

Production of *Plutella xylostella* bioinsecticide for brassicaceae plant based on bromelain enzyme extracted from pineapple peel and isothiocyanate extracted from broccoli stem and radish peel

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Abstract. Brassicaceae plant is a commodity of edible plant that is very important for Indonesia, with cabbage worm (*Plutella xylostella*) as its main pest. Pest control with biopesticide is needed to preserve environment and increase crops production. Several biological substance is toxic to cabbage worm, including bromelain enzyme and isothiocyanates. Extraction of bromelain is experimented by varying mixing duration (15, 30, 45, 60 mins) and solvent type (distilled water and phosphate buffer). Extraction of isothiocyanate is experimented by varying the solvent ratio (1:1, 1:2, 1:3), using dichloromethane as solvent. For the broccoli stem sample, phosphate buffer is added to maintain pH. Enzyme activity test is used with product tyrosin concentration as standard. Isothiocyanate is determined with GC-MS analysis. The most effective extraction method for bromelain is with distilled water as solvent with 15 min mixing time. The most effective extraction method for isothiocyanate is with 1:1 solvent ratio at 37°C temperature without adding phosphate buffer. Efficacy of each sample to cabbage worm is also experimented by feeding it broccoli leaf spread with sample and observing the mortality rate. Bromelain and isothiocyanate proved to be toxic to cabbage worm and can be used as alternative bioinsecticide with the highest mortality of cabbage worm is by isothiocyanate from radish peel, reaching 100%.

1 Introduction

Brassicaceae plants, namely broccoli, cabbage, radish, etc. is a group of horticulture plants that by statistics is the largest plant group produced in Indonesia in recent years, reaching 1,513,315 tonnes in 2016. The plants are commonly attacked by pests, like *Plutella xylostella* (cabbage worm) and *Crociodolomia pavonana* (cabbage crop worm) (Sipahutar, 2017). Cabbage worm is a highly pesticide resistant pest (Pracaya, 2009). The correct use of pesticide, be it in the type, dosage, volume, or the way it is applied, will be a good tool to minimize the pest attack. However, there are negative effects of using chemical pesticide to the plants, environment, and even the human who consume the plants. Pest control with biopesticide is needed to preserve environment and increase crops production (Ratnasari, 2017).

Several biological substance is toxic to cabbage worm, with two being bromelaine enzyme and isothiocyanates (Dent, 2000). Bromelaine enzyme degrade protein, lipin, and chitin inside insect's body (Manzoor, Nawaz, Mukhtar, & Haq, 2016) and is abundantly available in pineapple waste, namely its peel. Isothiocyanate is a metabolite toxic to insect which is

contained in plant, namely Brassicaceae plant like broccoli and radish, as its natural defense mechanism (Li & Xie, 2015). Therefore the extraction of these substance and its use as biopesticide can be an alternative way to produce an environmental friendly way to combat pests.

There are numerous research in the field of insecticide from plants, including bioinsecticide from *suren* seed with 50% mortality rate in 3 days and *suren* leaf with 92% mortality rate in 3 days (Darwiati, 2009), flavonoid as bioinsecticide from durian peel with 89.78% mortality rate in 7 days (Tunnisa, Mursiti, & Jumaeri, 2017), flavonoid as bioinsecticide from jatropha bark with 100% mortality rate in 5 days (Irawati, 2017), alkaloid as bioinsecticide from tobacco leaf with 60% mortality rate in 5 days (Wulandari, 2017), zingibain enzyme as bioinsecticide from red ginger with 20% mortality in 5 days (Afnan, 2017), and papain enzyme as bioinsecticide from papaya leaf and sap with 100% mortality in 5 days (Firda Nur, 2017).

