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Enhanced Production of Organosulfur Bioactive Compounds in Cell Suspension Culture of Single Garlic (*Allium sativum* L.) Using Precursor Feeding

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Abstract

Precursor feeding in cell suspension culture is one of the effective strategies used to induce plant bioactive compounds. The effect of feeding different glutathione precursors on cell suspension growth and organosulfur production in cell suspension of single garlic (*Allium sativum L.)* was investigated. The establishment of suspension culture was carried out by transferring callus to liquid MS media supplemented with 0.3 mg/L 2.4 D and 0.5 mg/L kinetin. The addition of glutathione precursor up to 15 mM on the culture media increased the cell suspension biomass and the accumulation of organosulfur compounds. The highest fresh weight (2.691 ± 0.006g), dry weight (1.738 ± 0.007g), settled cell volume (15% ± 0.0), and growth index (1.69 ± 0.006) were obtained from a media with 10 mM glutathione addition. HPLC analysis revealed 30 types of organosulfur compounds in the cell suspension culture of single garlic. The highest 12 essential organosulfur bioactive compound was detected from the media augmented by 12.5 mM glutathione (3 to 4 fold than the control). The docking molecular visualization results toward the ligands acting as a substrate with the targeted protein in the alliin biosynthesis through glutathione pathway showed interactions in the ligand-protein complex. Feeding glutathione is an effective means to increase the production of organosulfur bioactive compounds in single garlic cell suspension.

Keywords: Cell suspension, Precursor feeding, Organosulfur compound, Single garlic

1. Introduction

Single garlic (*Allium sativum* L) is garlic with only one clove in each bulb. Compared to the garlic with many cloves, single garlic is frequently used to solve different health issues due to its robust therapeutic properties (Bharat, 2014; Subramanian *et al*., 2020). Its unique smell and organosulfur compound content have carried various biological functions of garlic, such as being antioxidant (Sankaran *et al*., 2010; Rahman, 2012; Jang *et al*., 2017), antimicrobial (Nakamoto *et al*., 2020), anti-inflammatory (Tavakoli-Far *et al*., 2021) and immmunity within COVID-19 infection (Donma and Donma, 2020). Besides, it can also inhibit diabetes (Habtemariam, 2019), atherosclerosis (Lindstedt *et al*., 2021), hypertension (Ugwu and Suru, 2016; Saljoughian *et al*., 2017), and cancer (Pourzand *et al*., 2016; Zhang *et al.,* 2020).

The essential organosulfur compounds within garlic include alliin, allicin, allyl sulfide group, sulfide, vinyl dithiin, ajoene (Ramirez, 2017). Alliin is the parent in the forming of other groups of organosulfur compounds. Alliin is produced through the reaction between glutathione and allyl sources. The biosynthesis of the organosulfur compounds begins with the conjugation among glutathione and methacrylyl-CoA, resulting in S-(2-carboxypropyl) glutathione, an intermediate compound in the formation of alliin. After that, the glycyl group is eliminated from S-(2-carboxypropyl)glutathione, become S-(2-carboxypropyl) cysteine; meanwhile, the S-2-carboxypropyl group is transformed into S-2-propenyl group through an oxidative decarboxylation become γ-glutamyl-S-2-propenylcysteine (γ-glutamyl-S-allyl cysteine). The γ-glutamyl-S-allylcysteine experiences γ-glutamyl group omission, become S-allylcysteine. Oxygenation toward S-allylcysteine results in S-allyl cysteine sulfoxide or alliin (Yoshimoto *et al*., 2019). Hydrolysis of alliin by the alliinase enzyme will produce an intermediate compound of allyl sulfenic acid which then undergoes condensation to produce allicin (Borlinghaus, 2014). Allicin is an unstable compound and can quickly be degraded into allyl disulfide, ajoene, dithiin, and other sulfur compounds (Gruhlke, 2010).

In the pharmaceutical field, the plant cell and tissue culture can be an alternative source to attain bioactive compounds (Murthy, 2014; Espinosa-Leal *et al.,* 2018). One of the effective means and strategies to increase bioactive compound production is through the plant tissue culture with the precursor feeding (Gaosheng and Jingming, 2012; Isah *et al*., 2018; Guerriero *et al.*, 2018).

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The precursor is an exogenous or endogenous compound converted by the plant cells into secondary metabolites through biosynthetic pathways. The addition of precursor can be carried out based on the inclusion of intermediate compounds in the biosynthesis pathway of the bioactive molecule during the culture period as an additional substrate to enhance the production of the bioactive compounds within the cell culture (Isah *et al*., 2018; Singh and Sharma, 2020). Most of the intermediary substances can be selected as the precursor. The upstream precursor is transformed into a downstream compound using the specific enzyme catalysis. The precursor concentration determines the reaction speed; a high concentration frequently results in a more significant reaction pace than a lower concentration (Gaosheng and Jingming, 2012).

