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Utilization of cassava peel waste (*Manihot esculenta*) as substrate in the production of cellulase enzymes by *Aspergillus niger*

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Abstract

Cellulase enzymes is an enzyme that can hydrolyze cellulose into smaller sugar components. Cellulase enzymes are produced due to high demand, with 29.17% in the animal feed industry, 14.67% in the textile industry, and 26.17% in the paper industry. One method of producing cellulase enzymes is by utilizing the cellulolytic microorganism *Aspergillus niger*. This study aims to optimize cellulase enzyme production by *Aspergillus niger* through liquid fermentation on cassava peel substrate (*Manihot esculenta*) with pH treatments of 4.5 and 5.5 and temperatures of 29.5 and 30.5℃. The research method involved isolating pure cultures of *Aspergillus niger* from the laboratory of PT. Agritama Sinergi Inovasi. The fungus was grown on CMC agar medium to test its cellulolytic activity. The tested cellulolytic fungus can be used in fermentation in a medium containing cassava peel powder as the main cellulose substrate. The results of this study indicate that the cellulolytic fungus *Aspergillus niger* has the potential to produce cellulase enzymes. The highest enzyme activity value of 2.003 U/mL with a biomass of 0.535 mg/mL was obtained at pH 4.5 and 30.5℃. This study provides an important contribution in assessing the potential for cellulase enzyme production with variations in pH and temperature by *Aspergillus niger* fungi on cassava peel substrate as a basis for further development in organic waste management.

Introduction

Based on data from the Annual Food Report in 2023, the increase in production of cassava tubers *(Manihot esculenta)* in Indonesia increased by 12.13% every year. The impact of the increase in cassava production is the increase in cassava peel waste in Indonesia with a dry weight reaching 10-13% of the total weight of cassava (Marvie *et al.*, 2022). A total of 2.176 million tons of cassava peels each year, only 5% of which are used as compost and animal feed, the rest only become waste (Sari *et al.*, 2020). Therefore, cassava peels have the potential for cellulase enzyme production due to the relatively low cost, as cassava peels have a higher cellulose content compared to palm trunks and coconut husks (Saragih *et al.*, 2024). Cassava peels contain 10.38% starch, 7.65% lignin, 36.58% hemicellulose, and 43.63% cellulose (Artiyanti, A & Soedjono, 2018).

In Indonesia, cellulase enzyme production continues to increase due to the high demand of 29.17% in the animal feed industry, 14.67% in the textile industry, and 26.17% in the paper industry. Cellulase enzyme production has increased by 5% each year with a range of 2018-2024 (Gobal Info Research, 2024). Cellulase enzymes can be produced through the utilization of various microorganisms such as cellulolytic bacteria and fungi. According to Imaanual *et al.* (2006) fungi show high effectiveness in enzymatic production processes in general because they have superior cellulose degradation capacity compared to bacteria. Thus, the researcher determined that the microorganism used for cellulase enzyme production is

Aspergillus niger, because filamentous fungi have the potential to utilize cellulose material as a carbon and energy source and subsequently produce cellulase enzymes. Additionally, *Aspergillus niger,* is known for its ability to grow rapidly on a variety of lignocellulosic substrates and produce significant levels of extracellular cellulase enzymes under controlled fermentation conditions (Utami *et al.*, 2019). These advantages make A. niger one of the most efficient and economically viable candidates for industrial-scale cellulase production. By utilizing agricultural waste and potential microorganisms, it is expected that enzyme production can be more efficient, environmentally friendly and economical selling value.

