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UTILIZATION OF FISH PROTEIN HYDROLYSATE AS A NITROGEN SOURCE IN FERMENTED MEDIA OF CEPHALOSPORIN C PRODUCTION BY *ACREMONIUM CHRYSOGENUM* Biomcc 00141

Pemanfaatan Hidrolisat Protein Ikan sebagai Sumber Nitrogen dalam Media Fermentasi Produksi Sefalosporin C oleh *Acremonium Chrysogenum* Biomcc 00141

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ABSTRACT

Optimizing Indonesia's maritime resources as an effort to reduce reliance on imported raw materials for the pharmaceutical industry. This study investigates the use of fish protein hydrolysate, derived from trash fish flour, as a supplement in the fermentation process for producing cephalosporin C. This key raw material is used to create cephalosporin antibiotics through fermentation with the fungus *Acremonium chrysogenum* Biomcc 0141. Hydrolysate from trash fish flour is obtained through an enzymatic hydrolysis process using a protease found in pineapple hump extract which was optimized. The results of hydrolysis optimization on hydrolysate from trash fish flour produce a protein content of 3.37%. The protein hydrolysate obtained is then used in the cephalosporin C fermentation process. The addition of trash fish protein hydrolysate to cephalosporin C fermentation media showed a significant increase in the productivity of cephalosporin C, which is up to 85.39% higher than the fermentation process without the addition of fish protein hydrolysates.

Keywords: *Acremonium chrysogenum* Biomcc 0141*, Antibiotics, Cephalosporin C, Fermentation, Nitrogen*

ABSTRAK

Pengoptimalan potensi sumber daya bahari Indonesia sebagai usaha mengurangi kebergantungan Indonesia terhadap bahan baku import untuk industri farmasi. Dalam penelitian ini pemanfaatan hidrolisat protein ikan yang dibuat dari tepung ikan rucah digunakan sebagai suplemen dalam proses fermentasi produksi sefalosporin C. Cephalosporin C merupakan bahan baku utama untuk antibiotik sefalosporin yang diproduksi oleh kapang *Acremonium Chrysogenum* Biomcc 0141 melalui suatu proses fermentasi. Hidrolisat dari tepung ikan rucah didapatkan dari proses hidrolisis menggunakan protease yang terdapat di ekstrak bonggol nanas yang dioptimalisasi. Hasil dari proses hidrolisis yang dioptimalisasi pada hidrolisat dari tepung ikan rucah menghasilkan kandungan protein 3,37%. Hidrolisat protein tersebut kemudian digunakan dalam proses fermentasi sefalosporin C. Penambahan hidrolisat protein ikan pada media fermentasi menunjukkan peningkatan yang signifikan dalam produkstifitas sefalosporin C, yang mencapai 85,39% lebih tinggi daripada proses fermentasi tanpa penambahan hidrolisat protein ikan.

Kata kunci: *Acremonium chrysogenum* Biomcc 0141, Antibiotik, Sefalosporin C, Fermentasi, Nitrogen

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INTRODUCTION

The pharmaceutical industry, especially antibiotics in Indonesia, has enormous potential considering that antibiotics are the most widely used drugs in the world and Indonesia is a country with a large population. In Indonesia, around 90% of medicinal raw materials are imported (Kementerian Perindustrian Republik Indonesia 2018). The high dependence of the pharmaceutical industry in Indonesia on imported raw materials is a factor that hampers the development of the pharmaceutical industry. The key to overcoming this challenge is to exploreutilizing the potential of Indonesia's natural resources.

Fish protein hydrolysate (FPH) has been known to have applicative capabilities in various sectors, especially in the biotechnology sector, where FPH is commonly used as a source of nitrogen or supplementation for the growth of microorganisms in the fermentation industry (Yarnpakdee et al. 2015; Tang et al. 2023). One of them is in the fermentation of the fungus *Acremonium chrysogenum* which produces Cephalosporin C (CPC) as the main raw material for cephalosporin antibiotics. The CPC is the main source for the production of 7-aminocephalosporanic acid (7-ACA), an intermediate that is important for the manufacture of first-line antibiotics cephalosporin (Terreni et al. 2021; Liu et al. 2022).