A number of studies discovered that the use of precursor expand the bioactive compounds on some plants, such as 200 mg/L concentration of tryptophan improves the alkaloid production on the callus culture of god's crown (*Phaleria macrocarpa* [Scheff.]Boerl.) (Gusni *et al*., 2015), 150 mg/L concentration of tryptophan increases the thymol and proline production, while its 150 mg/L concentration multiply the coumarin production on callus culture of *Verbascum thapsus* L (Al- Jibouri *et al*., 2016). Simultaneously, 100 µM L-phenylalanine concentration increases the scopoletin production on the cell culture of *Spilanthes acmella* Murr (Abyari *et al*., 2016). This study aims to investigate the growth of cell suspension and production of organosulfur bioactive compound with the addition of various concentrations of glutathione, as a precursor, in the suspension culture of single garlic (*Allium sativum* L.).

This study aims to analyze the growth of cell biomass and the production of bioactive organosulfur compounds in cell suspension cultures of single garlic (*Allium sativum* L.) with the addition of glutathione as a precursor feeding at different concentrations. The results of this study were expected to obtain alternative methods to increase the production of organosulfur bioactive compounds in single garlic, which can help the availability of organosulfur bioactive compounds that have important benefits in the health field.

2. Materials and Methods

2.1. Establishment of cell suspension culture

The callus was induced from crown explant of single garlic variety Tawangmangu Baru, obtained from Magelang, Central Java. The explant was cultured on solid MS media with the addition of 0.3 mg/L 2.4-D and 0.5 mg/L kinetin. The cell suspension was initiated by inoculating 1 g of friable callus in 25 ml of liquid MS media, with the same growth regulator as callus induction media. The cell suspension was incubated at 25°C under continuous fluorescent white light (approx. 13.5 µmol/m^2.s) and agitated on a shaker at 100 rpm. It was routinely subcultured every two weeks by transferring 1 g of cell mass from the previous culture into 25 mL new liquid MS medium. The cell biomass was weighted using the SCV method every three days for three weeks to obtain the optimum growth rate. The optimum growth rate is considered to be a medium from which maximum cell mass and organosulfur compound production was obtained within the single garlic cell culture. The initiation and establishment of single garlic cell suspension cultures were presented in Figure 1.

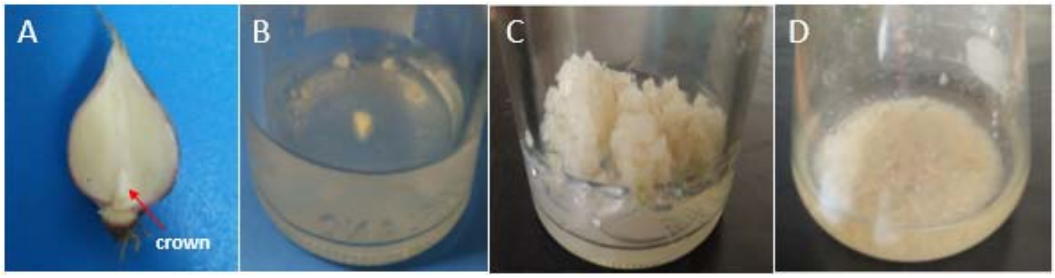


Figure. 1 Initiation and establishment of single garlic cell suspension: (A) Single garlic crown explant (B) Inoculation of explant in the MS media (C) Single garlic callus (D) Single garlic cell suspension.

2.2. Feeding of glutathione precursor to cell suspension culture

In the precursor feeding, glutathione with variations of 0, 5, 7.5, 10, 12.5, 15 mM was added to the suspension culture media. The liquid MS media with no glutathione addition was used as a control. The cell suspension was harvested and analyzed after two weeks to attain cell suspension kinetics growth and accumulation of organosulfur bioactive compound. The effect of precursor feeding on the cell suspension culture was evaluated based on the culture growth and the production of organosulfur compounds. Evaluation of cell suspension growth was carried out on the growth parameters, including the fresh weight, the dry weight, settled cell volume, and growth index. Measurement of fresh weight was carried out by weighing the cells that have been previously settled, while the dry weight was done by weighing the cells that have been previously dried in an oven (50°C, 12 h). Measurement of settled cell volume was done by pouring the cell suspension into a measuring glass and leaving it for 1 hour until all cells were settled. The volume fraction of cells precipitated was expressed as settled cell volume. The growth index is calculated based on the difference between the final weight and the initial weight divided by the initial weight of the cell (Loyola-Vargas and Ochoa-Alejo, 2012).