2 Methods

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2.1 Extraction

2.1.1 Bromelain from Pineapple Peel

The method to extract bromelain enzyme from pineapple peel is as follows:

1. Crushing 200 grams of pineapple peel using a blender, then using filter paper with vacuum filter to remove rough fibers
2. Preparing 4 beaker glass and mixing the filtrate with distilled water as solvent at 1:1 ratio in room temperature for 15 mins, 30 mins, 45 mins, and 60 mins.
3. Moving the mixture to centrifuge tubes and applying centrifugation at 4°C temperature and 10000 rpm rotary speed for 10 mins.
4. Removing the deposit and storing the extract solution supernatant in refrigerator for analysis.
5. After analysis, taking the best mixing time and repeating the method with buffer phosphate pH 7 as solvent for comparison.

2.1.2 Isothiocyanate from Broccoli Stem

The method to extract isothiocyanate from broccoli stem is as follows:

1. Crushing 200 grams of broccoli stem using a blender, then using filter paper with vacuum filter to remove rough fibers.
2. Preparing 3 beaker glass and homogenizing the filtrate with phosphate buffer pH 7,2 10:1 v/v at 42°C temperature.
3. Mixing the homogenate with CH₂Cl₂ solvent at 1:1, 1:2, and 1:3 ratio at room temperature for 30 mins.
4. Moving the mixture to centrifuge tubes and applying centrifugation at 4°C temperature and 10000 rpm rotary speed for 10 mins.
5. Removing the deposit and taking the bottom layer.
6. Storing the extract solution bottom layer in refrigerator for analysis.

2.1.3 Isothiocyanate from Radish Peel

The method to extract isothiocyanate from radish peel is as follows:

1. Crushing 200 grams of radish peel using a blender, then using filter paper with vacuum filter to remove rough fibers.
2. Preparing 3 beaker glass and mixing the filtrate with CH₂Cl₂ solvent at 1:1, 1:2, and 1:3 ratio at 37°C temperature for 1,5 hour
3. Storing the mixture in refrigerator at 4°C temperature for 2 hours.
4. Moving the mixture to centrifuge tubes and applying centrifugation at room temperature and 756 x g rotary speed for 15 mins.
5. Removing the deposit and take the bottom layer.

6. Storing the extract solution bottom layer in refrigerator for analysis.

2.2 Analysis

Three kinds of analysis are applied on this experiment to determine which extraction method variation gives the highest extract yield and the correlation with worm mortality to prove that bromelain and isothiocyanate can be used as bioinsecticide.

2.2.1 Enzymatic Activity Analysis for Bromelain Sample

Enzymatic activity analysis is used to determine the activity of the enzyme in the extract solution. Bromelain enzyme catalyses the hydrolysis of casein to tyrosin, and the analysis will be done by measuring the amount of tyrosin produced. First is to make a standard curve, with the method as follows:

1. Mixing 100 mg tyrosin to 100 ml of distilled water to make a 1000 ppm solution.
2. Diluting the solution to a variation of concentration from 0-600 ppm (20, 40, 60, 80, 100, 300, 600).
3. Adding 3 ml biuret solution to 1 ml tyrosin concentration, homogenizing the mixture, then incubating the homogenate at room temperature for 10 mins.
4. Adding 10 drops of folin reagent, shaking it well, then incubating the solution at room temperature for 30 mins.
5. Using UV-Vis spectrophotometer to measure the absorbance at 750 nm wavelength and making the curve to get the slope equation. The slope equation is used to calculate the concentration of tyrosin produced by each sample.

Then proceed with the analysis, with the method as follows:

1. Mixing 2 ml extract solution with 2 ml phosphate buffer pH 7 0,5 M.
2. Pre-incubating the mixture at 37°C temperature for 5 mins.
3. Adding 2 ml casein 2% to the mixture and incubating it at 37°C for 10 mins.
4. Adding 4 ml trichloroacetic acid (TCA) to stop the reaction to the mixture.
5. Moving mixture to centrifuge tube and applying centrifugation at room temperature and 1000 rpm rotary speed for 10 mins.
6. Incubating the mixture at 37°C for 50 mins.
7. Adding 3 ml folin reagent 50% then shaking it well.
8. Using UV-Vis spectrophotometer to measure the absorbance at 750 nm wavelength. To determine the enzymatic activity, the concentration of the produced tyrosin is calculated using a standard curve.

