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Palm Kernel Cake Reaction of Fermentation Results of Mannanase Activity Bacillus Subtilis ATCC 6633

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Abstract: Mannanase is an enzyme which is capable of breaking down mannan substrate by hydrolyzing the β-1.4-manosidic linkage between mannose and mannose. Mannanase can be applied to feed, paper, pharmaceutical, food, detergent, oil and gas industries. In the pharmaceutical industry, mannanase can be used for controlling drug release from composed matrix from cross-linked galactomannansThe objective of this study is to discover the activity of the bacterium Bacillus subtilis ATCC 6633 in producing mannanase through a fermentation process using PKC mediumThis kind of research is true experimental research with replications.The research results recommend to increase the utilization of palm kernel cake (PKC) as a medium and Bacillus subtilis ATCC 6633 as a source of mannanase, after production optimization, either in laboratory, pilot or commercial scale, by changing various factors which affect the productivity of Bacillus subtilis ATCC 6633 to determine the optimal time of fermentation, so that the result produces maximum mannanase activity.Bacillus subtilis ATCC 6633 is capable of producing mannanase through the fermentation process using PKC as a medium. Furthermore, the mannanase Bacillus subtilis fermented result activity ATCC 6633 was using 0.25% of PKC as a medium produced a mannanolytic index of 4.0 ± 0.98.

Keywords: Bacillus subtilis ATCC 6633, Enzyme, Mannanase, Mannan, Palm kernel cake

1. INTRODUCTION

Mannanase can be applied to feed, paper, pharmaceutical, food, detergent, oil and gas industries. For other fish and livestock feed industries, mannanase can be used as a mixture to increase the nutritional value and conversion of mannan-rich feed ingredients such as palm kernel cake (1,2). Mannanase can break down the mannan components found in pulp and eliminate lignin for bleaching (3). The hydrolysis activity of mannanase also facilitates removal of food or cosmetic stains which contain mannan components as additives (4). The oil & gas industry utilities galactomannan hydrolysis capability by mannanase to increase oil & gas flow during the drilling process. For bioethanol production, lignocellulose must be hydrolyzed into fermented sugar which can be done by cellulase, xylanase and mannanase (3). Mannanase can also produce prebiotics. Prebiotics are indigestible food ingredients (*oligosaccharides*) and have a beneficial impact on the growth and active probiotic microbes in the intestine (1). In the pharmaceutical industry, mannanase can be used for removing the biological layer, which is on the surface of the tool. Mannanase can also control the release of drugs from a composed matrix of the cross-linked galactomannan (4). Hydrolysis of glucomannan products with

mannanase, which has various molecular weights, have other biological functions, such as anti-tumor, immunoregulation and cytothesis (1).

Mannanase is obtained through a substrate which contains hemicellulose. Hemicellulose is composed of mannan, which can be classified into four subfamilies: mannan, glucomannan, galactomannan and galactoglucoman (1). Hemicellulose is a resource of nutrition because it contains mannan as carbon. Many natural ingredients contain mannans, such as palm kernel cake (PKC) copra, konjac powder, sesame meal, and soya bean meal. Materials above are sources of carbon for microorganisms during the fermentation process to obtain mannanase. This study uses PKC because it contains hemicellulose as a carbon resource of 22.08% (5). Another consideration using PKC, namely Indonesia's palm oil production in 2011 reached 22.51 million tons, so the amount of PKC produced was also abundant (6).

Potential sources of mannanase come from microorganisms, and its productivity is higher than other resources. In addition, production conditions that are more easily controlled and economical are other advantages when microorganisms are used (7). Mannanolytic microbes are mostly produced by Gram-positive, namely *Bacillus sp*. For Gram-negative, *Klebsiella oxytoca* can also produce mannanase. Fungi which acts as mannanolytics, such as genus *Aspergillus sp.*, *Penicillium sp.* and *Trichoderma sp.* In addition, from the group *Actinomycetes* like *Streptomyces sp.* also show a capability to produce mannanase (3).