Fish protein hydrolysate (FPH) has been reported can be produced from various types of fish (Liceaga-Gesualdo and Li-Chan 1999; Prihanto et al. 2019). Indonesia boasts a significant fish biomass; however, a substantial portion is discarded due to factors such as low market value stemming from overabundance or limited consumer preference, consequently generating what is known as 'trash fish'. The FPH from trash fish can be obtained by the hydrolysis process which has been reported used in producing FPH in various studies (Vázquez et al. 2017; Noman et al. 2022). The resulting hydrolysate protein from trash fish which at the same time aims to increase its economic value has been shown to have potential as a substitute for peptone in Escherichia coli growth media (Ann Suji et al. 2019; Vázquez et al. 2020). In this study optimization of fermentation, media was carried out by by utilizing hydrolysate made from the hydrolysis of Trash Fish Flour (TFF). The optimization process was done by Response Surface Methodology (RSM). The protein hydrolysate produced from the hydrolysis process under optimum conditions was then characterized by conducting the proximate test and amino acid contents analysis. The Hydrolysate produced was then used as a supplement on the media for CPC production by *Acremonium chrysogenum*.

MATERIALS AND METHOD

Location and Time

This research was conducted at the Balai Bioteknologi, Puspiptek Serpong, South Tangerang. Research activities were carried out from January 2019 to July 2019.

Optimization of the Hydrolysis Process using Response Surface Methodology (RSM)

The advantage of the RSM method is that it considers the interaction of the specified variables in determining the optimum point and can carry out experiments with more than one variable at a time (Bezerra et al. 2008; Malekjani and Jafari 2020).(Bezerra et al. 2008; Malekjani and Jafari 2020). Preliminary experiments were carried out using the One Factor At Time (OFAT) method where the data produced by this method was sequential, so the design used was Central Composite Design (CCD). The research design is generated by entering the midpoint, upper point, and lower point values for each variable. After that, experiments were carried out according to the CCD design produced in the Design Expert application version 11.0. The CCD experimental design was carried out with three independent variables, namely the concentration of pineapple tuber extract, solution pH and hydrolysis time with the response variable tested for optimization being total nitrogen which represents how much nitrogen is contained in the FPH.

Trash Fish Fluor (TFF) Preparations and Pineapple Hump Extracts (PHE)

Blanak fish (Moolgarda seheli) and Slanget fish (Anandontostoma chacunda), obtained from fish collectors in Cirebon, are used in the preparation of fish flour. The process of making the flour consists of washing, boiling, drying, and grinding the fish. The fish flour nutrient content is then analyzed by conducting the proximate tests carried out in proximate analysis as described by including moisture content, ash value, crude lipid levels, protein content with the Kjeldahl method, and total carbohydrates (Kurcz et al. 2018)(Kurcz et al. 2018). Protein levels are calculated by multiplying the total nitrogen value with a conversion factor (AOAC 2005).

The pineapple extract was taken from a local Indonesian pineapple known as HONI pineapple. The extraction process is started by crushing the pineapple hump with a blender with the addition of phosphate buffer 7,5. The pineapple slurry is then filtered using a muslin cloth. The filtrate was carried out further for purification by centrifugation at a speed of 6000 rpm at 4°C for 15 minutes. The supernatant is filtered with Whatman filter paper No. 1, and PHE is stored in the freezer at -30 °C. All the extraction process was maintained at a cold temperature. Proteolytic activity in PHE was measured by Sigma's universal protease assay with casein as a substrate (Sigma Aldrich 1999).

Microorganism

The isolates used in this research is *A. chrysogenum* fungus taken from the microbiology laboratory collection of the Biotechnology Research BPPT with strain code name Biomcc 00141 grown in slanted agar media containing maltose 70 g/L, malt extract 11,5 g/L, peptone 12,5 g/L, bacteriological agar 20 g/L and water and then stored in an incubator at 28 ° C for 14 days.

Table 1. Level Point of Each Variable at Central Composite Design

Variables	Code	Level Point				
		-alpha	-1	0	1	+ alpha
PHE Concentrations (%)	A	1,6364	3	5	7	8,3636
pH	B	4,3182	5	6	7	7,6818
Time (Hour)	C	0,6364	2	4	6	7,3636

The optimization of the hydrolysis process was done in Response Surface Methodology (RSM) with 3 independent variables that is PHE concentration, pH of the solution, and hydrolysis time. While for the dependent variable is the weight of flour: solution (1:10) and hydrolysis temperature is 55°C and total nitrogen as a response. Inactivation of proteases is carried out by increasing the process temperature to ± 85°C. The inactivated hydrolysate was then centrifuged and filtered with Whatman paper No. 1. The supernatant was analyzed for the total nitrogen content by the Kjeldahl method. The design experiments use the Central Composite Design (CCD). The range and levels of variables tested to optimize the hydrolysis process are present in Table 1. The optimization process with RSM is done using Design-Expert version 11.0 software. The TFH that has been carried out under optimum conditions is further analyzed for its nutrient content by conducting the proximate tests and analysis of amino acid content was done by High-Performance Liquid Chromatography (HPLC) instrument.

