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Enhancing Kojic Acid Production in *Aspergillus oryzae*: Leveraging Crude Cellulase from *Achatina fulica* for Strain Improvement via Protoplasting and UV Mutagenesis

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ABSTRACT

This study aims to prove the ability of crude cellulase enzymes from snails for protoplasting *Aspergillus oryzae* cells and its application for strain improvement with UV mutagenesis. Snail enzyme was obtained from *Achatina fulica* by dissolving its digestion track and fractionating it with ammonium sulfate. The activity of fractions was measured Spectrophotometrically and used for cell protoplasting for 2 hours, then irradiated with UV for 10, 15, and 20 minutes, respectively, with 5 cm in the distance. Screening of mutants is carried out with 1% $FeCl_3$, and the potential mutant strain was tested for kojic acid production in an aerobic state and determined by Spectrophotometry at 268 nm. The cellulase activity in crude snail enzyme was 11.5807 U/ml and increased to 16.3984 U/ml after fractionation. The best protoplast formation was obtained with a 60% fraction, which showed transparent performance under the microscope. The UV mutagenesis of protoplasts showed that the highest number of potential mutants was obtained from UV treatment for 15 minutes (41.67%). The potential mutants look dark brown (DBC), such as strain 10H3, and produced higher kojic acid concentration than the parent strain. In conclusion, UV mutagenesis of *Aspergillus oryzae* through protoplasting by crude cellulase of snail enzyme was effective and improved kojic acid concentration.

1. Introduction

Indonesia is a country known for being rich in biodiversity, having various living species, and a rich source of lignocellulosic biomass. The climate of Indonesia is almost entirely tropical, and its relative humidity ranges from 70 to 90%. Many fungal genera can grow in tropical humid conditions, one of which is *Aspergillus*.

This type of fungi grows within lignocellulosic materials and produces cellulase, which consists of exoglucanase, endoglucanase, and β-glucosidase, that hydrolyses The β -1,4 linkages of the cellulose into glucose (Namnuch *et al.*2021).

The diversity of the *Aspergillus* genus denotes a broad spectrum of metabolite products useful for industrial applications such as food additives, pharmaceuticals, and detergents. *A. oryzae* is considered one of the most essential biocatalyst in many countries around the globe. The fermented food industry in Japan uses this strain for the production of miso (soybean paste), shoyu (soy sauce), and tane-koji(seed rice malt). Tempeh, a typical food in Indonesia, is also produced by *A. oryzae* and *Rhizopus* sp. as a starter for inoculation (Daba *et al.* 2021).

Aspergillus oryzae produces organic acids such as kojic acid, which is used in industry as a food additive or in cosmetics pharmaceuticals. Various types of *Aspergillus* strains are known to produce Kojic Acid as its secondary metabolite, some of which are *Aspergillus oryzae, Aspergillus tamarii, Aspergillus parasiticus*, and *Aspergillus flavus*. Kojic acid (5-hidroxy-2-hydroxy methyl-4-pyrone) is an organic acid that is widely used as an analgesic, pesticide, and whitening agent in cosmetics (Ammar *et al.* 2017; Suryadi *et al.* 2022).

The cosmetics industry in Indonesia is experiencing rapid growth, with whitening agents being among the most sought-after products. Due to their high demand, these agents hold significant importance within the Indonesian cosmetics sector.

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Although kojic acid has been widely used in the industry, various efforts to optimize the composition of the medium and environmental conditions to increase kojic acid production by microorganisms are still being studied. In connection with this, mutations using UV radiation in the kojic acid-producing strains are considered simple and effective for increasing the rate of its production (Chaudhary *et al.* 2014).

The efficacy of the UV mutagenesis technique in enhancing strains relies on both the sensitivity of microorganisms to UV light and the capacity of UV rays to penetrate their cellular structures. The cell wall structure of *Aspergillus* is two-layered, and the dominant cell wall components are polysaccharides (Fontaine *et al.* 2000). It has a thick wall and is resistant to bad conditions. Therefore, protoplast formation or cell wall removal is needed to make penetration of UV-ray better (Suryadi *et al.* 2022). This treatment, followed by optimization of UV irradiation, will increase the effectiveness of mutations.