Materials and methods

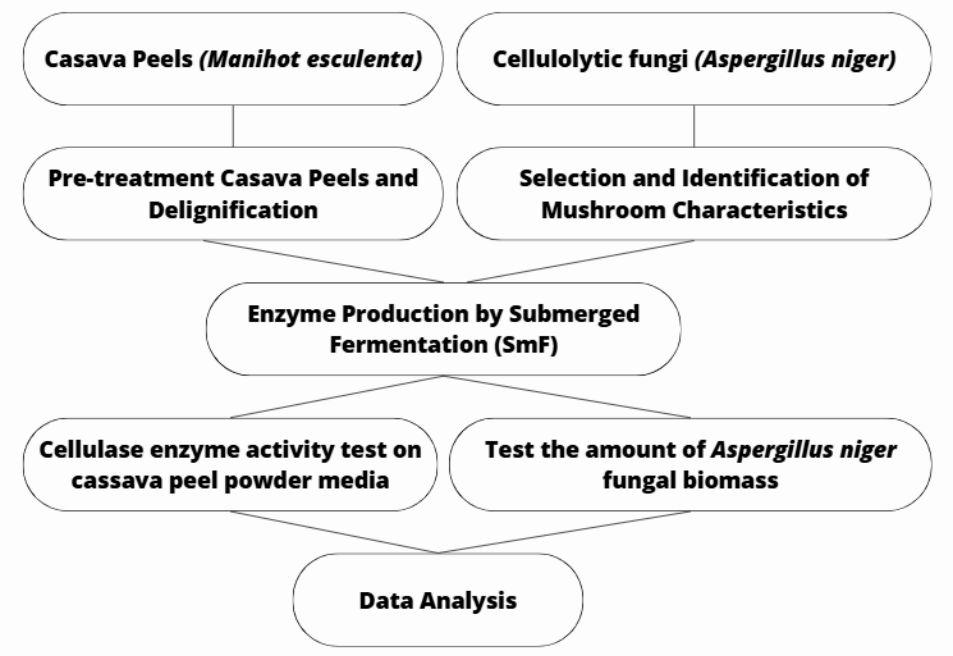

Figure 1. Flow diagram of cellulase enzyme production by *Aspergillus niger* on cassava peel substrate.

Research Design

The completely randomized design (CRD) method was used as the research approach. This design was chosen because it is suitable for conditions where experimental materials and environmental factors are homogeneous. Given that the research was conducted in a laboratory environment with a standard of uniformity in the use of tools, materials, and media, CRD was considered the most appropriate design.

Pre-treatment Cassava Peels and Delignification

Delignification of cassava peel powder was carried out by mixing the substrate and 6% NaOH solution in a ratio of 1:10. The purpose of NaOH is to damage the lignin structure in the crystalline and amorphous parts of the substrate. The mixture was then put into an autoclave at 121°C for 15 minutes. After that, the mixture was filtered using filter paper and washed with H_2SO_4 until the pH was neutral (pH 7). Finally, the delignification results were dried by putting them in an oven at 105°C for 8 hours. The delignification results will be used for enzymatic hydrolysis to determine the sugar content of the resulting hydrolysate.

Selection and Identification of Mushroom Characteristics

Fungal colonies were grown on Carboxymethyl Cellulose (CMC) agar. After that, it was stained with Congo red 0.1% and incubated for 30 minutes and rinsed with 1% sodium chloride solution. To identify the characteristics of fungi in this study, macroscopic, microscopic and biochemical tests were carried out including starch hydrolysis test, starch hydrolysis, gelatin hydrolysis, phosphate solvent test and carbohydrate fermentation test.

Enzyme Production by Submerged Fermentation (SmF)

Three inoculation loops of *Aspergillus niger* van Tieghem (ATCC 9029), a second-generation laboratory strain obtained from the Laboratory of PT. Agritama Sinergi Inovasi, were aseptically transferred into Potato Dextrose Broth (PDB) to initiate pre-culture growth., then incubate on a shaker. The enzyme production stage through Submerged Fermentation requires a medium containing $(NH_4)2SO_4$ 1.4 g; KH_2PO_4 2.0 g; urea 0.3 g; $CaCl_2$ 0.3 g; MgSO4 0.3 g; $FeSO_4.7H_2O$ 0.005 g; ZnSO4.$7H_2O$ 0.0014 g; $CoCl_2$ 0.002 g; peptone 0.75 g; cassava peels substrate 7.5 g dissolved in 0.2 M phosphate buffer with pH intervals of 4.5 and 5.5 as much as 1000 mL in a 2000 mL Erlenmeyer flask. 2% inoculum was added aseptically and the mixture was centrifuged for 96 h in a waterbath shaker at two different temperatures: 29.5°C and 30.5°C (Oktariani, 2017). Samples were collected directly from the fermentation broth during incubation. Following centrifugation at 10,000 rpm for five minutes, the crude enzyme extract was recovered as the clear supernatant and used for further analysis.

