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The Optimal Culture Media for Chitinase Production of *Lecanicillium lecanii* Based on Three Virulence Characters: Chitinase Activity, Sporulation, and Colony Growth

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Abstract

Optimization of culture media is an important factor in increasing the virulence of fungal. The aim of this study is to obtain culture media that optimal for chitinase production of *Lecanicillium lecanii* and also optimal to support other virulence characters, i.e. the production of conidia and colony growth. The study begins with selection of the best basal media that able to support three virulence fungal characters. The basal medium chosen was then tested against seven different carbon sources. The best carbon sources tested at 16 level concentrations to obtain the best concentration for chitinase production of *L. lecanii*. Data were analyzed using Anova and means showing statistical significance were compared using Least Significant Different (LSD) test. The effect of basal media composition on colony growth and the number of conidia were significant, but not on conidia size. Basal media I was the best basal media based on three virulence parameters of fungal and appropriate for chitinase analysis. N-acetyl-D-glucosamine as well as combination of glucose+ N-acetyl-D-glucosamine were the best carbon source for *L. lecanii* chitinase production. Combination of 0% N-acetyl-D-glucosamine + 1.5% glucose, 0.5% N-acetyl-D-glucosamine + 0% glucose, 0.5% N-acetyl-D-glucosamine + 0.5% glucose, 0.5% N-acetyl-D-glucosamine + 1% glucose, were optimum condition for *L. lecanii* chitinase production. This research found that of the four combined concentration of the carbon source, glucose does not act as a repressor on the chitinolytic system of *L. lecanii*.

Key-Words: Basal media, Diameter colony, Number of conidia, Carbon source, Chitinase activity

Introduction

Lecanicillium lecanii is one of the biological control agent that is already known for its potential in controlling various types of pests (Ladja, 2010; Florido 2009; Zare and Gams, 2008). Many kind of pests that have been reported to be successfully controlled by *L. lecanii*, among others, soybean pod sucking bug *Riptortus linearis* on egg, nymph and imago stages (Prayogo, 2009), larvae pathogen of the large elm bark beetle *Scolytus scolytus* (Barson, 2008), egg and larval nemathode of *Heterodera glycines* (Shinya *et al*., 2008), *Thrips palmi* (Cuthbertson *et al*., 2005), silverleaf whitefly *Bemisia argentifolii* (Gindin *et al*., 2000), and several other reports.

Although it has been known for its potency in controlling pests, the fungus *L. lecanii* and other entomopathogenic fungi not meet expectations as biological control agents. This is associated with their ability to kill pests that are relatively slower than chemical pesticides (Fang *et al*., 2009).

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Kao (2009) reported that *L. lecanii* cause mortality of 60% to *Thrips palmi* in 5 days after application. The results of recent studies reported that four isolates of *L. lecanii* isolates were selected as the most potential in controlling the eggs of *R. linearis* than 33 other isolates of *L. lecanii*, it takes 6 days to suppress 72% of the development of the eggs of *R. linearis* (Prayogo, 2011). One of the factors that determine the success of pest control using entomopathogenic fungi are the virulence of the isolates (Aiuchi *et al*. 2007; Khan *et al*., 2012). The virulence of isolates is determinded by degradative enzyme production by the entomopathogenic fungi. The role of such degradative enzyme is to degrade insect cuticle which acts as the first barrier against fungal infections (Xie *et al*., 2010). Chitinase is one of degradative enzymes that determine the pathogenicity and virulence of entomopathogenic fungi (Reyes *et al*., 2012). In *L. lecanii*, chitinase is the main degradative enzymes (Zhu *et al*., 2008). Research that examines three degradative enzymes in *L. lecanii* showed that chitinase is the highest enzyme secreted; while protease and lipase secreted in smaller amounts (Khan *et al*., 2012). Another physiological character that used as a

determinant of fungal virulence is the rate of colony growth and the number of conidia produced (Vu *et al*., 2007).