An enzyme that can break down the cell wall is needed for protoplast preparation. Besides commercial enzymes, there is also an alternative enzyme (biolytic) that can lyse cell walls, and it comes from the digestive glands of snails (*Achatina fulica*). An alternative lytic (biolytic) enzyme obtained from snails is easy to obtain, cheap enough, and works effectively on certain microorganisms (Wijanarka *et al.* 2015). This study aimed to find the effectiveness of crude snail enzyme for protoplasting *Aspergillus* cells and its application with UV mutagenesis for strain improvement of *Aspergillus oryzae* to produce kojic acid.

2. Materials and Methods

Aspergillus oryzae ATCC 10124 and *A. tamarii* IPBCC 11701 were obtained from IPB Culture Collection (IPBCC), Biological Department, Faculty of Mathematics and Natural Sciences, IPB Bogor. Stock cultures were incubated in potato dextrose agar (Difco) at 28°C. The *Achatina fulica* was obtained from snail collectors from Pangandaran, West Java.

2.1. Culture Maintenance and Initial Fermentation Medium

The mold isolate was taken and put on the PDA medium aseptically, then incubated for seven days at 28°C. Most were stored as stock cultures at 4°C, and the rest as working cultures at 30°C. Stock culture maintenance was for two months, while working culture was for two weeks (Suryadi and Sukarna 2018).

2.2. Enzyme Extraction and Fractionation

The method used is based on Dini *et al.* (2019) with modifications. The intestinal part of the snail was carefully cut, and the pieces were dissolved in 45 ml of cooled 1% NaCl. The mixed ingredients were stirred with a mixer for 10 minutes. After that, it was filtered and centrifugated at 3,000 rpm, 2°C for 30 minutes. The filtrate obtained was then stored in the refrigerator as a crude enzyme, and the cellulase activity was measured. Then, the crude extract obtained was precipitated with the addition of ammonium sulfate slowly at a temperature of 5 to 10°C to a saturation level of 0-40%, 40-60%, and 60-80% in a 50 ml of the crude enzyme named fraction 1, fraction 2, and fraction 3 sequentially (Dini *et al.* 2019).

2.3. Dialysis and Cellulase Activity Test

The method used is based on Mayasari (2016). Each fraction dialysis uses a cellophane (MW cut off 12,000-14,000 Da (Ward's Science, USA) moistened and soaked with 1 mM Na2EDTA and 20% $NaHCO_3$ solution for 10 minutes each. The two ends of the bag were tied with threads, and the bag was then immersed by hanging it on the beaker glass containing 200 ml of phosphate buffer pH 6.5 and stirred with a magnetic stirrer (100 rpm) for 24 hours. Check the dialysis process using a 1 M $BaCl_2$ solution; put 2 ml of buffer plus 0.1 M HCl with a few drops of $BaCl_2$ in a test tube. If there is no precipitation, dialysis is finished. Keep the temperature around 4°C with ice cubes. Cellulase activity was measured by Spectrophotometry at a wavelength of 510-550 nm. One unit of enzyme activity was defined as the amount of enzyme required to form 1 µmol of product per unit time for each mL of the enzyme (Murtiyaningsih and Hazmi 2017).

