTION

D.Y.R. collected and analyzed the data and wrote the manuscript and revised it, F.D. collected and analyzed the data and wrote the manuscript, G.P.C wrote the manuscript, A.J.N. wrote the manuscript, A.B.S. analyzed the data, E.S. analyzed the data, W.A.S.T. design the research and supervised all process.

ACKNOWLEDGMENTS

This study was funded by PDUPT (*Penelitian Dasar Unggulan Perguruan Tinggi*) from the Ministry of Research, Technology and Higher Education of Indonesia (to WAST).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Aleu, J. et al., 1999. Biotransformation of the fungistatic sesquiterpenoid ginsenol by *Botrytis cinerea*. *Journal of the Chemical Society - Perkin Transactions 1*, (6), pp.727–730. doi: 10.1039/A808044E.

Arano-Varela, H. et al., 2020. Verbascoside production in long-term *Buddleja cordata* Kunth cell suspension cultures. *3 Biotech*, 10(6), pp.1–9. doi: 10.1007/s13205-020-02222-4.

Bourgaud, F. et al., 2001. Production of Plant Secondary Metabolites: A Historical Perspective. *Plant Science*, 161, pp. 839-851.

Chen, H. & Chen, F., 2000. Effect of yeast elicitor on the secondary metabolism of Ti-transformed *Salvia miltiorrhiza* cell suspension cultures. *Plant Cell Reports*, 19(7), pp.710–717. doi: 10.1007/s002999900166.

Chodisetti, B. et al., 2013. Improved gymnemic acid production in the suspension cultures of Gymnema sylvestre through biotic elicitation. *Plant Biotechnology Reports*, 7(4), pp.519–525. doi: 10.1007/s11816-013-0290-3.

Damayanti, F. et al., 2020. Variation of 2,4-dichlorophenoxyacetic acid (2,4-D) concentration on kaffir lime callus growth as raw material for cell suspension. *AIP Conference Proceedings*, 2260. doi: 10.1063/5.0016420.

Dwimahyani, I., 2007. Metode Suspensi Sel Untuk Membentuk Spot Hijau Pada Kultur In-Vitro Galur Mutan Tanaman Jarak Pagar (Jatropha curcas L). *Jurnal Ilmiah Aplikasi Isotop dan Radiasi*, 3(2), pp.55–79. doi: 10.17146/jair.2007.3.2.559.

Fajarina, S. et al., 2021. Evaluation of Anticancer Bioactive Compounds and Cytotoxicity of Kaffir Lime (Citrus hystrix Dc.) Callus Extract Post Preservation. *Indonesian Journal of Pharmacy*, 32(2), pp.179–192. doi: 10.22146/ijp.1062.

Gueven, A., 2003. Plant Tissue Cultures in Production of Secondary Metabolites. *Scientific Works of UFT*, pp.553–556.

Habibah, N.A. et al., 2017. Flavonoid production, growth, and differentiation of Stelechocarpus burahol (Bl.) hook. f. and th. cell suspension culture. *Pakistan Journal of Biological Sciences*, 20(4), pp.197–203. doi: 10.3923/pjbs.2017.197.203.

Jamil, S.Z.M.R., et al., 2018. Metabolite profiles of callus and cell suspension cultures of mangosteen. *3 Biotech*, 8(8), pp.1–14. doi: 10.1007/s13205-018-1336-6.

Khanpour-Ardestani, N., Sharifi, M. & Behmanesh, M., 2015. Establishment of callus and cell suspension culture of Scrophularia striata Boiss.: an in vitro approach for acteoside production. *Cytotechnology*, 67(3), pp.475–485. doi: 10.1007/s10616-014-9705-4

Klis, F.M. et al., 2002. Dynamics of cell wall structure in Saccharomyces cerevisiae. *FEMS Microbiology Reviews*, 26(3), pp.239–256. doi: 10.1111/j.1574-6976.2002.tb00613.x.

Leksonowati, A., Witjaksono & Ratnadewi, D., 2017. Induction of Callus Culture and Cell Suspension Culture of Aquilaria malaccensis Lam. *Berita Biologi*, 16(1), pp.1–11. doi: 10.14203/beritabiologi.v16i1.2687

Montes de Oca, R. et al., 2016. Yeast : Description and Structure Chapter 2 Yeast : Description and Structure. *Yeast Additive and Animal Production*, (February), pp.3–13.