The virulence enhancement of the fungus can be achieved by optimizing the inoculum preparation, among which through the optimization of the culture media (Jholapara *et al*., 2013)**.** Furthermore, it was stated that the composition of the culture media is the major factor for growth and metabolite production by microorganisms. Chitinase production of the fungus is believed to be able to increase sharply when it is grown in culture media with optimal composition (Rebecca *et al*., 2013). Until now, there have been no research reports, especially in the entomopathogenic fungus *L. lecanii*, which examines the optimum culture media for the production of chitinase which also optimum for other virulence parameters. Most studies only focused to optimize the production of chitinase. Therefore, this study aimed to obtain the optimal culture media for chitinase production of *L. lecanii* and also optimum to support other virulence parameters, i.e. the production of conidia and colony growth.

Material and Methods

Fungal strains and inoculums preparation

L. lecanii was obtained from Indonesian Legumes and Tuber Crops Research Institute. The microorganism was maintained on potato dextrose agar slants, sub-cultured regularly every 1 month and incubated at room temperature for seven days. Conidia were harvested with distilled water and sieved through filter paper into sterile vials. Conidia were counted using haemocytometer to calibrate a suspension of 1 x 10^6 conidia/mL. The spore suspension was used as inoculum.

***R. linearis* cuticle treatment**

The cuticle of *R. linearis* was dried in oven at 100ºC until completely dry. The cuticle was then ground finely and sieved. Then, it was sterilized at 121ºC for 15 min, and kept at room temperature for later used.

Effect of basal media composition on colony growth

To determine the most appropriate basal media for the growth of *L. lecanii*, seven different media composition were tested as follows (g/100 ml): (I) K_2HPO_4, 0.1; urea, 0.3; MgSO4, 0.05; FeSO4, 0.01; yeast extract, 0.01; glucose, 0.5 (Dommes, 2013; *modification*); (II) glucose, 0.5; K_2HPO_4, 0.1, peptone, 0.5, $MgSO_4$ 0.01; (III) peptone, 0.406; K_2HPO_4, 0.1; KCl, 0.05; $MgSO_4$, 0.05; $FeSO_4$, 0.01; glucose, 0.5; (IV) $CaCl_2$, 0.00005; KH_2PO_4, 0.015; $MgSO_4$, 0.0012; NaCl, 0.0025; Na_2HPO_4, 0.0065; NH_4Cl, 0.0012 (Lawati, 2013, *modification*); (V) peptone, 0.406; K_2HPO_4, 0.1; KCl, 0.05, $MgSO_4$, 0.05; FeSO4, 0.0001 (Gao, 2011, modification); (VI) K_2HPO_4, 0.07; KH_2PO_4, 0.03; $MgSO_4$, 0.05; $FeSO_4.7H_2O$, 0.001 (Ng'ang'a *et al*., 2011; modification); (VII) glucose, 0.2; K_2HPO_4 0.1; peptone, 0.5; $MgSO_4$ 0.01; SDS 0.25 (Wu *et al*., 2010, *modification*). In media I, IV, V, VI and VII was added 1 g of *R. linearis* cuticle as complex carbon source; while media II and III only use glucose as carbon source. All medium was added 1.5 g bacto agar. Media were auctoclaved at 121ºC for 15 min and 15 ml each medium was poured into 9 cm Petri dishes. The plates with diffusion hole 7 mm were inoculated with 65 µl conidial suspension and incubated at room temperature with 5 replications. Colony growth on each basal medium is determined on the fifth day after inoculation. Data resulted from this treatment was analyzed qualitatively.

Determination of the basal media I, II, and III as the best basal media for chitinase analysis based on three parameters: colony growth, number and size of the conidia

Selection of basal media IV, VI, and VII in this analysis was based on the results of previous experiments. The composition of the basal medium III, V, and VI were in accordance with the previously described. Carbon source used on this experiment was 0.5% glucose and 1% *R. linearis* cuticle. mm hole were inoculated with 65 µl conidial suspension and incubated at room temperature. The experimental design used was completely randomized design with 4 replications on colony growth parameters, 40 replications on number of conidia parameters, and 20 replications on size of conidia parameters. All parameters was mesured at the seventh day after inoculation. Colony growth was determined by measuring two sections of colony diameter. The number of conidia was counted using an improved Neubauer haemocytometer. The size of conidia were measured by using ocular and stage micrometer. Observations were carried out by using a microscope Olympus CH20. Basal media that showed the best results from the three parameters will be used in subsequent analyzes of chitinase activity.