2.4. Protoplast Formation

The method used was using our previous study (Suryadi *et al.* 2022). Cells with a density of approximately 107 were inoculated in 50 ml of YES medium in Erlenmeyer, then incubated for 48 hours at 28°C with 180 rpm. Cells were separated by centrifugation from the media at a speed of 5,000 rpm for 15 minutes. The precipitated mycelial

cells were taken and washed two times with 0.6 M potassium chloride by centrifuging it again at 5,000 rpm for 15 minutes. The precipitated cells were then suspended in 10 ml of the enzyme in phosphate buffer pH 6 containing 0.6 M potassium chloride and incubated for 2 hours at 28°C with 100 rpm. Then, filtration was carried out against the incubated protoplasts using lens paper, and the filtrate was centrifuged for 10 minutes at a speed of 5,000 rpm. The precipitate obtained was suspended with 1.0 ml phosphate buffer pH 6 containing 0.6 M potassium chloride. The formation of protoplasts was observed under a microscope and was stored at 4°C.

2.5. Mutagenesis of Protoplast

As much as 100 µL of protoplast suspension was pipetted into several Petri dishes containing a PDA medium with 0.6 M KCl. UV radiation was performed with a wavelength of 254 nm for 10, 15, and 20 minutes, respectively, and 5 cm in distance. Once mutated, the Petri dishes were protected from light for one night to avoid photoreactivation and then incubated at 28°C for three days. The incubated petri dishes were cultured to maintenance culture and screened with $FeCl_3$ reagent and UV-Vis Spectrophotometry (Ren *et al.* 2018).

2.6. Screening of Mutants and Culture Maintenance

The results of mutant screening with $FeCl_3$ reagent, which gave dark red-brown results, were measured by UV-Vis spectrophotometry. The result that gave the greater absorbance of kojic acid at a wavelength of 268 nm was chosen (4). Selected mutant cells were subcultured on PDA sloping agar by being transferred aseptically and then incubated at 28°C for seven days. Most were stored as stock cultures at 4°C, and the rest as working cultures at 30°C.

2.7. Kojic Acid Fermentation and Measurement

The fermentation method is based on Suryadi and Sukarna 2018 with little modifications. Pre-Culture was prepared using suspension spores of selected strains by inoculating the spores in 100 ml of YES medium (10%v/v) in 250 ml Erlenmeyer.

Incubation was at 28°C with 180 rpm by shaking for 24 hours. Fermentation was performed in 100 ml of various fermentation mediums (Table 1) in 250 ml Erlenmeyer with 10% (v/v) inoculum, followed by incubation at room temperature for eight days at 180 rpm. Then, the cell biomass and the concentration of kojic acid produced were observed. Kojic acid standard solutions were prepared between 4 and 12 ppm and measured with a UV-Vis Spectrophotometer at its maximum wavelength (269 nm). The standard curve obtained was used to calculate kojic acid concentration in the fermentation culture.

Table 1. Fermentation medium

Medium	C source conc. (%)	N source conc. (%)	KH_2PO_4 (%)	$MgSO_4 \cdot 7H_2O$ (%)
Yeast extract sucrose	5	0.25	0.1	0.05
Yeast extract molasses	5	0.25	0.1	0.05

3. Results

3.1. Partial Purification of Crude Enzyme

The giant African snails were from Snail collectors in Pangandaran, Indonesia. After the snail's shell is broken, the soft part is blended. The extract was then filtered, and the amount of crude enzyme collected was 107.8 ml (Figure 1A). The filtrate obtained was a crude enzyme that will be used to isolate protoplasts after partial purification. The crude enzyme was partially purified with ammonium sulfate (40%, 60%, and 80%), then dialysis was carried out, and cellulase activity was measured for each fraction. As shown in Figure 1B, C, and D), each fraction has a different color density based on the protein precipitated.

3.2. Cellulase Enzyme Activity Test

Based on absorbance measurements using a UV-Vis spectrophotometer at a wavelength of 510-550 nm, the regression equation of the standard solution of glucose was: $y = 0.115 + 0.0035x$ with $r = 0.9877$ (x as absorbance and y as concentration). This equation measures the concentration of reducing sugars to obtain the value of cellulase activity. The results show that fraction 2 had the highest cellulase activity (Table 2).