Cellulase enzyme activity test on cassava peel powder media

A total of 0.01 g of cassava peel powder was added to 1 ml of buffer with pH 4.5 and 5.5 of *Aspergillus niger* fungus and 1 ml of crude extract enzyme in a 10 ml test tube incubated for 30 minutes at 29.5 and 30.5°C. After mixing, the reaction was stopped by incubating at 100°C for 15 minutes. Then the suspension was centrifuged for 10 minutes. Then the sample was measured with a 540 nm spectrophotometer. Cellulase activity was measured as 1 unit amount of enzyme producing 1 µmol of glucose per minute (Susilowati and Haryono, 2018). All treatments were carried out in triplicate (n = 3) to ensure the reproducibility and statistical reliability of the results.

Test the amount of *Aspergillus niger* fungal biomass

A total of 1 mL of fermentation medium from each sample was transferred into a cuvette. Distilled water was used as a blank to measure light absorbance at 610 nm using a spectrophotometer. Each treatment was performed in triplicate (n = 3). (Stephanie, 2019)

Data Analysis

Data analysis used the SPSS 27 for Windows program for this study. The first step is to test the normality and homogeneity of the research data. If the resulting data is normal and homogeneous, the Two Way Anova (Analysis of Variance) test is continued. However, if the resulting data is not homogeneous and abnormal, then use a non-parametric test, namely the Mann-Whitney test.

Results and discussion

1. Cellulolytic Activity Test of *Aspergillus niger*

The cellulolytic activity of *Aspergillus niger* was determined by comparing the clear zone diameter and colony diameter on CMC agar media. The clear zone contained in the media shows the ability of *Aspergillus niger* to hydrolyze CMC which is the main carbon source for selective cellulolytic media (Sari *et al.*, 2017). In Table 1 the results of the Cellulase Activity Index (IAS) produced by *Aspergillus* which is classified as an index with a high ratio because according to research by Sari et al., (2017) the calculation of the Cellulase Activity Index (IAS) in the extracellular ratio with a value ≤ 1 is low, 1-2 is classified as medium and a value ≥ 2 is high. Based on this, it can be said that *Aspergillus niger* isolates are proven to have a better ability to hydrolyze cellulose compared to *Trichoderma sp* which has a cellulolytic index of 0.403 and *Cunninghamella sp* of 0.754 in Setiawan & Sumardi (2024).

Table 1. Cellulase Activity Index Result

Isolate Code	Cellulase Activity Index	Enzyme Ratio
AN 2	3.5	High
AN 4	3	High
AN 9	2.6	High

Characteristic Identification of *Aspergillus niger* Fungus

Based on the table of microscopic and macroscopic observations of cellulolytic fungal isolates *Aspergillus niger* above, macroscopically *Aspergillus niger* shows the characteristics of black conidiospores with reverse colony or yellow colony surface which has a velvety texture. This is in accordance with research Praja & Yudhana (2018) who reported that *Aspergillus niger* is characterized by black colonies with a velvety texture and pale yellow to brownish underside.

Table 2. Results of identification of fungal characteristics

Deskripsi	*Aspergillus niger*
Colony on medium	
Colony color	Black
Colony reverse	Yellow
Texture	Velvety
Conidia	
Shape	Round
Color	Black
Conidiophores	
Shape	Cylindrical
Color	Hyaline (Colorless)
Hyphae	Non-ceptate
Vesicle	
Shape	Globose
Fialid	Biseriate (Growing from metula)
Additional Traits	
Growing zone	Available
Radial growth	Available