Preparation of colloidal chitin

Colloidal chitin was prepared according to the method described by Roberts and Selitrennikoff (1988) with some modification. Ten grams of chitin powder from shrimp shell (Sigma, USA) was slowly added into 200 ml concentrated HCl with vigorous stirring for 1 hour and kept overnight at 4ºC.The solution was filtered through glasswool. The resulting filtrate was then added with 100 ml of distilled water and neutralized with NaOH 12N. The chitin suspension was centrifugated at 3000 rpm for 60 min at 4ºC. The precipitate added with distilled water, stirred to

dissolved residual salt, and then centrifugated again at 3000 rpm for 60 min at 4ºC. The precipitate (colloidal chitin) stored at 4ºC until used.

Effect of carbon source on chitinase activity

Study the effect of carbon source on chitinase activity of *L. lecanii* was carried out quantitatively by Schales method. The best basal medium (I) that gives optimum results against fungal growth, number and size of conidia based on previous experimental results were used as a basal medium for chitinase activity analysis of *L. lecanii*. As carbon source, glucose, N-acetyl-D-glucosamine, glucose + N-acetyl-D-glucosamine, glucose + colloidal chitin, glucose + chitin powder, glucose + cuticle of *R. linearis* were added to the basal medium. All the carbon source were used at concentration of 1 g/100 mL. Erlenmeyer flask containing 35 ml medium were sterilized (121ºC for 15 min). The flask were inoculated with 1 ml of 1 x 10^6 conidia/mL and incubated at room temperature for 5 days on rotary shaker. Thereafter, culture were centrifugated at 3000 rpm for 10 min in a cooling centrifuge (4ºC).The clear supernatant was considered as crude enzyme to assay chitinase activity (Rattanakit *et al*., 2007). The experimental design was a group randomized design with 4 replications.

Effect of N-acetyl-D-glucosamine and glucose concentration on chitinase activity

N-acetyl-D-glucosamine and glucose as the best source of carbon based on the previous experiment result was used on this test. 35 mL of sterile basal medium prepared with different N-acetyl-D-glucosamine + glucose concentration: 0%+0%, 0%+0.5%, 0%+1%, 0%+1.5%, 0.5%+0%, 0.5%+0.5%, 0.5%+1%, 0.5%+1.5%, 1%+0%, 1%+0.5%, 1%+1%, 1%+1.5%, 1.5%+0%, 1.5%+0.5%, 1.5%+1%, 1.5%+1.5%,. The flask was inoculated with 1 ml of 1 x 10^6 conidia/mL and incubated at room temperature for 5 days under the shaking condition. The experimental design was a group randomized design with 6 replications.

Chitinase activity assay

Analyzes of chitinase activity were conducted on the fifth day after incubation. Culture were centrifugated at 3000 rpm for 10 min in a cooling centrifuge (4ºC). The clear supernatant was considered as crude enzyme to assay chitinase activity (Rattanakit *et al*., 2007). Chitinase activity was measured by the released of N-acetyl-D-glucosamine equivalent from colloidal chitin by following method of Spindler (1997). The reaction mixture consisted of 0.45 mL of crude enzyme and 0.9 mL 1% (w/v) colloidal chitin in phosphat buffer (0.05 M, pH 5.6) and incubated at 37ºC for 30 min. The reaction was stopped by heating the solution in boiling water for 10 min and then cooled. Thereafter, the reaction mixture was centrifuged at 3000 rpm for 5 min. To the supernatant, 3 ml Schales reagents and 1.5 ml distilled water was added, and then heated in boiling water for 10 min for colour development. After cooling, the developed color, as indication to the quantity of released N-acetyl-D-glucosamine, was read at 420 nm in Ganesys 10UV Spectrophotometer.

Standard graph was prepared with curve for authentic N-acetyl-D-glucosamine to convert the absorbency values to micromoles of N-acetyl-D-glucosamine liberated from colloidal chitin.