3.3. Protoplast Formation

The activity of the cellulase enzyme on the fractions has been obtained, and these fractions are used to make protoplasts of *A. oryzae* cells. The

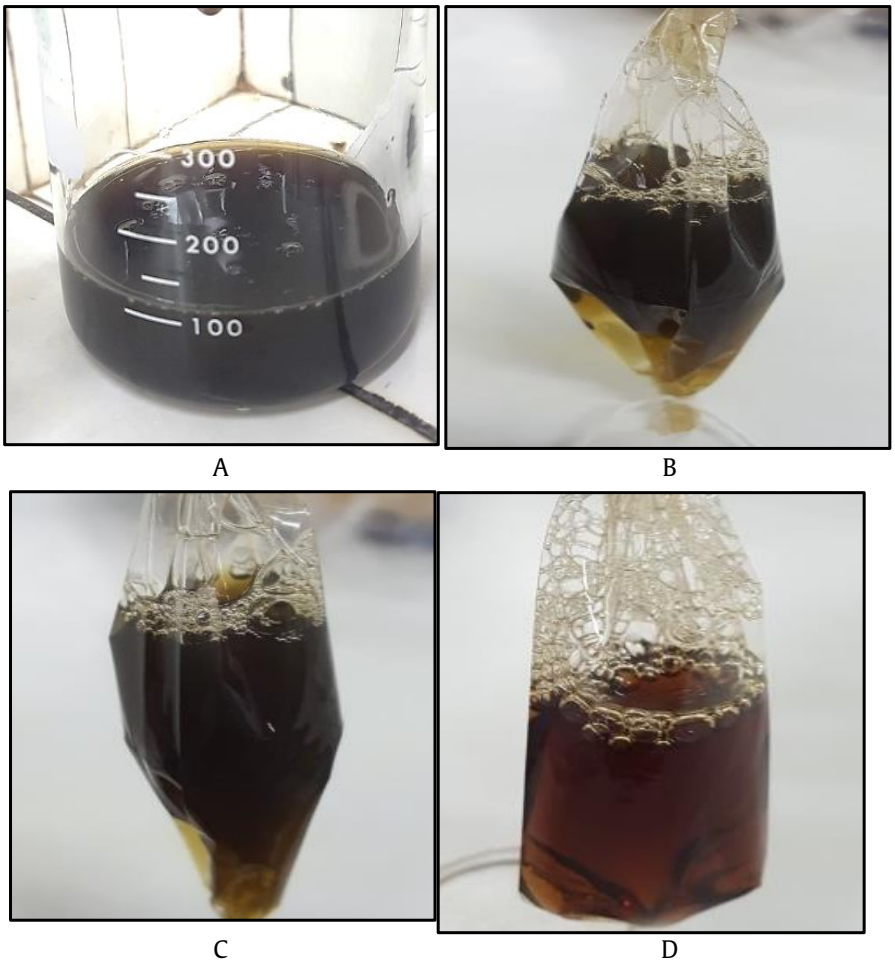


Figure 1. (A) Snail crude enzyme, (B) fraction 1 (dark greenish brown), (C) fraction2 (reddish brown), (D) fraction 3 (greenish brown)

Table 2. Cellulase activity in crude and fractions (Student's t-test, df =3; p =0.002)

Source of enzyme	Cellulase activity (U/ml)
Crude enzyme	11.58
Fraction 1	11.60
Fraction 2	16.39
Fraction 3	11.55

results of the protoplast formation are shown in Figure 2. It showed that the formation of protoplasts using fraction two is better than fractions one and three, shown by the formation of transparent spheres observed through a microscope at 100x magnification. Therefore, protoplasts from fraction two are continued in the mutagenesis process.