2.3. Analysis of organosulfur content

The analysis of the organosulfur compound on the cell suspension was carried out using the HLPC method. Extraction of the single garlic cell suspension was carried out with a slight modification of the method described by Al-Jibouri *et al*. (2016). A 100 mg of cells were taken and crushed using pestle and mortar. The crushed cells were extracted with 95%

methanol. The solution was stirred until homogenized and incubated overnight at cold temperature. The collected extracts were centrifuged and filtered out using Whatman No.1. The aliquots from the filtrate were filtered again using 0.22 µm syringe filters. Organosulfur content in the extract was estimated by high-performance liquid chromatography (HPLC). The instrument used was HPLC Shimadzu with Shim-pack VP ODS (5 µm 150 x 4,6 mm) column type as the stationary phase. A detector type SPD 20-A UV-Vis with 210 nm wavelength was used. The mobile step used was 10 mM Potassium dihydrogen phosphate: Acetonitrile (1:1)(v/v), isocratically, with a flow speed of 1mL/minute.

2.4. Statistical Analysis

The experiment was analyzed using one-way ANOVA. The average score was compared using Duncan's Multiple Range Test, at a 5% significance level ($p < 0.05$), using SPSS software version 25.

2.5. Molecular docking in the biosynthesis of organosulfur compounds

The ligand preparation was completed by examining the compound activities using Pass Server (http://www.pharmaexpert.ru/passonline/) database. The potential effect of a compound on the targeted protein as its interaction partner was analyzed and predicted using the STITCH (http://stitch.embl.de) database. The ligand's three-dimension structure collection was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) database. The protein preparation was carried out using the protein with a role in the alliin biosynthesis path. It was analyzed and predicted using the Plant Metabolic Pathway (https://plantcyc.org/) and STITCH (http://stitch.embl.de) database to discover its interaction with other compounds. The three-dimension structure collection for the targeted protein was completed by modelling it into the SWISS-MODEL Repository database (https://swissmodel.expasy.org/). After that, molecular docking was completed using PyRx 0.8 software to gain the best binding affinity. The compound with the lowest binding affinity was saved in the PDB format. The interaction of that compound was visualized in three dimensions using PyMol software. The two-dimension scheme and type of receptor interaction with formulated ligand were visualized using Discovery Studio software.

3. Results

3.1. The effects of glutathione precursor feeding on cell suspension growth

The addition of glutathione as a precursor in the culture medium showed a significant effect on the suspension culture growth of single garlic. As compared to control, the addition of glutathione with a 5 to 15 mM concentration range can enhance the fresh weight, dry weight, settled cell volume, and growth index. The fresh weight, dry weight, settled cell volume, and growth index increased from 5 mM to 10 mM and subsequently decreased at 12.5 mM and 15 mM glutathione concentration, as presented in Figure 2.

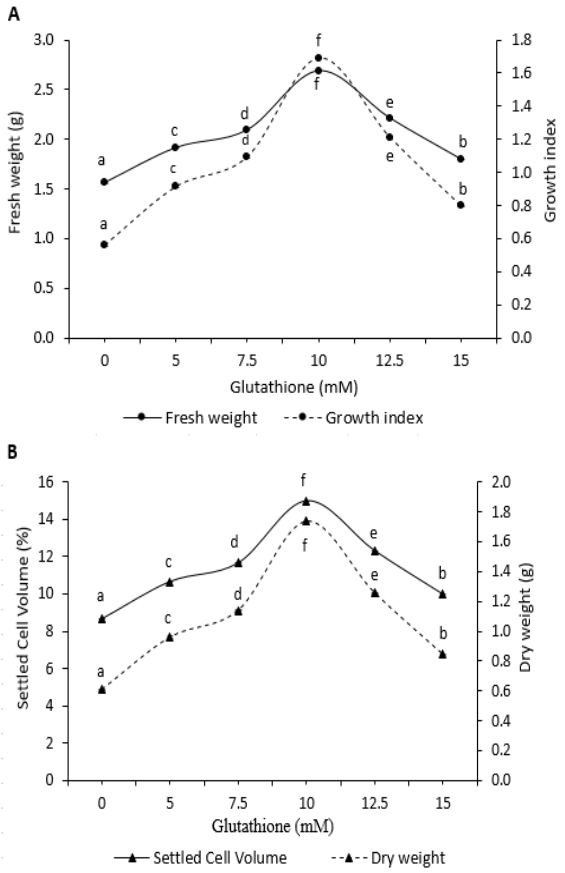


Figure 2. The effect of glutathione precursor feeding in cell suspension culture of single garlic: (A) Fresh weight and growth index (B) Settled cell volume and dry weight. The different letters show significant differences in the Duncan's Multiple Range Test ($p<0.05$) on each parameter.