2.2.2 Gas Chromatography Mass spectrophotometry for Isothiocyanate Sample

All samples from broccoli stem and radish peel extraction are analysed at PUSLABFOR (*Pusat Laboratorium Forensik*) using GC-MS to determine the existence and percentage of isothiocyanate in the samples.

2.2.3 Efficacy to Cabbage Worm

Efficacy to cabbage worm is carried with 4 concentration variation for the crude bromelain enzyme, which is 0% (negative control), 25%, 50%, and 100%, and 4 variation for the isothiocyanate extract, one for each volume ratio variation. The test is carried out to cabbage worm grown in Insect Physiology and Toxicology Laboratory, Plant Protection Department, Bogor Institute of Agriculture. The steps are as follows:

1. Preparing one set of petri dish, a sheet of 5x5 cm broccoli leaf, a sheet of paper towel, and 5 worms for each sample.
2. Smearing 1 ml of sample on a sheet of leaf and feeding it to the cabbage worms on top of a paper towel inside the petri dish for easier observation.
3. Observing the growth or mortality of the worms being fed every 24 hours for 7 days.
4. Replacing the leaf with a fresh one to feed the worms every 48 hours.

3 Result and Discussion

3.1 Bromelain Enzymat Extraction from Pineapple Peel

Enzymatic catalytic activity is defined as a measured property by the increase of conversion rate (ie reaction rate times volume) from a specific chemical reaction produced by enzyme in a specific measuring system (Cornish-Bowden, 2014).

Enzymatic activity is analysed by reacting enzyme sample with casein as substrate, where tyrosin will be produced. Tyrosin concentration of each sample is analysed with UV-Vis spectrophotometer. To get the relation between enzymatic activity with tyrosin concentration, the following formula is observed:

$$V = -\frac{d[S]}{dt} = \frac{d[P]}{dt} \dots \text{(Cornish-Bowden, 2014)}$$

V is conversion rate, [S] is substrate concentration, [P] is product concentration, and t is reaction time. Enzymatic activity is proportional with product concentration, which is tyrosin. Therefore, the sample with the highest tyrosin concentration, is assumed to have the highest enzymatic activity.

3.1.1 Effect of Mixing Duration to Extraction Result

Mixing duration is varied to know the effect of mixing duration to extraction result and get the optimum duration. Extraction is carried with distilled water as

solvent and mixing duration variation of 15, 30, 45, and 60 minutes. During extraction, bromelain enzyme is transferred from the pineapple peel juice (as feed solution) into solvent which is distilled water because of direct contact for a time. The longer the contact duration between solvent and feed, the better contact between the two phase is achieved, therefor component transfer from feed into solvent will be higher. This reaction is carried until equilibrium is reached (Ngatijo, Pranjono, Sri Galuh, & Windaryati, 2005).

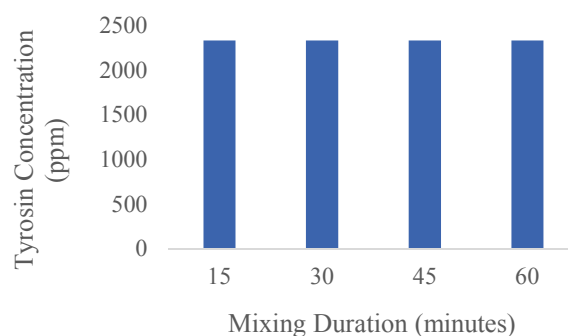


Fig. 1 Mixing duration variation against produced tyrosin concentration

All variations prove to have the same results, 2338.7 ppm tyrosin, which suggest that all sample have the same enzymatic activity and that mixing duration between 15-60 minutes do not have effect to the enzymatic activity and tyrosin concentration. The data suggested that equilibrium is reached within the first 15 minutes. Therefore, 15 mins mixing time is picked to be the most efficient time and then is carried to the next step, which is changing the solvent to phosphate buffer.