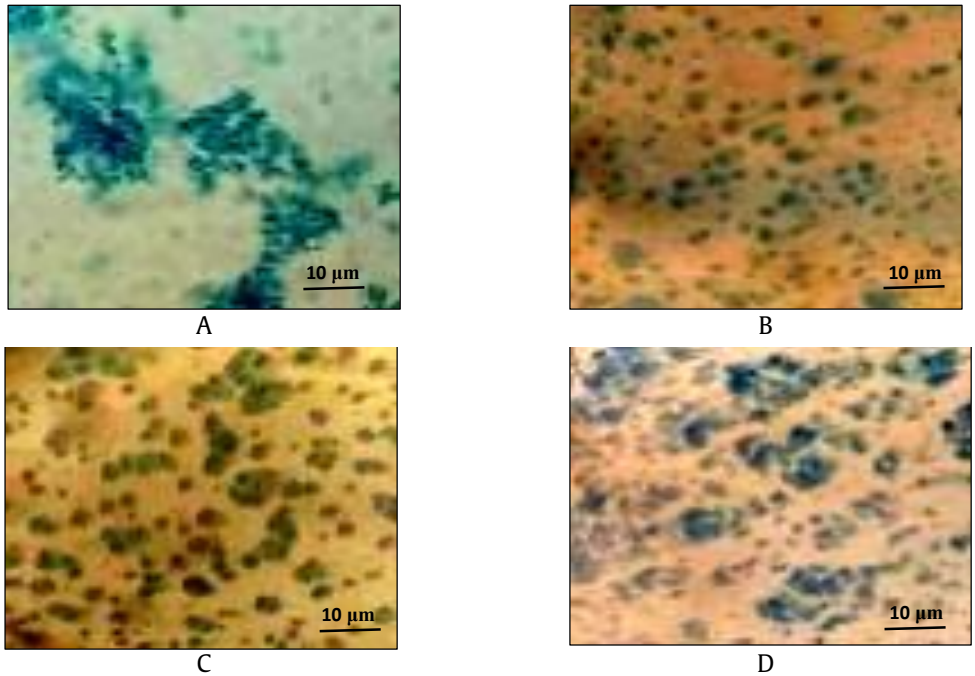
3.4. Mutagenesis of Protoplast

The selected protoplast suspension was pipetted into three Petri dishes containing a PDA medium with 0.6 M KCl. These petri dishes containing protoplast were then treated with UV radiation with a wavelength of 254 nm for 10, 15, and 20 minutes, respectively. The colony of the mutated strain, which had 15 minutes of treatment, had a greener look than the other two (see Figure 3). These Petri dishes containing protoplast were then subcultured into 96-well plates for further screening.

3.5. Screening of Mutants with $FeCl_3$ Reagent

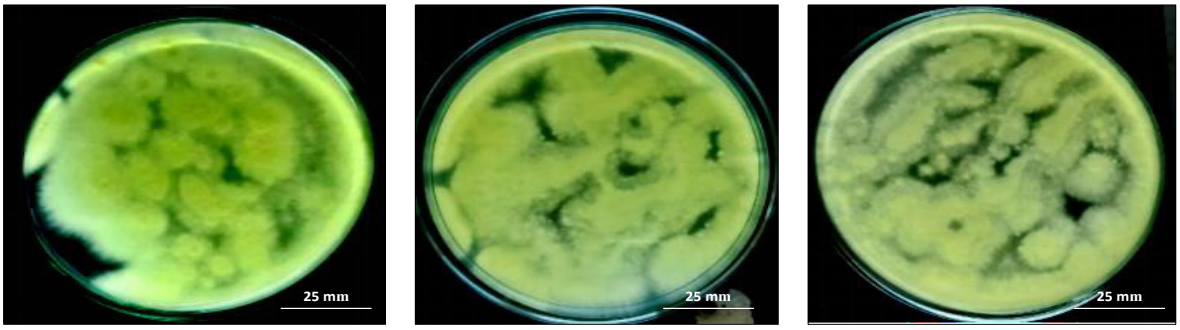
After the protoplasts treatment with UV rays, the mutants were sub-cultured into 96-well plates (Figure 4) and incubated for four days at 28°C. Then, the mutants screening was with a drop of $FeCl_3$, and the mutants with the deepest brick red (reddish-brown) color were selected.