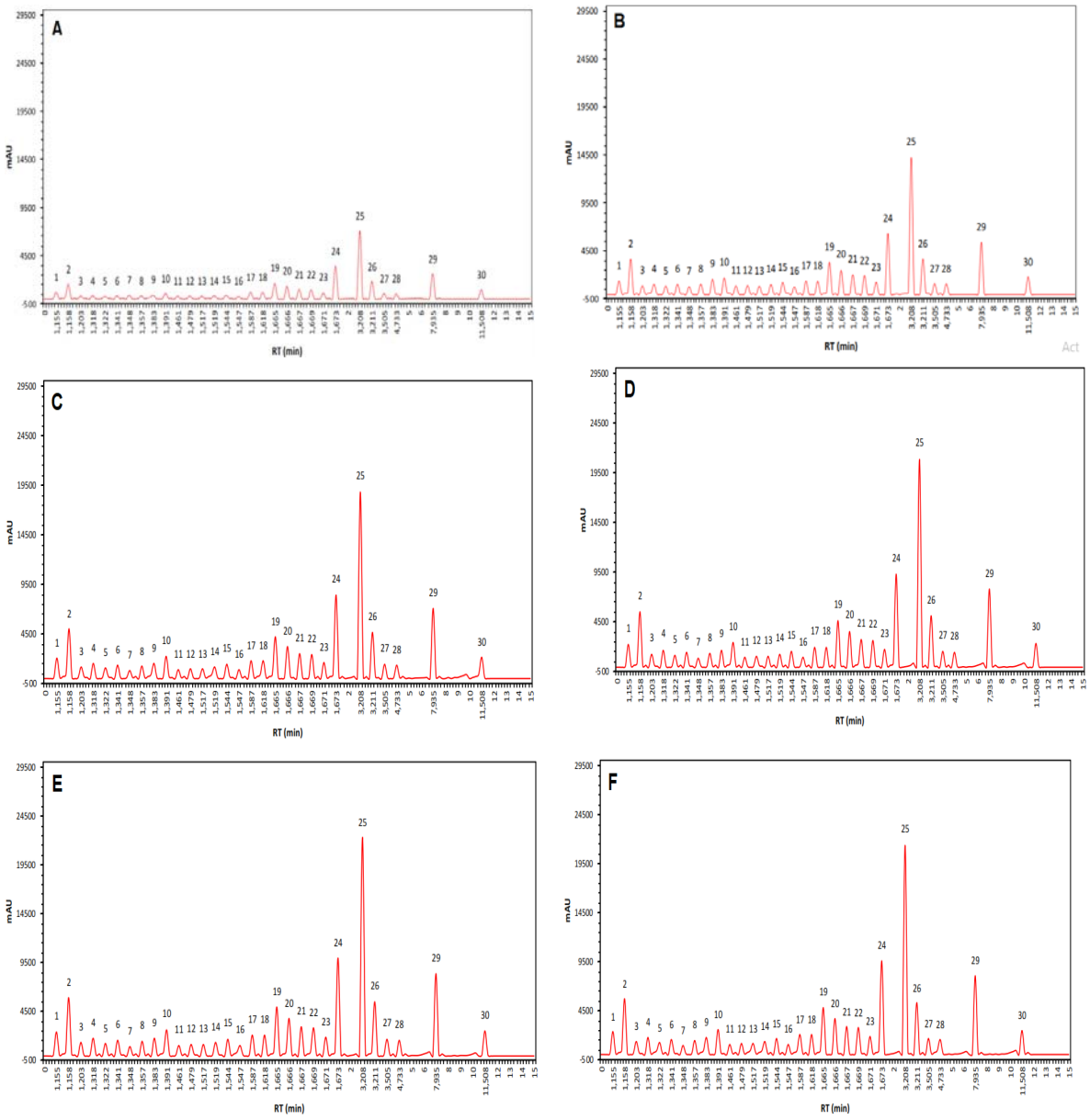
The cell mass, including fresh weight, dry weight, and settled cell volume without the addition of glutathione to the culture medium, were 1.562 ± 0.006 g, 0.608 ± 0.007 g, 8.7 ± 0.0 %, respectively. The highest cell suspension growth was produced at the addition of 10 mM glutathione for all growth parameters, of about 2.691 ± 0.006 g on fresh weight, 1.738 ± 0.007 g on dry weight, and 15 ± 0.0 % on settled cell volume. The growth index represents the same increases and decreases in the cell fresh weight at each glutathione concentration added to the culture medium. The maximum growth index (1.692 ± 0.006) was achieved at the addition of 10 mM glutathione, which showed the occurrence of faster growth compared to control (0.562 ± 0.006).