3.1.2 Effect of Solvent Type to Extraction Result

To study solvent type effect to extraction result, 2 variations of solvent type is used. The use of distilled water as solvent in the experiment is due to the fact that enzyme has high solubility in water, which practice is commonly refered to as isolation of enzyme. Bromelain is a water soluble enzyme due to its polar characteristic (Costa, Fernandes, Romao, & Ventura, 2014).

Phosphate buffer pH 7 is suggested as solvent to compare the extraction result with pH keeping. Phosphate buffer can keep system's pH value with small change in pH value (Bonner, 2007). Using buffer solution sometimes prove to be very efficient as solvent for some proteins and enzymes without causing denaturation (Bonner, 2007). A good solvent for enzyme extraction should be able to keep the pH value around the optimum pH value of the target enzyme. Bromelain has optimum pH value of 4.0, 4.5, and 6.8 (Harrach, et al., 1995). Both solvent is tested in the extraction method and the resut is reacted with casein to get tyrosin. The result is then analysed with UV-Vis spectrophotometer.

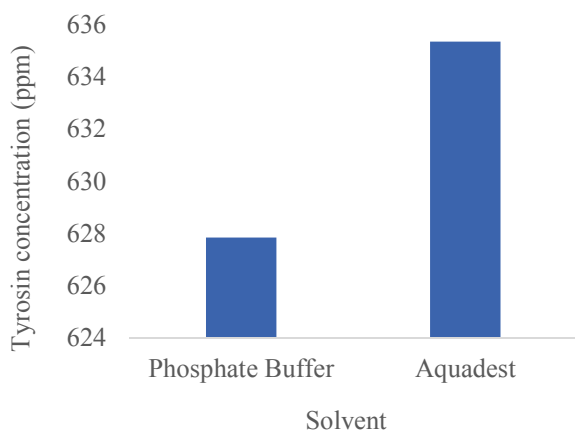


Fig. 2 Solvent type against produced tyrosin concentration

The data shows that sample with distilled water (aquadest) as solvent produced 635.35 ppm tyrosin, slightly higher than sample with phosphate buffer as solvent with 627.85 ppm tyrosin. This shows that the enzyme in the sample with distilled water as solvent has higher enzymatic activity. This suggests that polarity holds an important factor in the bromelain extraction and bromelain is more soluble in water than in phosphate buffer, and that both properties are more important as factors than keeping pH value. The experiment proves water to be more effective as bromelain solvent than phosphate buffer.

3.2 Isothiocyanate Extraction from Radish Peel and Broccoli Stem

Extraction of isothiocyanate from radish peel and broccoli stem. One of the important factors that affect extraction result is the volume ratio of feed : solvent.

Extraction result is analysed with GC-MS instrument. Analysis is carried out without standard solution to identify the content of the sample. Analysis result is presented as some possible compounds detected, the similarity percentage with data bank, area percentage according to peak height of the component, and retention time at which the compound is detected. The comparison of area percentage between samples will be used to determine which sample has the highest isothiocyanate content.

Isothiocyanate extraction from radish peel and broccoli stem is carried out using dichloromethane (CH₂Cl₂) as solvent because of its non-polar property as an organic compound that is able to solve isothiocyanate (MacBean, 2010). In choosing solvent, another important factor is boiling point. Dichloromethane's boiling point is at 40.2°C (Manual of Fumigation for Insect Control, 2018), which is good as solvent for isothiocyanate with much higher boiling point. A few drops of solvent with low boiling point like dichloromethane will evaporate in a few seconds at room temperature (Suryana, 2013). Dichloromethane is also a chlorohydrocarbon compound with the lowest toxicity (Back and Cotton, 1935 in (Manual of Fumigation for

Insect Control, 2018)) and is only dangerous to insects in high exposure (United States Environmental Protection Agency, 2017).