Mutant strains were named by their position in 96 well plates (alphabetic A-H from up to down, and no.1-12 from left to right).



A B C D

Figure 2: Protoplast formation using snail enzyme. (A) Control, (B) fraction 1, (C) fraction 2, (D) fraction 3



A B C

Figure 3. Colonies of mutated Strain after irradiation of UV light for 20 (A), 15 (B), and 10 (C) minutes respectively and after 7 days of incubation

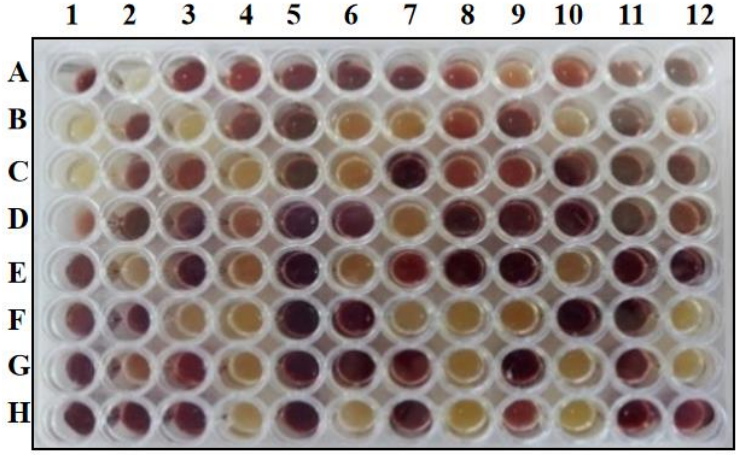


Figure 4. The sub cultured mutants of selected treatment (15 minutes UV irradiation) after incubated and visualized with a drop of $FeCl_3$. M utant strains was named by its position in 96 well plates (alphabetic A-H from up to down, and no.1-12 from left to right)

The qualitative screening above showed that all UV treatments from 10 to 20 minutes could produce high-performance mutants, indicated by their deep reddish-brown color (DBC). Based on the number of potential mutant strains obtained, UV treatment for 15 minutes was selected as the best technique that produced the highest number of high kojic acid producer mutants. (41.67%; see Table 3). Irradiation with UV for 10 minutes and 20 minutes produced more mutants with lower kojic acid concentrations. Among the potential mutants obtained from each 96-well plate (a total of 112 mutants), strains 10C7, 10D10, 10H3, 15D11, 15G5, 15H5, 20A8, and 20G11 were selected. These mutants were for further quantitative screening.

3.6. Screening of Mutants with UV-Vis Spectrophotometry

The quantitative screening of selective mutants was carried out by measuring kojic acid absorbance at a wavelength of 269 nm. The standard curve of kojic acid obtained has the regression equation, y = 0,0551x + 0,0113 with r = 0,9968. The screening results showed that all potential mutants selected by screening with $FeCl_3$ had higher concentrations of kojic acid than the parent strain (1.0244 g/L). The two strains with the highest concentration of Kojic Acid (20G11 and 10H3) from the quantitative screening were then continued to further the fermentation process (Table 4).

3.7. Kojic Acid Fermentation

The two best mutants from quantitative screening were continued to the fermentation process. The medium used in fermentation consisted of 2 variations of carbon sources, namely sucrose (5%) and molasses (5%). The results after fermentation for eight days are in Table 5. These results show that the fermentation medium using yeast extract and molasses as a source of nitrogen and carbon produced higher kojic acid than using yeast extract and sucrose. Moreover, from these results, the 10H3 strain produced higher kojic acid than the parent strain and 20G11 strain.