Statistical analysis

The data obtained from the experiments were analized using *SPSS* statistical programme and means showing statistical significance were compared by Least Significant Different (LSD) test.

Media were auctoclaved at 121ºC for 15 min and 15 ml of each medium was poured into 9 cm Petri dishes. The plates with diffusion 7.

Results and Discussion

Effect of basal media composition on colony growth

Seven different basal media composition was tested on the colony growth. *L. lecanii* was able to grow widened and thickened on basal medium I, II, and III (Fig. 1a, 1b, 1c).Other basal medium composition is not appropriate to support the growth of colonies of *L. lecanii*.In the basal medium IV, V, and VI; the colonies were only able to grow widened but not thickened (Fig. 1d, 1e, 1f), while at the basal medium VII, colony grows only in the hole diffusion, but does not show the widening and thickening (Fig. 1g).

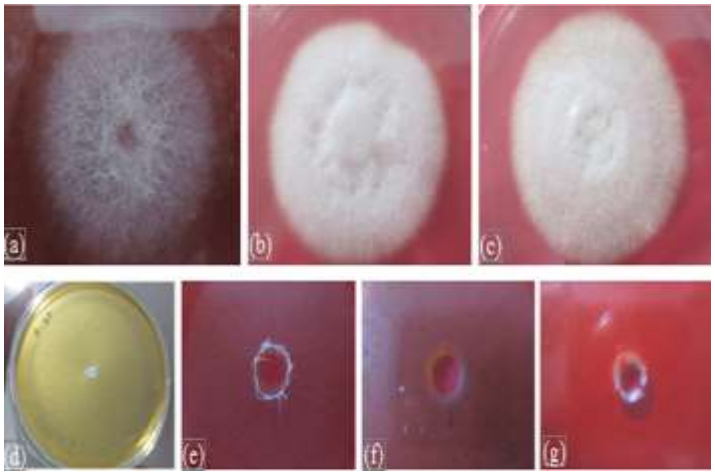

Fig. 1: Colony growth of *L. lecanii* after 7days after inoculation on: (a) basal media I, (b) basal media II, (c) basal media III, (d) basal media IV, (e) basal media V, (f) basal media VI, (g) basal media VII

In general, each microorganism requires a source of carbon, nitrogen, inorganic phosphate, sulfur, trace metals, water, and vitamins. Jonathan and Fasidi (2001) states that the carbon is an essential nutrient in the culture medium and the amount must be more than the other essential nutrients. Evaluation of seven basal media that has been tested, it is known that glucose is an essential carbon source for *L. lecanii* growth. Colonies of *L. lecanii* that only grows wider without thickening (Fig. 1e) can then grow widened and thickened (Fig. 1c) after the replacement of complex carbon sources (cuticle *R. linearis*) with a simple carbon source (glucose). *L. lecanii* can only utilize complex carbon sources if in the culture medium is also added with simple carbon source. This condition ws showed by the growth of *L. lecanii* on the basal medium I (Fig. 1a). In the basal medium VII, inability of *L. lecanii* to grow thickened and widened even though on the media has been added glucose may due to the presence of SDS. This compound is not common to be added to the culture media. *L. lecanii* can grow well after SDS elements eliminated on the basal medium II, which is a modification from the basal medium VII. The presence of SDS is believed interfere the absorption of nutrients in the culture medium so that the fungus cannot grow well. The needs of glucose for fungal growth has also been previously reported in *Tricholoma terreum* (Kibar and Peksen, 2011), *Phlebopus portentosus* (Kumla *et al*., 2010), some strains *Suillus* and *Boletinus* (Hakateya and Ohmasa, 2004).