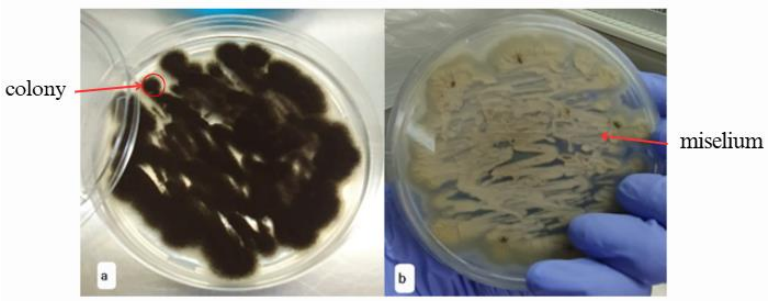

Figure 2. Macroscopic characteristics of *Aspergillus niger*

The microscopic structure shows black round conidia with cylindrical conidiophores that are hyaline (colorless). According to Nompo *et al* (2019) the characteristics possessed by *Aspergillus niger* are long conidiophores that are not septate with globose (round) vesicles at the end. These conidiophores carry radially arranged conidia, forming a black round head-like structure. Observations also show that *Aspergillus niger* has a distinctive characteristic with a growing zone which is an active area in fungal colonies, where new cells continue to be produced and extend the colony at the edges (Priyanta *et al*., 2019). As well as the characteristics of colonies that have radial growth, which means that fungal colonies spread out from the center In the research of Sari *et al* (2017) showed that *Aspergillus niger* has non-ceptate hyphae, which each conidiophora supports each conidia with a phialid formed in the metula.

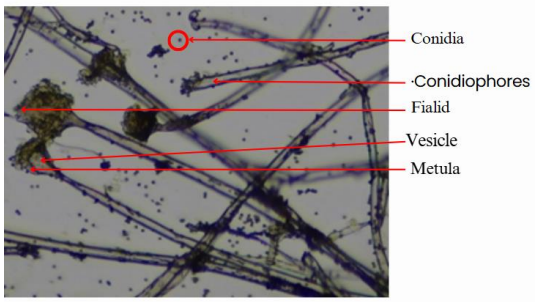

Figure 3. Microscopic characteristics of *Aspergillus niger*

Based on the observation of biochemical tests, *Aspergillus niger* isolates have the ability to degrade starch and lipids, and can dissolve phosphate in Pikovskaya media and ferment glucose and xylose. However, it cannot break down gelatin protein into smaller peptides because the gelatin structure consists of polypeptide chains bound by hydrogen bonds that cannot be broken down by *Aspergillus niger* (Susilowati and Haryono, 2018).

Biomass and Cellulase Enzyme Activity

Fungal biomass production and enzyme activity are influenced by several environmental and physiological factors, including temperature, pH, nutrient availability, oxygen levels, and the growth phase of the fungus. Optimal temperature and pH are critical for enzymatic reactions and fungal metabolism. At suitable pH levels, fungi such as *Aspergillus niger* can efficiently utilize substrates, leading to increased enzyme secretion and cellular growth. Furthermore, enzyme production is typically correlated with the fungal growth phase, where maximum enzyme activity often occurs during the exponential phase due to high metabolic rates and active cell division. According to Ariyani *et al.* (2015), this phase is characterized by the rapid synthesis of primary metabolites, including enzymes that facilitate substrate breakdown and biomass accumulation. Therefore, monitoring enzyme activity in relation to biomass can provide valuable insights into the fungal growth kinetics and its metabolic potential.

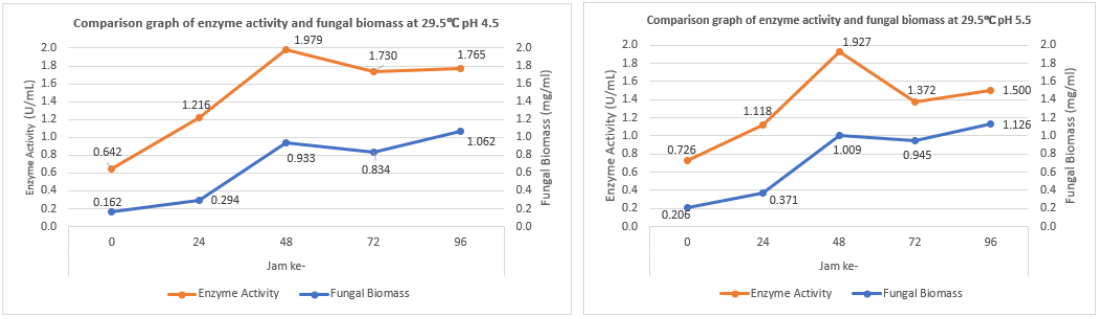

Figure 4. Comparison graph of enzyme activity and fungal biomass at 29.5℃ pH 4.5 and pH 5.5