4. Discussion

Aspergillus oryzae was grown in a PDA medium for culture maintenance and incubated for seven days at 28. Its colony expressed a yellowish-green color similar to reference (Park *et al.* 2017). (data not shown). The environmental and food conditions of *Achatina fulica* affect the enzymes produced by the digestive glands. *Achatina fulica* was fed with leaves and vegetables. Feeding was carried out routinely for three days so that Achatina fulica conditions were in a homogeneous condition. Bacteria in the snail digestive tract have cellulolytic activity. The production of enzyme extract was obtained by removing the shell, then blending the soft parts of

Table 4. Results of mutants quantitative screening

Strain	Mutant strain number	Kojic acid (g/L)
Parent strain	-	1.02443
20’	20G11	6.2328
	20A8	4.6588
15’	15H5	4.4494
	15G5	2.9283
	15D11	5.0837
10’	10H3	6.7238
	10D10	4.4673
	10C7	5.3884

Table 5. Result of kojic acid fermentation

Strain	Kojic acid concentration (g/L)	
	Yeast extract-sucrose	Yeast extract-molasses
Parent strain	3.177	3.997
20G11	5.859	6.928
10H3	6.926	7.339

Table 3. Number of mutant type and cases prosentation

Mutation time	Number of mutant after $FeCl_3$ addition			Cases prosentation (%)		
(minutes)	YC	TBC	DBC	YC	TBC	DBC
10	14	47	35	14.58	48.96	36.46
15	25	31	40	26.04	32.29	41.67
20	14	35	37	14.58	36.46	38.54

*YC: yellow color, TBC: thin brown color DBC: deep brown color

the body and adjusting to low temperatures so that the enzyme does not experience changes (Wijanarka *et al.* 2015).

UV irradiation rays penetrate microbial cells depending on the level of cell sensitivity. *Aspergillus oryzae* has a thick and complex cell wall consisting mainly of polysaccharides. So, it is necessary to remove cell walls with the formation of protoplasts to increase the effectiveness of UV light mutagenesis (Wan *et al.* 2004; Ren *et al.* 2018). The absence of the cell wall makes protoplasts very sensitive to the osmotic pressure of the environment. It is necessary to provide an osmotic stabilizer to avoid crenation or lysis of the cell membrane. This solution comprises inorganic salts, sugar, and sugar alcohols (Wahyudi *et al.* 2006). The removal of *Aspergillus oryzae's* cell wall will need the help of enzymes that follow the cell wall composition of the microorganism. Filamentous fungi such as *Aspergillus oryzae* have a cell wall mainly consisting of chitin, glucan, and mannoprotein structures. The commercial lysing enzyme system is commonly used to lyse the cell wall of the mold. This enzyme is usually a mixture of several different enzymes, including β-glucanase (cellulase), protease, mannanase, and chitinase, which act effectively and synergistically in lysing the cell wall (Wan *et al.* 2004; Salazar and Asenjo 2007)

An alternative source of lytic enzymes is present in the intestinal fluid of snails (*Achatina fulica*). Intestinal fluid from snails contains enzymes with cellulase activity. Cellulase is an enzyme that plays a vital role in nature by recycling polysaccharides, a primary component of the A*spergillus oryzae's* cell wall. Bacteria in the snail digestive tract have a cellulolytic activity of cellulose, which catalyzes the hydrolysis of 1,4-β- d-glucoside bonds in cellulose.

This study obtained cellulase activity in crude enzyme at 11.58 U/ml (Table 2). This crude extract cellulase activity was much higher than those obtained by Mulyaningrum and Suryati in 2008 (*A. fulica* cellulase 0.729 units/ml) or by Septiani *et al.* in 2019 (fungus cellulase, 0.374 U/ml). The crude enzyme obtained was precipitated with the addition of ammonium sulfate slowly at a temperature of 5 to 10°C to a saturation level of 0% -40%, 40% -60%, and 60% -80% s in a 50 ml crude enzyme. Ammonium sulfate precipitation is the most commonly used method for protein purification and fractionation. It can separate proteins by altering their solubility in a high salt concentration (Wingfield 1998). Adding ammonium sulfate to the enzyme solution at the optimum saturation level will increase the charged electricity around the enzyme protein, pulling the water coat away from the protein molecule. Hydrophobic interactions among protein molecules at high ionic conditions will reduce protein solubility so that the enzyme protein precipitates.