Mannanase production from bacteria requires a relatively shorter time than production from the mold. Harvesting enzymes are completed during the final log phase. In this phase, the number of microbial cells and enzymes were produced at their optimal point. Microbial growth curves were performed to discover the final log phase. Mannanase production is done by growing isolates on selective media containing mannan then incubated at a certain pH, temperature and time. Enzyme isolation was done by centrifugation at 4°C to avoid enzyme denaturation. Supernatant which was obtained is a rough mannanase. Time, pH, substrate concentration and temperature factors need to be controlled to maximize mannanase production. Other treatments, such as agitation, aeration, and type of substrate, can also affect mannanase production (5).

The results of other studies, according to Jiang et al., (2006), *Bacillus subtilis* WY34 produced mannanase that were grown on konjac powder as a source of carbon at 50°C. The mannanase activity was produced in the amount of 1543.4 units/mg. After purification through the process of precipitation with 40-80% of ammonium sulfate, Superdex™ 75 and Q-Sepharose fast flow, mannanase activity increased into 8,302.4 units/mg. Mannanase is optimally produced at pH 6.0 and stable in the pH range of 5.5-10.1. The optimal temperature for mannanase activity is 65°C and stable to 60°C, but 90% of the activity is lost at 70°C after 30 minutes of incubation. In this study, *Bacillus subtilis* ATCC 6633 was selected as a Mannanase-producing microorganism because it was proven by Jiang (2006), that *Bacillus subtilis* can produce mannanase with konjac powder as a source of mannan (8).

This study intends to determine the activity of mannanase produced by Bacillus subtilis ATCC 6633 through the fermentation process using PKC as a medium, and determine the optimal concentration of PKC to produce mannanase through the fermentation process by Bacillus subtilis ATCC 6633 bacteria.

This research is expected to increase the selling value of PKC to increase Indonesian commodities in general, and for the researchers, it is useful to know the optimal PKC concentrations, which are required to produce mannanase with the most significant specific activity.

2. METHODS

This kind of research is true experimental research with replications. Observations were made by measuring the diameter of the clear zone (mm) produced for 120 hours. The choice of optimal media concentration is based on the diameter of the clear zone, which is converted to a mannanolytic index (MI). To observe the difference in mannanase activity which is produced through the fermentation process using PKC and LBG as a substrate, statistical data processing using SPSS (Statistical Product and Service Solution) method, namely one way ANOVA test and continued with HSD test at 95% confidence level or significance level $\alpha = 0.05$. The decision making is based on the level of the significance level. If the significance level is lower than 0.05, there are significant differences. Conversely, if a significance level is greater than 0.05, there is no significant difference between the treatment groups.

3. RESULT

Observation Results of Growth of *Bacillus subtilis* ATCC 6633 on PKC and LBG Agar Media

Screening for growth of *Bacillus subtilis* ATCC 6633 was done on 1% of LBG and 1% of PKC (9). On the first day, two media have not shown growth. On day 5, the growth of bacterial colonies began to appear.

Table 1.Growth Observation Results of *Bacillus subtilis* ATCC 6633 on agar media LBG and PKC

Media	Day 1	Day 5
LBG 1%	-	Growth indicated
PKC 1%	-	Growth indicated
PKC 2%	-	Growth indicated
PKC 3%	-	-
PKC 4%	-	-
PKC 5%	-	-

The Optimization Results of LBG Concentration as Media

Optimization of LBG concentration as a medium for the production of mannanase was carried out using the agar-disc method. The test media contained LBG 1% and 0.8% of NB. After 24 hours incubation and given Congo Red stain, there was no clear zone showing mannanase activity around the iron cylinder. When the bacterial colony is released, a clear zone emerges due to the Congo Red stain, which does not color underneath the area.

Optimization was again carried out by decreasing the concentration of LBG used to 0.5% and the incubation time prolonged to 72 hours. The method used is agar-well diffusion with Congo Red stain and iodine (10). The incubation results showed that the iodine stain provided a clear zone of mannanase activity. In contrast, Congo Red did not show a clear zone of mannanase activity around the iron cylinder.