Nitrogen is also an essential element necessary for the growth of fungi. In this study, the best growth of colonies of *L. lecanii* occurs in the basal media using nitrogen sources such as yeast extract and peptone. Yeast extract and peptone are organic nitrogen that has a better role in supporting fungal growth than inorganic nitrogen. That is because in the organic nitrogen-containing growth factors that can trigger cell growth (Jholapara *et al*. 2013). Previous research has revealed that the peptone and yeast extract was the best nitrogen source for the fungal growth, among others, *Streptomyces heteromorphus* 4075 (Kotra *et al*., 2013), *T. terreum* (Kibar and Peksen, 2011), *Streptomyces* sp. PTK19 (Thiagarajan *et al*., 2011), *P. portentosus* (Kumla *et al*., 2010), *Phlebopus portentosus* strain CMUHH121-005 (Thongklang *et al*., 2010).

Determination of the media I, II, and III as the best basal medium for chitinase analysis based on three parameters: colony growth, number and size of the conidia

Observations at 7 days after inoculation showed that the growth of fungus colonies on basal media I (Fig. 2a) is not as thick as the colonies that grown on basal media II (Fig. 2b) as well as basal media III (Fig. 2c). Morphology of colonies on those three kinds of basal media showed the same color, i.e. white pale (Fig.2).

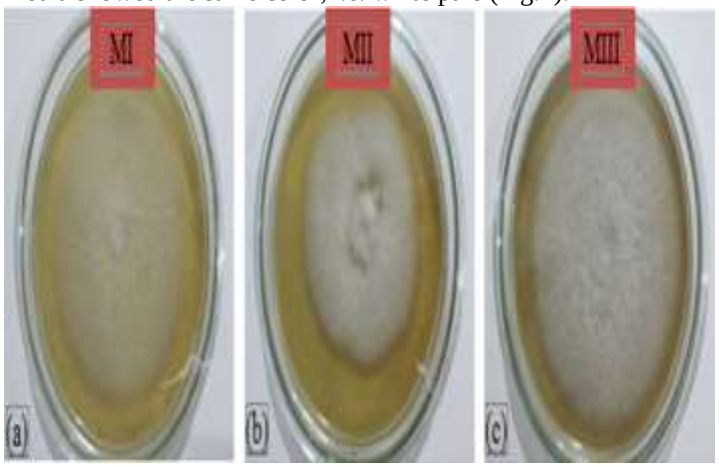

Fig. 2: Colony morphology of *L. lecanii* at 7 days after application. (a) Colony morphology *L. lecanii* on basal media I; (b) Colony morphology *L. lecanii* on basal media II; (c) Colony morphology *L. lecanii* on basal media III

The analysis of variance revealed that there were significant differences on basal media composition for colony growth on 7 days after inoculation ($F_{2,6}$ = 9.87, P = 0.05). Basal media I as well as basal media III were the best basal medium composition for the optimum colony growth of *L. lecanii*; while the lowest colony growth were observed for basal medium II (Fig. 3).

The effect of basal media composition on number of conidia were significantly difference ($F_{2,78}$ = 161.09, P = 0.01). The highest number of conidia was observed when the fungus was cultured in media basal I with the average number of conidia 45 x 10^6. Meanwhile, the lowest number of conidia was recorded when the fungus cultured in basal media II (with the average number of conidia 9.9 x 10^6) as well as basal media III (with the average number of conidia 10.4 x 10^6) (Fig. 3).

In the third parameter, the observation at 7 days after inoculation showed that the size of conidia *L. lecanii* on basal medium I, II, and III were not different ($F_{2,38}$ = 2.339, P = 0.05). The average size of conidia of *L. lecanii* on basal medium I, II, and III, respectively i.e. 4.625 µm x 11.875 µm, 3.875 µm x 10.375 µm dan 3.75 µm x 11.75 µm (Fig. 3).

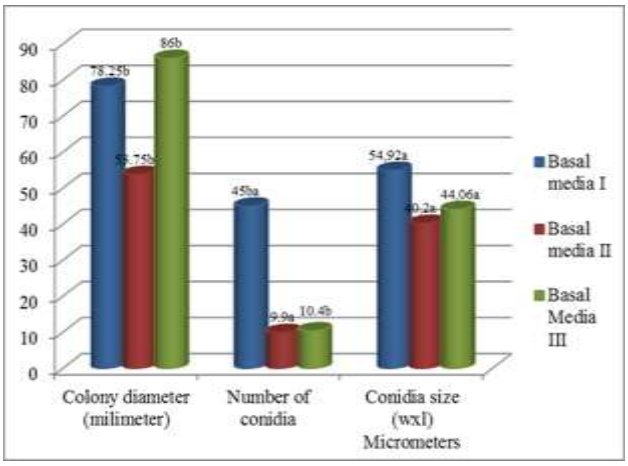

Fig. 3: The parameters determining the best basal media: diameter of colony, number and conidia size