3.2. The effects of glutathione precursor feeding on organosulfur production

The HPLC chromatogram analysis demonstrated that the single garlic cell suspensions, with or without precursor, identified 30 types of organosulfur compounds. The different peak area of each compound represents the organosulfur bioactive compound level difference in each glutathione concentration. From those 30 compound types, 12 essential organosulfur compounds for human health, namely alliin, allicin, ajoene, dithiin groups (2-vinyl 1,3-

dithiin; 3-vinyl 1,2-dithiin) and allyl sulfide group (2 propenyls 1 propenyl disulfide; 1 propenyl allyl disulfide; allyl methyl disulfide; allyl propyl disulfide; allyl trisulfide; allyl methyl trisulfide; diallyl heptasulfide) (**Figure 3**). However, among those 12 compounds, alliin, allicin, and ajoene are responsible for the characteristic taste and odor of single garlic.

The addition of glutathione on the culture media significantly affected the increase of 12 organosulfur bioactive compound levels. The organosulfur bioactive compound level improves from the 5 mM to 15 mM glutathione addition. Meanwhile, the highest level was detected at 12.5 mM precursor addition (**Figure 4**). In the suspension culture, alliin, allicin, and ajoene levels were found to have more considerable than the other organosulfur compounds. These three compounds experience a significant increase in cell suspension due to the provision of the glutathione precursor. Their higher increase was detected at 12.5 mM precursor concentration. Additionally, on the control, the alliin, allicin, and ajoene compounds were 9.88, 4.81, and 3.70 mg/g, respectively. The compounds level increased three times higher (30.90, 13.90, and 11.64 mg/g, respectively) at the 12.5 mM precursor concentration. The same pattern of increase in levels was also showed in other organosulfur compounds, such as 2-propenyl 1-propenyl disulfide, allyl propyl disulfide, allyl methyl disulfide, 2-vinyl 1,3-dithiin, 3-vinyl 1,2-dithiin, E1-propenyl allyl disulfide, allyl methyl trisulfide, allyl trisulfide, and diallyl heptasulfide. The compounds level of allyl propyl disulfide, allyl methyl trisulfide, allyl trisulfide, and diallyl heptasulfide before treatment were 0.98, 0.99, 0.78, and 1.35 mg/g respectively, and increased three times higher after the administration of glutathione (2.93, 2.94, 2.36, and 3.52 mg/g, respectively). Meanwhile, the compounds level of 2-propenyl 1-propenyl disulfide, allyl methyl disulfide, 2-vinyl-1,3-dithiin, 3-vinyl-1,2-dithiin, and E1-propenyl allyl disulfide before treatment were 0.36, 0.83, 0.42, 0.46, 0.58 mg/g respectively, and also increased four times higher after the administration of glutathione (1.47, 3.68, 1.62, 1.90, and 2.35 mg/g, respectively).



Peak number

1	= 1,2-Epithiopropane	16	= **2-Propenyl 1-propenyl disulfide**
2	= Allyl mercaptan	17	= **Allyl propyl disulfide**
3	= Dimethyl disupfide	18	= **Allyl methyl trisulfide**
4	= 2,5-Dimethylthiophene	19	= Deoxyalliin
5	= 3-Methyl 2-cyclopentene 1 thione	20	= Allyl 2-propenethiosulfinate
6	= Isobutyl isothiocyanate	21	= Trans 1-propenyl allyl thiosulfinate
7	= 2,5-Dimethyltetrahydrothiophene	22	= 1-propenyl allyl thiosulfinate
8	= Propyl disulfide	23	= 2-propene 1-sulfinothioic acid 1-propenyl ester
9	= 1,3-Dithiane	**24**	**= Allicin**
10	= **Allyl methyl disulfide**	**25**	**= Alliin**
11	= 2,3,4-Trithiapentane	26	= Cycloalliin
12	= 1,2-Dimercaptocyclopentane	27	= **Allyl trisulfide**
13	= **2-Vinyl 1,3-dithiin**	28	=3,5-Diethyl 1,2,4-trithiolane
14	= **3-Vinyl 1,2-dithiin**	**29**	**= Ajoene**
15	= **E1-Propenyl allyl disulfide**	30	= **Diallyl heptasulfide**

Figure 3. Chromatogram of organosulfur bioactive compounds in cell suspension of single garlic with differences in glutathione concentration (A) 0mM. (B) 5mM. (C) 7.5mM. (D) 10mM. (E) 12.5mM (F) 15mM.