Moscatiello, R., Baldan, B. & Navazio, L., 2013. Plant Cell Suspension. *Plant Mineral Nutrients*, 953(July), pp.195–207. doi: 10.1007/978-1-62703-152-3_5

Murthy, H.N., Lee, E.J. & Paek, K.Y., 2014. Production of secondary metabolites from cell and organ cultures: Strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell, Tissue and Organ Culture*, 118(1), pp.1–16. doi: 10.1007/s11240-014-0467-7

Namdeo, A.G., 2007. PHCOG REV .: Review Article Plant Cell Elicitation for Production of Secondary Metabolites : A Review. *Review Literature And Arts Of The Americas*, 1(1), pp.69–79.

Pasitvilaiturm, M. & Pankasemsuk, T., 2012. Endosperm culture of Jatropha curcas L. *Chiang Mai University Journal of Natural Sciences*, 11(1 SPECIAL ISSUE), pp.97–102.

Pereira, P.S. et al., 2007. Enhanced triterpene production in Tabernaemontana catharinensis cell suspension cultures in response to biotic elicitors. *Quimica Nova*, 30(8), pp.1849–1852. doi: 10.1590/S0100-40422007000800010.

Ramirez-Estrada, K. et al., 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules*, 21(2). doi: 10.3390/molecules21020182

Ratnasari, J., Siregar, A.H. & R. E., R., 2001. Pengaruh Pemberian Elisitor Ekstrak Khamir Saccharomyces Terhadap Kandungan Ajmalisin dalam Kultur Agregat Sel Catharanthus roseus (L .) G . Don. *Berita Biologi*, 5 (April), pp.349–355. doi: 10.14203/beritabiologi.v5i4.1118.

dos Santos, A.L.W. et al., 2010. Biochemical and morphological changes during the growth kinetics of Araucaria angustifolia suspension cultures. *Brazilian Archives of Biology and Technology*, 53(3), pp.497–504. doi: 10.1590/S1516-89132010000300001.

Sierra, M.I. et al., 1992. Stability of alkaloid production in cell suspension cultures of Tabernaemontana divaricata during long-term subculture. *Plant Cell, Tissue and Organ Culture*, 28(1), pp.59–68. doi: 10.1007/BF00039916.

Sitinjak, R.R., Siregar, A.H. & Rizkita, R., 2000. Pengaruh Pemberian Ekstrak Saccharomyces cerevisiae Hansen terhadap Kandungan Gosipol pada Kultur Kalus Gossypium hirsutum L. *Berita Biologi*, 5(2), pp.131–136. doi: 10.14203/beritabiologi.v5i2.1133.

Sparapano, L. & Bruno, G., 2004. Cupressus callus and cell suspension cultures: Effect of seiridins on their growth and sensitivity. *In Vitro Cellular and Developmental Biology - Plant*, 40(6), pp.617–625. doi: 10.1079/IVP2004581.

Sukmara, E., Sukamto, L.A. & Bintang, M., 2014. Induksi dan Karakter Pertumbuhan Kalus Triploid dari Endosperma Avokad (Persea americana Mill.). *Current Biochemistry*, 1(1), pp.20–28.

Thorat, A.S. et al., 2017. Plant regeneration from cell suspension culture in Saccharum officinarum L. and ascertaining of genetic fidelity through RAPD and ISSR markers. *3 Biotech*, 7(1), pp.1–12. doi: 10.1007/s13205-016-0579-3.

Trejo-Tapia, G. et al., 2008. Effect of screening and subculture on the production of betaxanthins in Beta vulgaris L. var. "Dark Detroit" callus culture. *Innovative Food Science and Emerging Technologies*, 9(1), pp.32–36. doi: 10.1016/j.ifset.2007.04.009.

Tunjung, W.A.S. et al., 2015. Anti-Cancer Effect of Kaffir Lime (Citrus hystrix DC) Leaf Extract in Cervical Cancer and Neuroblastoma Cell Lines. *Procedia Chemistry*, 14, pp.465-468. doi: 10.1016/j.proche.2015.03.062.