Optimization Results of Mannanolytic Test Media Composition

The composition of the mannanolytic test media is carried out without NB to stimulate cells to produce metabolites (mannanase). After 72 hours of incubation, a clear zone of mannanase activity has not yet been evident.

Optimization Results of Composition Starter Mannanolytic Test

Mannanolytic test was carried out using agar-well diffusion method and Congo Red stain. After 120 hours of incubation, the NB test media did not show a clear zone of mannanase activity around the iron cylinder (negative control). In contrast, 0.5% of LBG of media produced a clear zone showing mannanase activity.

Mannanase Activity Test

Mannanase activity test was carried out using agar-well diffusion method on 0.5% of LBG test media and concentration variation in PKC. After incubation for 120 hours, the test media containing 0.5%, 0.75%, and 1% of PKC show no clear zone of mannanase activity. For test media, 0.5% of LBG and 0.25% of PKC showed a clear zone of mannanase activity.

4. DISCUSSION

This research began with a reduction of particle size from the PKC using a grinder. Reducing the particle size is aimed to increase the surface area of PKC particles. The outer surface of PKC particles is a factor that can affect the speed of the fermentation process of mannanase production by Bacillus subtilis ATCC 6633. Palm Kernel Cake (PKC) used contained fiber (mannan) in the amount of 17.5%. Mannan contained in PKC fiber functions as a substrate and induces mannanase production (5). Besides PKC, LBG also contains galactomannan > 80%, which can also function as a substrate (11).

The microorganism used to produce mannanase is Bacillus subtilis ATCC 6633 obtained from the Assessment Service Unit (AST). The selection of Bacillus subtilis ATCC 6633 is based on preliminary research that *Bacillus subtilis* can be used as a mannanase-producing microorganism (12).

Screening for growth of Bacillus subtilis ATCC 6633 was performed on LBG 1% and PKC media (1%, 2%, 3%, 4% and 5%) with the streak plate method. Before use, all media tested were sterilized by autoclave at 121°C for 15 minutes to avoid contamination. On day 1 there was no growth in all media (LBG and PKC). New growth was seen on day 5 in 1% of LBG, 1% of PKC, and 2% of PKC. The new growth observed on day 5 could be due to Bacillus subtilis ATCC 6633 taking longer to break down LBG and PKC, which are polymers into carbon and nitrogen sources for bacterial growth. LBG concentrations of 1% and PKC 1% (maximum) will be used as a screening of mannanase activity while PKC 2% is not used because PKC with concentrations of greater than 1% (b/ v) can obstruct bacterial growth. Palm Kernel Cake (PKC) with a level of 1% can obstruct bacterial growth, and it cannot produce mannanase (9).

The next step is to optimize the concentration of LBG used. According to Olaniyi et al., (2013), the LBG concentration used for screening of mannanase activity is 1% (b/v). Incubation results for 24 hours provide a clear zone under the colony that was released after being given Congo Red stain and rinsing with 1% NaCl (6). The clear zone of mannanase activity is expected around the Bacillus subtilis ACC 6633 colony is not under the colony that was released during flushing with Congo Red (9). The clear zone under the bacterial colony is caused because the Congo Red stain cannot penetrate the area covered by the Bacillus subtilis ATCC 6633 colony. After that, the second optimization is continued by changing the concentration of LBG used to 0.5% using the agar-well diffusion method and incubation time for 72 hours. In the second optimization, Congo Red stain has not provided a clear zone of mannanase activity around the colony of Bacillus subtilis ATCC 6633. In contrast, iodine stain gives a clear zone of mannanase activity qualitatively. According to Mabrouk et al., (2008), iodine stain can be used to show a clear zone of mannanase activity. The clear zone that does not appear with Congo Red staining is caused by the length of submersion is only 15 minutes, and the concentration of Congo Red used is 0.1% (13). Congo Red concentration of 0.1%

causes the resulting red color does not contrast with the clear zone of mannanase activity which will complicate the observation. The submersion of the medium with Congo Red stain takes ± 30 minutes to bind together to mannan on PKC or galactomannan on LBG (10). The NB content in the medium test can also reduce the ability of mannanase production because Bacillus subtilis ATCC 6633 does not lack carbon and nitrogen sources so that the secretion of secondary metabolites (mannanase) is reduced.