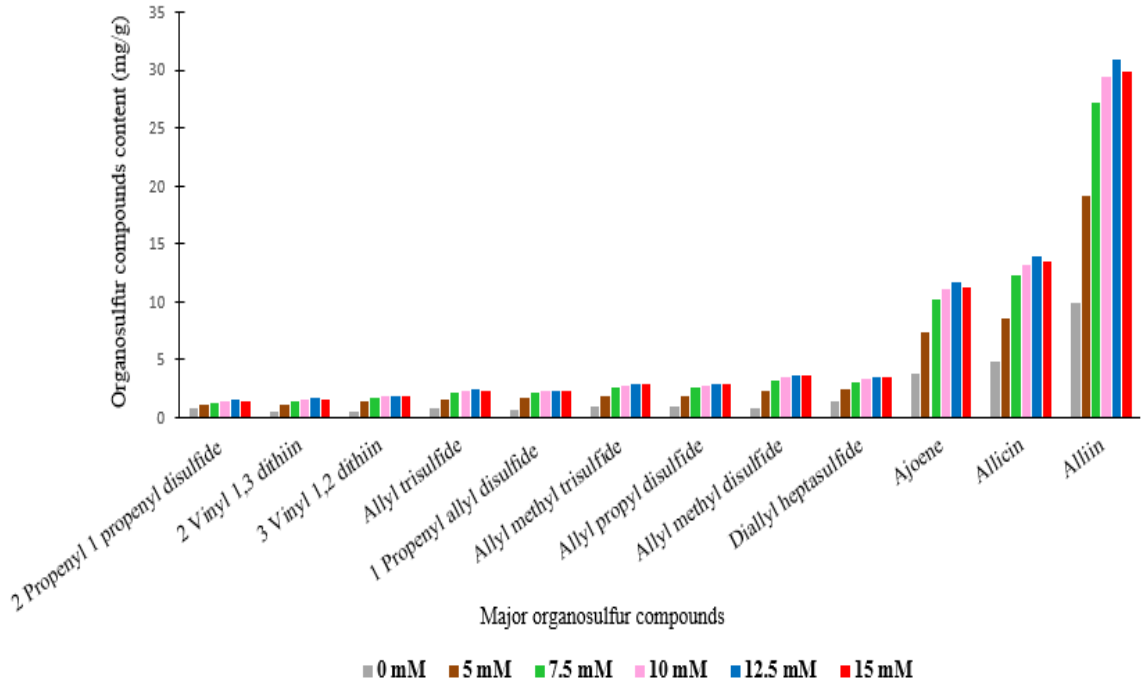


Figure 4. Differences in concentration of glutathione on organosulfur bioactive compounds content in single garlic suspension culture

3.3. The molecular docking visualization in the biosynthesis of organosulfur compounds

As a precursor, glutathione role that improves the organosulfur bioactive compound on the suspension culture can be predicted from the molecular docking between the ligands and enzymes that catalyze the reaction as a targetted protein. The biosynthetic pathways that are possible in the biosynthesis of alliin (as the parental of other organosulfur compounds) from the glutathione include some reaction stages, namely conjugation glutathione, the glycyl group removal, the S-alk(en)yl group modification, γ-glutamyl group removal, and S-oxygenation catalyzed by a specific enzyme. Glutathione transferase U24 (GSTU24) is predicted to catalyze glutathione conjugation and methacrylic acid into S-2 carboxypropyl glutathione. The release of glycyl group on S-2 carboxypropyl glutathione into γ-glutamyl-S-allylcysteine is presumed to be catalyzed by glutathione-γ-glutamylcysteinyltransferase (AT5G44070). Simultaneously, the discharge of the glutamyl group on γ-glutamyl-S-allylcysteine is possibly catalyzed by γ-glutamyltranspeptidase (AsGGT1). AsFMO1, which is a coding gene of garlic, is forecasted to catalyze S-allylcysteine, generating alliin. Besides, alliin conversion to be allyl sulfonate and amino acrylate is predicted to be catalyzed by alliin lyase.

The presence of glutathione precursors interaction as a ligand, GSTU24 as the targeted protein, through in silico method, is illustrated by the complex formulated those two (**Figure 5**). The molecular docking results reveal that interaction that formulates the complex among glutathione, and GSTU24 is observed in the initial reaction. The interaction can be seen from the bonds glutathione on the active side of GSTU24. That interaction emerges because of the hydrogen bond and van der Waals on the amino acid residues that bind the glutathione on the active bond side (**Figure 5A**). The docking results on the different reactions also indicate interactions with the formation of the complex between ligand and the targetted protein, such as between methacrylic acid-GSTU24, S-2 carboxypropyl glutathione-AT5G4407, γ-glutamyl S allylcysteine-AsGGT1, S-allylcysteine-AsFMO1, and alliin-alliin lyase1 (**Figure. 5B-F**).