The cellulase enzyme obtained from precipitation was not pure and contained salt residues, thus making the dialysis method necessary. The final limit of dialysis is the absence of $BaSO_4$ precipitates when the buffer solution with $BaCl_2$ salt is added. Dialysis is using a cellophane bag with a pore size of 12-14 kDa in 200 ml phosphate buffer pH 6.5 with six times changes of solvent. Based on literature studies, the molecular weight of the cellulase enzyme is between 26-200 kDa. So that the cellulase enzyme cannot get out through the pores in the dialysis tube, but ammonium sulfate in the form of ions can quickly exit through the membrane (Sonia and Kusnad 2015).

The cellulase activity of the crude enzyme was 11.5807 U/ml, while the fraction 1, 2, and 3 resulted in cellulase activity of 11.6045, 16.3984, and 11.5540 U/ml, respectively. One unit is the enzyme needed to break 1 µmol cellulose into reducing sugar per minute under test conditions (Dini *et al.* 2019). Fraction 2 (a saturation level of 60%) had the highest cellulase activity, thus making it better in protoplast formation. Increasing concentrations of ammonium sulfate cause an increase in the electrical charge around the protein, which will attract the water mantle from the colloidal protein, and the hydrophobic interaction between protein molecules will reduce protein solubility. Most cellulase enzymes were assumed to be precipitated in this 60% saturation level.

In fermentation, carbon and nitrogen sources have dominant roles because these nutrients are directly associated with the cell biomass and metabolite formation. The source of nitrogen used in this fermentation process was yeast extract (El-Aasar 2006; Suryadi *et al.* 2022). Yeast extract produces the highest kojic acid production compared to other nitrogen sources. Another study also demonstrated these findings that yeast extract is a complex nitrogen source containing vitamins that can act as a precursor of kojic acid formation (Ammar *et al.* 2017). The carbon sources used in

this fermentation process are sucrose and molasses. Both carbon sources contain glucose, which acts as a precursor of kojic acid (Wan *et al.* 2005).

This study found that the highest kojic acid was produced in a medium with molasses as a carbon source (Table 5). Molasses is a byproduct of the sugar-making process. Molasses is a thick liquid obtained from the separation of sugar crystals. Molasses contains mostly sugars, amino acids, and minerals. The sucrose in the molasses varies between 25–40%, and the reducing sugar content is 12–35%. An essential component in molasses is TSAI (Total Sugar as Inverti), a combination of sucrose and reducing sugar. Molasses has TSAI levels between 50-65%. TSAI levels are significant for the fermentation industry because the greater the TSAI level, the more profitable it will be. According to other studies, molasses contains about 44% as total sugar (glucose, sucrose, and fructose) and 0.46% as total nitrogen in addition to detectable amounts of some vitamins such as riboflavin and thiamin (El-Kady *et al.* 2014). The glucose in the molasses acts as a precursor to kojic acid, and fructose contributes to microbial cell growth. In addition, using molasses as a carbon source is also more economical due to its cheaper price than commercial sucrose.

From the fermentation results, one of the mutant strains, the 10H3 strain, produced higher kojic acid than the parent strain and 20G11 strain. This concentration was 7.3394 g/L or about 1.83 times higher than the parent strain of *Aspergillus oryzae*. This result showed that the mutation process after protoplast preparation in the parent strain was effective and generated potential mutant cells that can produce higher kojic acid levels than the parent strain.

In conclusion, the considerable high activity of cellulase from crude snail enzyme presents a viable alternative as a lysing agent for protoplast formation in *Aspergillus oryzae* cells that is suitable for penetration with UV irradiation. This technique has proven simple, inexpensive, and effective for strain improvement in the future.

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