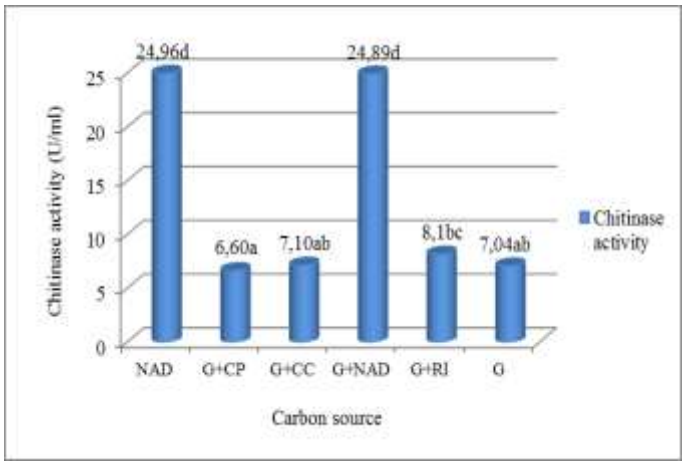

Fig. 4: The effect of carbon source on chitinase activity of *L. lecanii*

The main nutritional elements which have a great influence on the colony growth and conidial formation are source of carbon and nitrogen of the culture medium (Sun and Liu, 2006). The main difference basal media composition I, II, and III were the macronutrient composition of carbon and nitrogen sources. The basal media I has more carbon source (i.e. glucose and cuticle of *R. linearis*) than basal media II and III which only has glucose as carbon source. The quantity of carbon sources believed to be able to providing more energy for fungus to grow and conidia formation. This is in accordance with the statement of Jonathan and Fasidi (2001) which states that the fungus requires a carbon source in an amount more than other nutrients. Associated with the nitrogen source, yeast extract seems more appropriate to support the growth and metabolism of *L. lecanii* than peptone. The results of this study corresponds to Kotra et al (2013) who found that yeast extract was the best nitrogen source for *S. heteromorphus* 4075.

Effect of carbon source on chitinase activity

Analysis of variance result showed that the treatment is significantly influenced chitinase activity of *L. lecanii* ($F_{5,15}$ = 641,99, P = 0.01). The highest chitinase activity was recorded when the fungus cultured in basal medium I with N-acetyl-D-glucosamine as carbon. The results were not significantly different when the fungus was cultured on glucose + N-acetyl-D-glucosamine. Meanwhile, the lowest chitinase activity was recorded when the fungus cultured in basal medium I with glucose+chitin powder, glucose+chitin colloid, and glucose as source carbon. Chitinase activity at three different carbon source were not significantly different (Fig. 4).

It is clear that the chitinolytic system of *L. lecanii* following the induction-repression mechanism. N-acetyl-D-glucosamine is the best inducer for chitinase activity of *L. lecanii*. Related to glucose, as the sole carbon source in the culture media, glucose with concentration 0.5% acts as a repressor of chitinase *L. lecanii*; but if its presence in the culture media with N-acetyl-D-glucosamine, glucose does not seem to act as a repressor. These findings are new facts related chitinolytic system of *L. lecanii*. In previous studies, Reyes *et al*. (2012) reported that glucose acts as a repressor in chitinolytic system of *L. lecanii*. The addition of glucose to the culture medium led the activation of regulatory proteins CreA/Cre1 to bind with sequences (SYGGRG) and represses gene transcription of chitinase (Stapleton and Dobson, 2003). The presence of glucose along with other carbon sources in the culture media could be expected to interfere glucose binding with regulatory proteins CreA/Cre1.