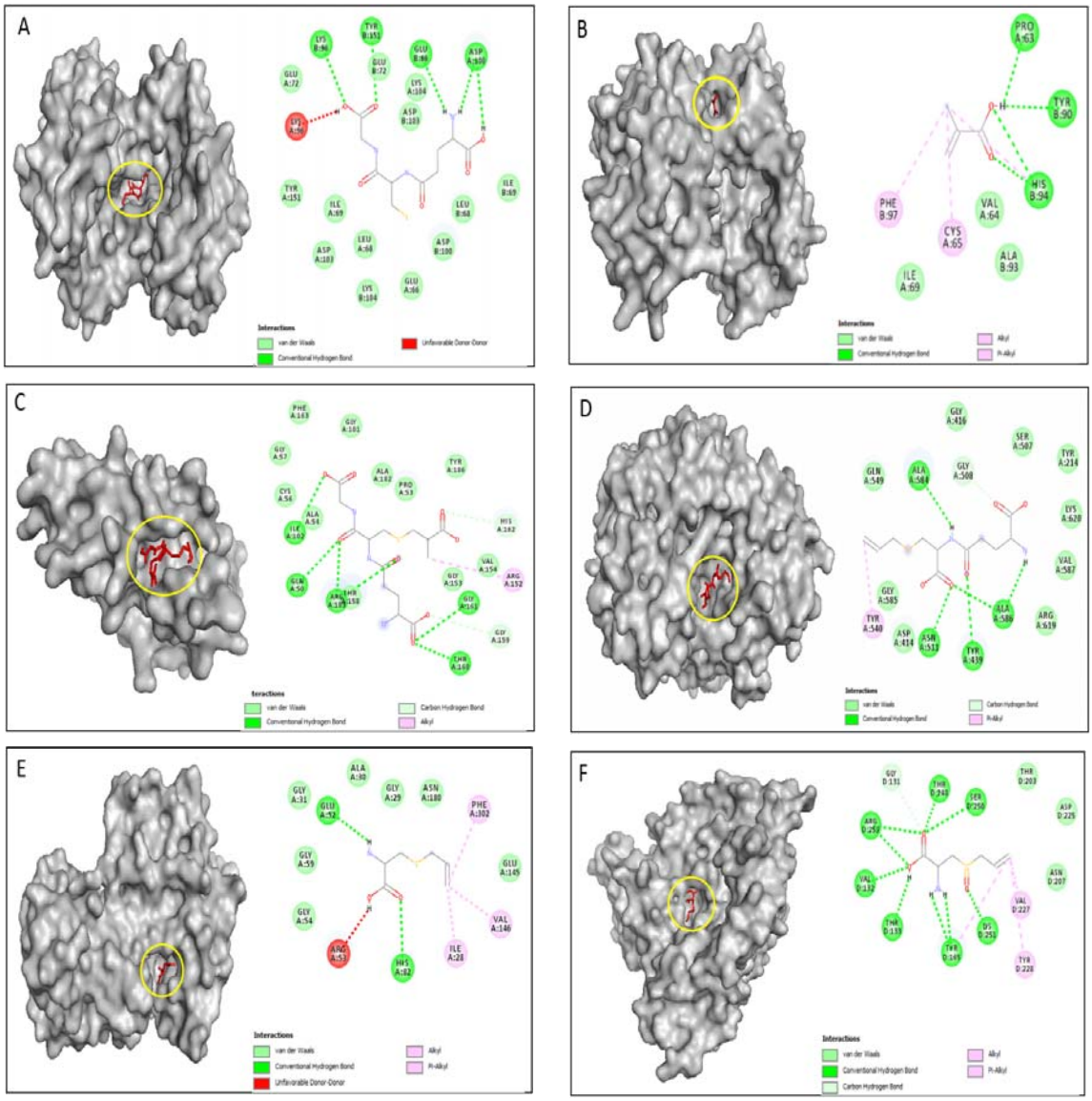


Figure. 5 Formed complex between ligand (red) - receptor (grey) and the bound appears in the interactions between ligand-receptor (the yellow circle): (A) Complex of glutathione and GSTU24 (B) Complex of methacrylic acid with GSTU24 (C) Complex of S-2 carboxypropyl glutathione and AT5G4407 (D) Complex of γ-glutamyl S-allylcysteine and AsGGT1 (E) Complex of S-allylcysteine and AsFMO1 (F) Complex of alliin with alliin lyase1.