3.2.1 Effect of Feed : Solvent Volume Ratio to Isothiocyanate Extraction from Broccoli Stem

Table 1 GC-MS Analysis Result of Radish Peel Extract

| Sample | Feed : Solvent Volume Ratio | Possibility of Compound | Similarity Percentage |
|--------|-----------------------------|---|-----------------------|
| 1 | 1:1 | Disulfide, dimethyl / 2,3-Dithiabutane / Dimethyl disulfide / Methyl disulfide / (Methyldithio)methane / Dimethyl disulphide (CH ₃ S) ₂ | 96% |
| | | Disulfide, dimethyl | 91% |
| | | Disulfide, dimethyl | 91% |
| 2 | 1:2 | Disulfide, dimethyl / 2,3-Dithiabutane / Dimethyl disulfide / Methyl disulfide / (Methyldithio)methane / Dimethyl disulphide (CH ₃ S) ₂ | 91% |
| | | Disulfide, dimethyl | 91% |
| | | Disulfide, dimethyl | 91% |
| 3 | 1:3 | Disulfide, dimethyl / 2,3-Dithiabutane / Dimethyl disulfide / Methyl disulfide / (Methyldithio)methane / Dimethyl disulphide (CH ₃ S) ₂ | 94% |
| | | Disulfide, dimethyl / 2,3-Dithiabutane / Dimethyl disulfide / Methyl disulfide / (Methyldithio)methane / Dimethyl disulphide (CH ₃ S) ₂ | 94% |
| | | Disulfide, dimethyl | 94% |

In all of the samples tested, there is no isothiocyanate detected. There is, however, in the 7.6-7.61 minute retention time, a compound detected to be one of the following: dimethyl disulfide, 3-dithiabutane, methyl disulfide, or (methyldithio)methane. GC-MS uses high temperature (up to 150°C) in the analysis so it is possible that the isothiocyanate in the sample is degraded by thermal degradation. Some of the possible direct

derivative of allyl isothiocyanate are diallyl disulfide and diallyl sulfide.

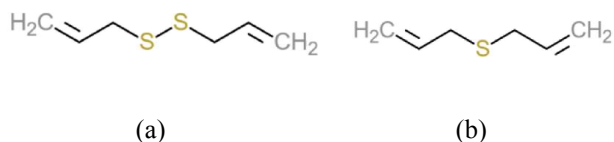


Fig. 3 (a) Diallyl disulfide (b) diallyl sulfide

These compound can be degraded also, by reduction, to the detected compound in the sample, dimethyl disulfide and methyl disulfide, which is detected in the sample.

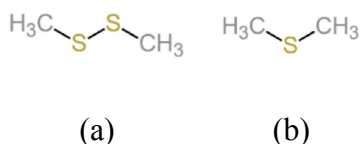


Fig. 4 (a) Dimethyl disulfide (b) dimethyl sulfide

Solvent and reaction temperature are some of the factor in extraction. The detection of allyl isothiocyanate derivative show that the used method, including dichloromethane as solvent and reaction temperature at 37°C, is proven to be successful in extracting isothiocyanate.

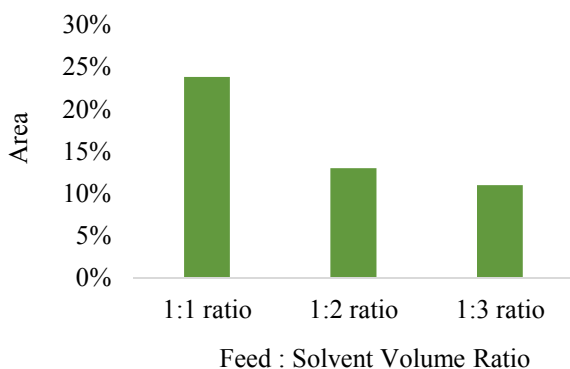


Fig. 5 Comparison of area percentage of the targeted compound in sample.

By looking at the area percentage of the compound in the sample, we can assume the amount of isothiocyanate in the sample. The compound has 23.82% area in the 1:1 solvent ratio sample, 13% area in the 1:2, and 11.02% in the 1:3. This possibly happen because the more solvent is added, other compound with higher solubility in dichloromethane is solved more than isothiocyanate, resulting in higher area for those other compounds. In

the sample of 1:2 and 1:3, the compound with increasing area percentage is buthane and 2-butanon.