Figure 4 shows the results of enzyme activity values and fungal biomass at 29.5 ℃ pH 4.5 and 5.5 where there is a directly proportional relationship between fungal biomass and the value of enzyme activity produced. The highest enzyme activity at pH 5.5 was 1.927 U/mL with a fungal biomass of 1.009 mg/ml while at pH 4.5 had an enzyme activity value slightly greater than pH 5.5 of 1.979 U/mL with a fungal biomass of 0.933. Based on Figure 1, the significant growth phase phenomenon of enzyme activity and biomass of *Aspergillus niger* in hours 0 to 48 occurred because the cells were in the exponential phase, because according to Ariyani *et al.* (2015) in this phase there is high enzyme protein synthesis due to optimal nutrient availability and induction by cellulose substrates Cahyani *et al.* (2015) explained that cellulase production reaches a peak during exponential growth because cellulase gene expression is maximally induced. This phenomenon is in line with the research of Lubis *et al.* (2024) found that the increase in *Trichoderma sp*

biomass is directly proportional to the increase in activity up to a certain phase, until it decreases after reaching the optimal point.

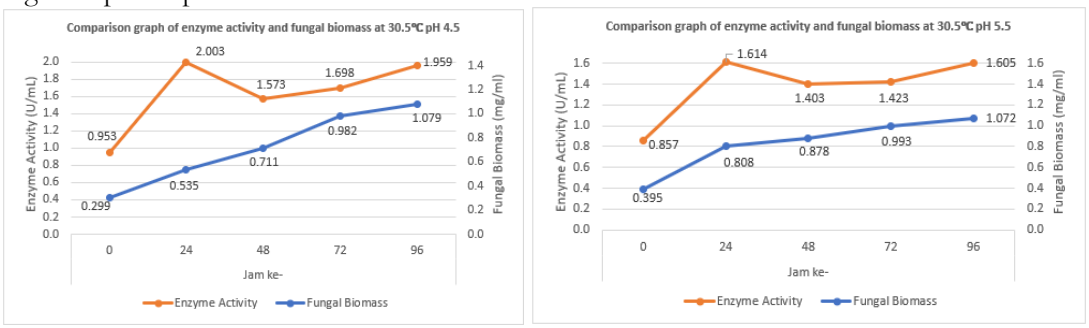

Figure 5. Comparison graph of enzyme activity and fungal biomass at 30,5℃ pH 4.5 and pH 5.5

Based on the graph data in Figure 5, the comparison between the enzyme activity produced and the biomass of *Aspergillus niger* fungus 30.5 ℃ pH 4.5 shows that during the fermentation process for 24 hours the *Aspergillus niger* fungus experiences exponential growth or an increase in cell number, where in this case the fungus does not require a long time to adapt to the conditions and treatment of fermentation media so that the fungus grows well so that the enzyme activity reaches a peak at the 24th hour with the resulting enzyme activity value of 2.003 U/mL with a fungal biomass of 0.535 mg/ml. While at pH 5.5 showed the same pattern as pH 4.5 where the biomass and activity of the fungus were directly proportional until the 24th hour with enzyme activity reaching a peak at the 24th hour with a value of 1.614 U/mL with a fungal biomass of 0.808 mg/ml.

In this study, it can be said that the optimum pH for cellulase enzyme production by *Aspergillus niger* with cassava peel is at pH 4.5 compared to 5.5 by producing higher enzyme activity because according to the research of Qur'ania *et al.* (2023) the optimal pH for the production of filamentous fungi ranges from 4-5. In conditions of pH 4-5 is the optimal pH for the growth of *Aspergillus niger*, so that it can produce maximum activity. This is reinforced by the research of Lubis *et al.* (2024) that in general fungi can grow and produce various kinds of enzymes in the acidic pH range, these acidic pH conditions can facilitate the release of cellulase enzymes, while high pH can cause inhibition of the release of cellulase enzymes. The optimal temperature of this study was at 30.5 ℃ because the highest activity value was at that temperature with an activity value of 2.003 U/mL, as well as the control treatment which had an optimum value at 30.5 ℃ with a value of 0.260 U/mL. This can occur because it can accelerate the enzymatic reaction of substrate degradation which is in line with this study, the highest activity was at the 24th hour compared to the temperature of 29.5 ℃, the highest activity was at the 48th hour. Supported by research by A. R. Sari *et al.* (2017) explained that the optimum temperature used in the production of cellulase enzyme is 30℃. Research by Namnuch *et al.* (2021) cellulase enzyme production using *Aspergillus flavus* as the cellulolytic fungus produced the highest activity value at 30℃ compared to other treatments at 25℃ and 28℃. This is because cells find it difficult to degrade substrates at temperatures that are too low, so the products produced are low. In contrast to the research of Laura *et al.* (2020) cellulase enzyme production by *Trichoderma koningii* produces the highest activity value at 28 ℃ because in their research the *Trichoderma koningii* fungus is not strong with high temperatures because it can affect its enzymatic activity.