Fermentation Process

Inoculum preparations were done by suspending 14 days old *A. chrysogenum* was isolated with 6 mL physiological saline solution and distributed as much as 0,5 mL into 250 mL flasks containing 30 mL of sterile inoculum medium. The compositions in inoculum media were 34 g/L of local Corn Starch Liquor (CSL), 10 g/L of sago sugar, local sucrose 35 g/L, $CaCO_3$ 5 g/L, liquid paraffin 1,75 g/L, DL-Methionine 0,5 g/L, Ammonium acetate 5,5 g/L, 0,6 mL /30 mL local palm oil. The flasks were incubated on a shaker incubator at 220 rpm at 28 °C for 72 hours. The inoculum incubated on the starter medium was inoculated as much as 10% v/v into the fermentation medium which

had added as much as 5% v/v of Trash Fish Hydrolysate (TFH). The materials used in fermentation media were (g/L) local CSL 110 g/L, local sago sugar 50 g/L, urea 2,1 g/L, maltodextrin 50 g/L, $MgSO_4$ 9 g/L, $CaCO_3$ 15,5 g/L, corn starch 28 g/L, technical KH_2PO_4 3,3 g/L, technical DL-methionine 2 g/L, trace element stock 10 g/L, technical liquid paraffin 4 g/L, ammonium sulfate 5 g/L, palm oil 0,6 mL/30 mL and RO water. The inoculated medium was then transferred to a shaker incubator to be incubated with agitation at 220 rpm and a temperature of 25°C for 144 hours.

The CPC in the culture supernatants was determined using HPLC as described by Hassanein et al. (2009)(2009). The Mobile phase was acetate buffer pH 4,75 (980 mL): acetonitrile (20 mL) with a flow rate of 2 mL/min. Detection was carried out by a UV detector at 254 nm and CPC in samples.

RESULT AND DISCUSSION

Optimization of Hydrolysis Process using Response Surface Methodology

The Hydrolysis process was carried out enzymatically by utilizing proteolytic activity in PHE. The specific PHE activity obtained is 0,303 U/mg these results are quite similar to the crude extract bromelain from the previous study by Setiasih et al. (2018). The variable formulation used to determine the optimum hydrolysis conditions was carried out based on the CCD experiments that had been done. The results of the data design for CCD produce 20 running experiments consisting of point variations on each variable. These 20 runs are the experiments that must be carried out to obtain the optimum point for each variable. The interactions between variables were described in a 3-dimensional graph in Figure 1.

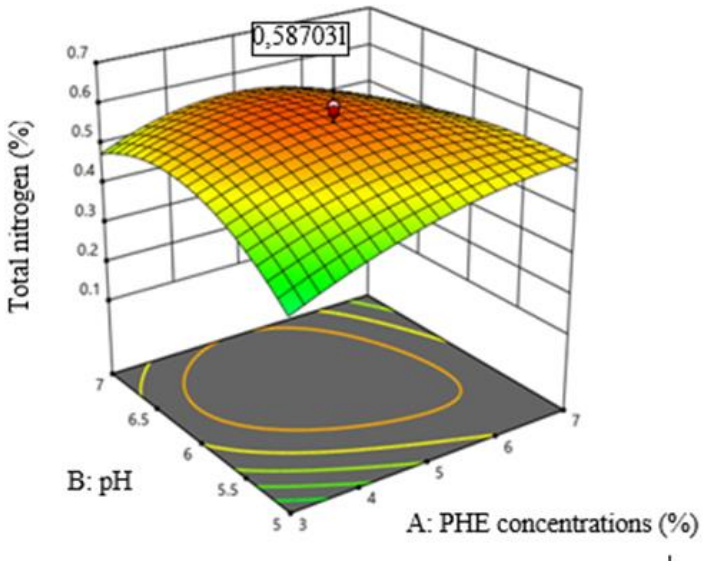

Figure 1. The flag in the red area shows the location of the highest response point on the 3-dimensional surface response graph

The green area is the area with a low response while red indicates where the best response results are. The predicted results in the optimum point shown in Figure 1 are 0,57803 % with the confidence of the predictions 95%. Figure 1, indicates that the PHE concentrations and pH levels have a significant effect on the percentage of total nitrogen produced. The edge of the surface sloping down when the level of both variables used lowered, means that these two factors influence each other in the formulations. After doing the model validation and response estimation, the next step is to confirm the analysis results. Using the confirmations option in the Design Expert 11.0 program it will mathematically determine the optimal value of the variables of PHE concentrations, pH, and hydrolysis and generate the results. From the confirmation results, it was determined that the optimum condition of the hydrolysis process of fish meal to get the response optimum of total nitrogen was at the

pineapple hump extract concentration of 5,71%, pH 6, and hydrolysis time for 5 hours 16 minutes. The optimum level points obtained in this experiment are still in the same range as those reported in previous studies using the enzyme bromelain in pineapple for the hydrolysis process (Aharonowitz and Demain 1978 and Koesoemawardani et al. 2011).