3.2.2 Effect of Feed : Solvent Volume Ratio to Isothiocyanate Extraction from Broccoli Stem

Same with radish peel sample, there is no isothiocyanate detected in broccoli stem sample. However, there is also no isothiocyanate derivative detected in these sample, different from the sample from radish peel. There are two possibility, either there is very little isothiocyanate in the sample. When the concentration of a compound in a sample is very low compared to the other compound, the difference in peak height can make the much lower one unread. This very low concentration of isothiocyanate derivative can be because the method used which mix the sample with 10 times volume phosphate buffer make the ultimate ratio of broccoli stem essence:solvent to be very high in solvent, up to 20 times the volume. Looking at the data of the radish peel extract, the higher the solvent ratio, the lower the isothiocyanate extract. This is proportional with the data mentioned because the solvent ratio is very high.

Other than the solvent ratio, operation temperature can affect the extraction. In the method used for this sample, the extraction is held at room temperature. It can be assumed that the optimum temperature for extraction is 37°C.

Last, the difference of compound in broccoli stem and radish peel can give difference in the extraction process. Other compound in broccoli stem can be solved too with higher solubility in dichloromethane, making less isothiocyanate extracted from broccoli stem.

3.3 Efficacy of Bromelain Enzyme and Isothiocyanate Extract to Cabbage Worm

Efficacy test is done by smearing 1 ml of sample on the bottom side of fresh broccoli leaf with 5x5 cm size and letting it dry out. Cabbage worm eats the bottom epidermis of the leaf so it will be exposed directly. Solvent with low boiling point like dichloromethane need a very short time to be dry because the solvent can immediately evaporate, unlike water which has a high boiling point. In the negative control, 20% of the worm died so a sample will be considered effective in killing worm if 40% or more worm died.

3.3.1 Bromelain

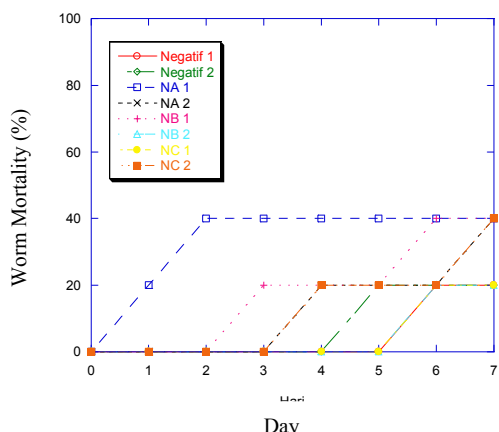


Fig. 6 Worm mortality percentage of bromelain extract sample with dilution variation.

All sample proved to have 40% mortality rate on worm over 7 days. The difference lies in the speed at which it is reached. The sample with 100% liquid extract concentration (without dilution) reached 40% mortality on the second day. The sample with 50% liquid extract concentration on the sixth day and the sample with 25% liquid extract concentration on the seventh day. This suggests that the concentration of enzyme in the extract is not yet in the lethal dosage to cabbage worm. Further purification, for instance the removal of solvent, can be essential to more accurately determine the lethal dosage of the enzyme to cabbage worm. However it can be proved that bromelain is toxic to cabbage worm because the concentration of bromelain is proportional to the rate in which the mortality rate is achieved.

3.3.2 Isothiocyanate

3.3.2.1 Radish peel extract

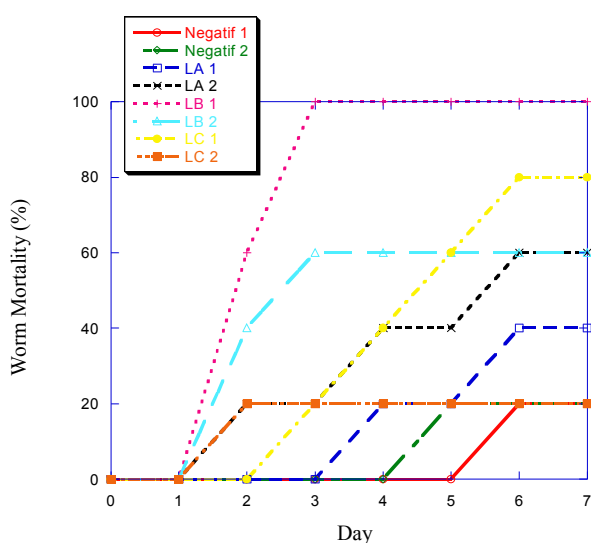


Fig. 7 Worm mortality percentage of isothiocyanate from radish peel extract sample with solvent ratio variation. LA=1:1, B=1:2, LC=1:3