4. Discussion

Feeding precursor in cell suspension cultures increased the growth of cultures and the content of organosulfur compounds. Glutathione is a tripeptide (γ-glutamyl-cysteine-glycine) with a thiol group, which has a role as the main regulator of cellular redox. Cellular redox potential is important mediator of several cell processes such as cell growth, proliferation, and differentiation. According to Ogawa (2005), Maher (2011), and Nahar *et al.* (2015), glutathione plays a role in modulating cell proliferation, growth, development, cell cycle, gene expression, and protein activation. Kerk *et al.* (1995) further stated that in the early stage of the cell cycle (G1), cells have a low glutathione content. The lack of glutathione availability at this stage can cause the cell cycle to stop (Potter *et al*., 2004). In the regulation of cell proliferation, glutathione is involved in the continuation of the cell cycle. Sequestering of glutathione in the nucleus occurs in the early stage of the cell proliferation cycle, where glutathione in the nucleus affects the gene transcription process, including cell division, redox regulation and regulation of transcription factors. Distributed glutathione into the nucleus causes a reduction in the accumulation of glutathione in the cytoplasm, whereas the availability of glutathione in the nucleus and cytoplasm in a balanced state is needed during the cell cycle (Diaz Vivancos *et al*., 2010; Diaz Vivancos *et al*., 2015; Schnaubelt *et al*., 2015). By feeding the glutathione, there will be an increase in the amount of glutathione needed by cells to be able to develop from the initial stage (G1) to the synthesis stage (S) (Kerk and Feldman, 1995). The glutathione also affects the production of endogenous cytokinin, growth regulators that play a role in promoting cell division in meristematic tissue. As reported by

Synkova *et al*. (2004), overproducing of cytokinins was detected in plants with high glutathione and ascorbate enzyme activity. Glutathione added to culture media contributes to stimulating growth when applied at levels appropriate to physiological levels. Precursor feeding with a too high concentration can inhibit cell growth and enzyme activity, which can be toxic to cells (Gaosheng and Jingming, 2012). A number of studies reported that the use of glutathione was able to increase callus multiplication in *Phoenix dactylifera* L (Al-Mayahi *et al*., 2020).

The increase in organosulfur compounds in single garlic cell suspension culture cannot be separated from the involvement of glutathione in the biosynthesis of alliin organosulfur compounds, which are parental to other organosulfur compounds. The administration of glutathione involved in the biosynthetic pathway into the culture media can increase the alliin organosulfur compound. It is based on the fact that any intermediate compounds present at the beginning or in the secondary metabolite biosynthetic pathway can increase the end product. In the biosynthetic pathway of alliin organosulfur compounds, glutathione is upstream and involved in the reaction's initial stage. According to Gaosheng (2012), upstream precursors can be converted into downstream compounds after being catalyzed by specific enzymes. The precursors added at the beginning or during the culture period can serve as additional substrates for increasing the high production of metabolites in cultivated plant cells, tissue, or organ cultures (Isah *et al*., 2018). In the conjugation reaction between glutathione and methacrylic acid to produce S-2 carboxypropyl glutathione, the enzyme glutathione transferase (GSTU24) is predicted to catalyze the reaction. The increase in S-2 carboxypropyl glutathione produced at the initial stage of the reaction will increase a product in the next reaction stage and increase the organosulfur compound as the end product. The stimulation given to the metabolic pathways in plant cell culture can produce bioactive compounds with a significant increase (Wang *et al*., 2001).

Glutathione as a precursor feeding can be seen in the visualization of molecular docking between glutathione and GSTU24. Glutathione and methacrylic acid can bind to the active site of GSTU24 by hydrogen bonding and van der Waals interactions, with a binding affinity of -6.5 and -4.6 kcal/mol, respectively. This interaction is dominated by hydrogen bonds, which are stronger than the other bonds. The formation of glutathione and methacrylic acid complexes on GSTU24 macromolecules, then glutathione has the potential to become a GSTU24 substrate to produce S-2 carboxypropyl glutathione. Plant glutathione S-transferase enzyme has various roles in endogenous metabolism; one of them is an enzyme that catalyzes glutathione conjugation (Dixon *et al*., 2010; Obeidat *et al*., 2017). The next reaction stage reveals an interaction formulating complex between the ligand and the target protein, namely S-2 carboxypropyl glutathione and AT5G4407, γ-glutamyl S allylcysteine and AsGGT1, S-allylcysteine and AsFMO1, alliin and alliin lyase1. The formation of the complex between the substrates as a ligand, and the enzymes that catalyze it as target protein at each reaction stage of the metabolic pathway, shows that organosulfur bioactive compound can be produced and increased by the addition of glutathione precursor.

5. Conclusion

The results of this study showed that glutathione as precursor feeding added to the culture medium is an effective method to increase the suspension cell growth and production of bioactive organosulfur compounds in single garlic cell suspension culture, which can enhance the availability of organosulfur compounds with important health benefits. Cell suspension culture of single garlic with glutathione precursor has the potential for scale-up studies at the commercial level by the pharmaceutical industry to further enhance the medicinally important bioactive organosulfur compounds. The molecular docking visualization on every reaction stage in the alliin biosynthesis through the glutathione pathway showed the presence of interaction between ligands as a substrate and enzymes as the target protein.

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