Based on this, the use of cassava peel agricultural waste substrate *(Manihot esculenta)* for cellulase enzyme production can increase the value of the resulting activity because according to Artiyanti, A & Soedjono (2018) cassava peel has 43.63% cellulose so that it can stimulate the production of cellulase enzymes in larger quantities. In the research of Ariyani *et al.* (2015) used rice straw 39.00%, banana peel 17.36% and pineapple peel 33.25% as substrates for the production of cellulase enzymes, which are relatively low when compared to the cellulose content of cassava peel. And the lignocellulosic bonds of cassava peel are easier to degrade because cassava peel only has 7.6% lignin (Prasetyo *et al.*, 2018). This statement can be an advantage for agricultural waste substrates to be utilized further regarding cassava peel because in the research of Ariyani *et al.* (2015) the agricultural waste used was pineapple peel which showed lignin levels before delignification of 10.81% and after delignification of 8.39%, when compared to cassava peel before the delignification process of 7.6%, especially in this study the substrate underwent a pretreatment process with 6% NaOH so that it only required lower energy for hydrolysis. Another factor

that makes cassava peels effective for cellulase enzyme production is due to the additional nutrient content in cassava peels as in the research of Kumar *et al.* (2017) explained that cassava peels contain 4-6% and essential minerals that support microbial growth and more optimal enzyme production.

Data Analytics

Table 3. Statistical Test Results

Statistical Test		α	Significance
Normality Test *(Kolmogrov-Smirnov)*		<0.05	0.121
Homogeneity Test *(Levene's Test)*		<0.05	0.563
Two Way Anova Test *(Test of Between-Subsjects Effect)*	Temperature	<0.05	0.578
	pH		0.328
	Temperature*pH		0.763

Based on the results of the statistical test above, the factors of temperature and pH influence are not significantly different from the enzyme activity produced, as well as there is no interaction between the two factors of temperature and pH. This is because the pH and temperature range tested is very narrow, while the degradation of lignocellulosic substrates such as cassava peels will be clearly visible in a larger pH range and a minimum temperature difference of 5-10 ℃. According to Gi *et al.* (2018) enzymes that work on cassava peel substrates such as cellulase and amylase enzymes require greater conditions to show significant variations in activity. In the research of Sulistyowati *et al.* (2023) explained that some enzymes that work on lignocellulosic substrates such as cassava peels show good stability in the pH range of 4-6 and temperatures of 25-35°C. This explains why small changes between pH 4.5 and 5.5 and temperature 29.5°C and 30.5°C did not result in significant changes in activity. Another reason why temperature and pH do not significantly affect enzyme activity is due to the phenomenon of enzyme adsorption on lignocellulosic substrates such as cassava peels, according to Baig and Turcotte (2016) this adsorption creates a microenvironment that tends to stabilize on the substrate surface, so that enzyme activity is not significantly affected by small changes in environmental pH and temperature.

Conclusion

Based on the findings, data analysis, and discussion, it can be concluded that the specific characteristics of *Aspergillus niger* fungal isolates include black conidiospores, yellow colony reverse, a velvety texture, as well as radial growth with a visible growing zone. The cellulolytic fungus *Aspergillus niger* is capable of producing cellulase enzymes from cassava peel substrate *(Manihot esculenta),* with the highest enzyme activity observed at 30.5°C and pH 4.5, reaching 2.003 U/mL and a fungal biomass of 0.535 mg/mL.

Author Statements

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Generative AI: not applicable

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