The extract gives 60-100% mortality rate to cabbage worm. The highest mortality rate, at 100%, is reached by the sample with 1:2 solvent ratio. The isothiocyanate derivative in that sample has 13% area in the GC-MS reading. The second highest mortality rate, at 80%, is reached by the sample with 1:3 solvent ratio, with 11.6% area of isothiocyanate derivative detected. This proves that the higher the concentration of isothiocyanate, the higher the mortality rate, suggesting that isothiocyanate is toxic to cabbage worm and can be used as insecticide. There is an anomaly however in the sample with 1:1 solvent ratio where the mortality rate is only 60% while having 23.82% area of isothiocyanate derivatives. Cabbage worm has a very short life cycle and the older they get the more resistant they are. It is possible that some of the worms have entered fourth instar in which they are resistant to most pesticides (Sastrosiswojo, 1987). This can be solved by purification methods such as separation. By removing the solvent before applying to use as bioinsecticide, the concentration of the bioinsecticide can be controlled and more accurate lethal dosages can be analyzed and achieved.

3.3.2.2 Broccoli stem extract

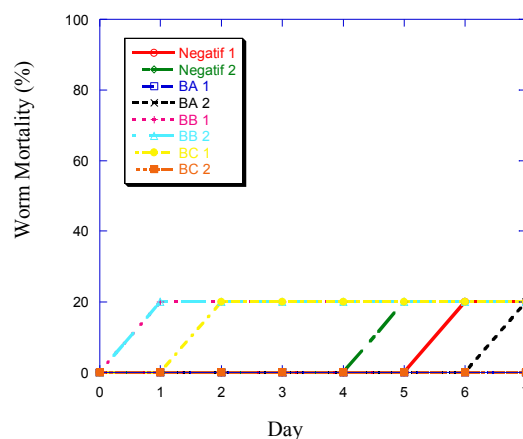


Fig. 8 Worm mortality percentage of isothiocyanate extract from broccoli stem sample with solvent ratio variation. BA=1:1, BB=1:2, BC=1:3

The extract gives very low mortality rates, at 0-20%. This result is proportional to the GC-MS analysis of broccoli stem extract, in which the isothiocyanate derivatives detected are very low. This result proves that there are no other compounds in the sample that are toxic to cabbage worms and further proves that isothiocyanate is the one that is deadly to cabbage worms.

4 Conclusion

1. Optimum mixing duration for extracting bromelain enzyme in pineapple peel is 15 minutes.
2. Bromelain enzyme extraction with distilled water as solvent from pineapple peel gives higher activity than with phosphate buffer.
3. Extraction of bromelain enzyme with distilled water and optimum mixing duration of 15 minutes gives bromelain enzyme which is capable of producing 635.5 ppm tyrosin from 20000 ppm casein substrate.
4. Optimum feed : solvent ratio from for isothiocyanate extraction is 1:1 at 37°C.
5. Isothiocyanate extraction from radish peel at 37°C with optimum ratio 1:1 gives isothiocyanate derivative with 23% area in GC-MS analysis result.
6. Isothiocyanate extraction from broccoli stem did not give satisfactory result because the addition of excessive phosphate buffer.
7. Bromelain enzyme from pineapple peel and isothiocyanate from radish peel proved to be deadly to *Plutella xylostella* and can be used as alternative insecticide. Best result is achieved by radish peel extract with feed : solvent ratio 1:2 which is proven effective in killing cabbage worm with 100% mortality rate on the second day.

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