The next optimization uses the medium of the mannanolytic test without using NB using agar-disk method. After being given the Congo Red stain, there is still no clear zone of mannanase activity around the Bacillus subtilis ATCC 6633 colony. Another factor that causes the no appearance of the clear zone of mannanase activity is the concentration of NaCl and the length of submersion when rinsed. According to Kasana et al., (2008), the length of submersion with NaCl is 15-20 minutes and can be repeated three times so that the clear zone looks increasingly clear (10).

The final optimization is the starter composition used for the mannanolytic test. There are two starters used, namely 0.8% of NB and 0.8% of NB + 0.5% of LBG. With LBG in the starter composition, it can maintain the activity of β-mannanase and trigger the mannanase production (9). The results obtained after incubation for 120 hours is a medium test that only contains 0.8% of NB cannot be stained by the Congo Red stain and a medium test that contains 0.5% of LBG produced a clear zone of qualitative mannanase activity around the block with the act of two different starters. Medium test that only contains 0.8% of NB cannot be stained with the Congo Red stain because it does not contain the linkage of β-1.4-D-Mannopiranosil which will bind together with Congo Red that gives the red color (1). Qualitatively, the diameter of the clear zone with a starter act that contains 0.8% of NB is smaller than the act with a starter that contains 0.8% of NB and 0.5% of LBG. The clear zone diameter of mannanase activity with the starter that contains 0.8% of NB and 0.5% of LBG greater because Bacillus subtilis ATCC 6633 does not experience phase lag that can inhibit growth for mannanase production. The iodine stain also gives the same results as Congo Red, which is to produce a clear zone of mannanase activity.

After various optimizations are carried out, it is followed by the test of mannanase activity. The test of mannanase activity was carried out using the method of agar-well diffusion on the medium test of 0.5% of LBG and the various of PKC concentrations (0.25%; 0.5%; 0.75%; and 1%). The negative control used contained 0.8% of NB and 0.25% of PKC without inoculum Bacillus subtilis ATCC 6633 does not provide a clear zone of mannanase activity, around the bacterial block or colony. The medium test of 0.5% of LBG and 0.25% of PKC provide a clear zone of mannanase activity, while other medium tests such as PKC 0.5%; PKC 0.75%; and PKC 1% do not produce a clear zone of mannanase activity. The clear zone that does not appear can be caused by metal content in high PKC, which acts as an inhibitor so that mannanase activity is reduced. According to Sumardi, (2005) metal content of Fe^{3+} may decrease mannanase activity. In addition, there is a notion that Bacillus subtilis ATCC 6633 is sensitive to bacterial growth-inhibiting compounds found in PKC (9). According to Mabrouk, et al., (2008), Bacillus sp. tends to rapidly metabolize carbon sources that are easily digested and have an impact on decreasing enzyme secretion (mannanase) for mannan hydrolysis of PKC. The mannanase test in the medium test of 0.5% of LBG gave the average mannanolytic index (MI) of 2.6 ± 0.22 and on the medium of 0.25% of PKC for 4.0 ± 0.98 (13). According to the research results Utami et al., (2013), PKC can be used by Brevibacillus borstelensis as a medium for production mannanase with the activity of 0.032 units/ml, and *Bacillus subtilis* can produce mannanase activity with MI of 4.5 (14). Medium tests that contain 0.5% of LBG have a clear zone of mannanase activity that is smaller than the medium test of 0.25% of PKC. According to the research results of Liu et al., (2015), metal K^{+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+},

and Na+ that allegedly contained in PKC as trace elements were able to increase mannanase activity compared to LBG which does not contain trace elements (15).

5. CONCLUSION

Bacillus subtilis ATCC 6633 is capable of producing mannanases through the fermentation process using PKC as a medium. Furthermore, the mannanase activity of the fermented Bacillus subtilis ATCC 6633 using 0.25% of PKC as a medium that produced a mannanolytic index of 4.0 ± 0.98.

6. REFERENCES

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