Besides glucose, low chitinase activity was also detected in the culture medium with chitin and cuticle of *R. linearis* as carbon source. Microorganisms, including *L. lecanii*, cannot use the substrate (chitin and cuticle *R. linearis*) with a complex polymeric structure because it is insoluble (Reyes *et al*., 2012). *L. lecanii* can only utilize chitin when it already hydrolyzed to soluble oligomers or N-acetyl-D-glucosamine. This finding corresponds with Matsumoto (2006).

Effect of N-Acetyl-D-glucosamine and glucose concentration on chitinase activity

The analysis of variance revealed that there were significant differences on the effect of interactions between glucose and N-acetyl-D-glucosamine concentration to the chitinase activity of *L. lecanii*

($F_{9,30}$ = 302.41, P = 0.01). The highest chitinase activity was found when *L. lecanii* were cultured in basal medium I with a combination of the concentration of N-acetyl-D-glucosamine + glucose as follows: 0% + 1.5%, 0.5% + 0%, 0.5% + 0.5%, 0.5% + 1%. Chitinase activity of *L. lecanii* was detected very low when cultured in basal medium I with concentration of carbon source 1% N-acetyl-D-glucosamine + 1.5% glucose (Fig. 5).

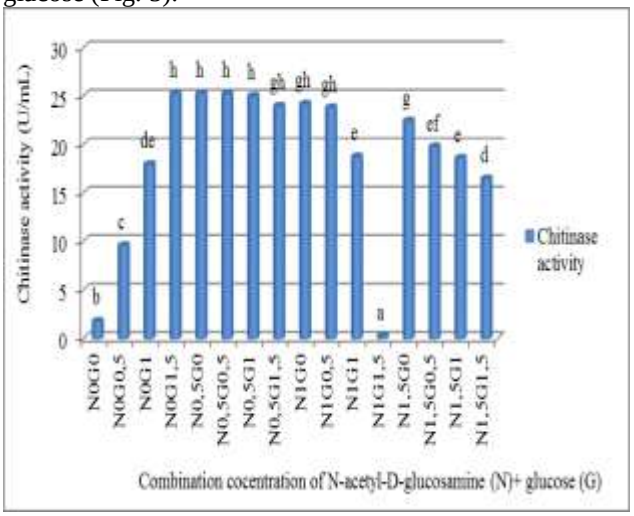

Fig. 5: The effect of combination concentration of N-acetyl-D-glucosamine + glucose on chitinase activity of *L. lecanii*

In chitinolytic system of *L. lecanii*, concentration combination of N-acetyl-D-glucosamine and glucose has reached the optimum conditions at four concentration combination, i.e. 0% +1.5%, 0.5% + 0%, 0.5% + 0.5%, 0.5% + 1%. At these concentration combinations, substrate can bind optimally at the active site of enzyme. On the other concentration combinations, substrate is not able to bind optimally to the enzyme active site. Natsir *et al*. (2010) explains that at the low substrate concentration, only a few substrates that bind to the active site of the enzyme. Conversely, at higher substrate concentrations, all the active sites of enzyme will be saturated by the substrate.

As general conclusion of this research, the optimal culture medium composition for the production of chitinase *L. lecanii* and to support three fungal virulence characters (g/100 mL) i.e.: N-acetyl-D-glucosamine + glucose (0% +1.5%, 0.5% +0%, 0.5%+0.5%, 0.5%+1%), K_2HPO_4, 0.1; urea, 0.3; MgSO4, 0.05; FeSO4, 0.01; yeast extract, 0.01.

Based on the results of this experiment, further studies need to be conducted to uncover how the combination of both substrate (glucose + N-acetyl-D-glucosamine) binding to the enzyme active site substrate so that it can bind to the active site of enzyme. Depth studies should also be conducted to reveal how the work both substrate in relation to the substrate protein work CreA/Cre1 so that the repression of chitinase genes does not occur.

Further studies that also important to be done is to determine the optimal concentration of yeast extract for chitinase production of *L. lecanii*. This is because yeast extract is an essential nitrogen source in the culture medium. Optimization of the concentration of micronutrients and other culture conditions also need to be done in an effort to optimize the production of chitinase *L. lecanii*.

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