Analysis of Trash Fish Hydrolysate

Proximate test analysis on trash fish hydrolysate to determine the nutrient levels was conducted on TFF and after it became hydrolysate. The protein levels for fish are obtained by multiplying the total nitrogen value with a coefficient of 6,25 According to the results of other studies the use of fish flour originating from fresh fish or flat total proteins is always above 50% (Assadad et al. 2015). The test results on TFF and after it became hydrolysates can be seen in Table 1. All test variables in the table experienced changes in the levels of moisture content, ash value, crude lipid, protein, and total carbohydrates.

Table 1. The difference in proximate test results in TFF and when it became hydrolysate

Parameters	Fish flour	Hydrolysates
Moisture content (%)	9,29	**95,9**
Ash Value (%)	7,95	**0,34**
Crude Lipid (%)	5,97	**<0,02**
Proteins levels (%)	70,47	**3,73**
Total Carbohydrate (%)	6,32	**0,03**

The most significant change was shown in the water content, this is because the hydrolysate is in liquid form. However, another significant change was protein levels, which decreased. The protein content obtained in this study was not significantly different from the previous study conducted by Haditjaroko et al. (2015) protein of 4.61% with longer hydrolysis time. The difference in protein levels obtained might be due to the difference in bromelain enzymes used with a longer hydrolysis time that took 7 hours (Kahiro et al. 2018).

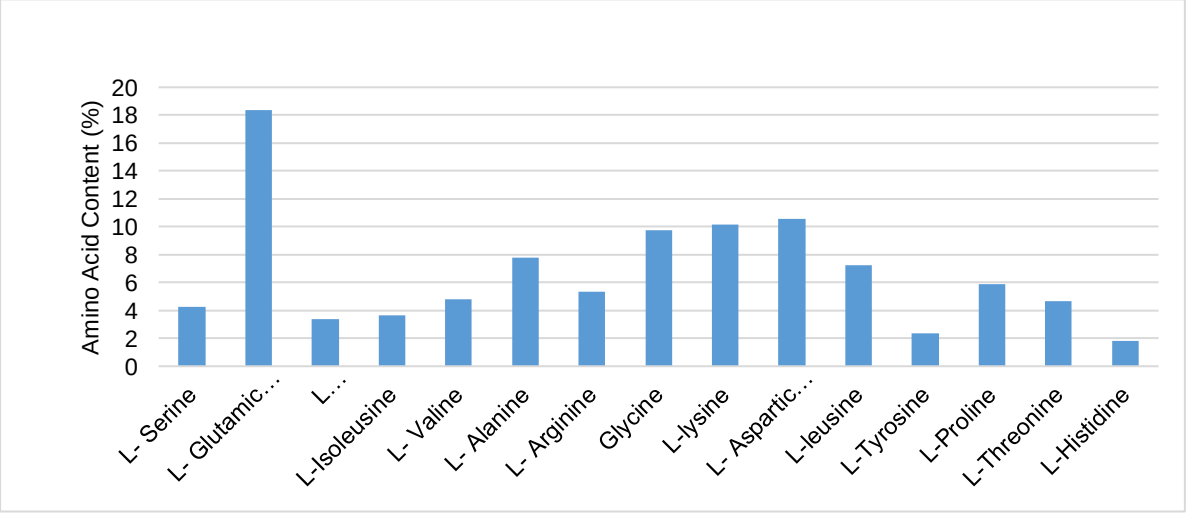

Figure 1. Amino Acids Content in TFH by HPLC Detection Instrument

Amino acid detection data shown in Figure 5 shows that glutamic acid is the amino acid that dominates the hydrolysate product followed by aspartic acid. The analysis of amino acid in the optimum TFH also showed the presence of valine with a concentration. Valine is one of the CPC precursors included in amino acids that have an essential role in the regulation of β-lactam antibiotics other than lysine, methionine,

and glutamate (Demain and Newkirk 1962; Liu et al. 2018).

The Cephalosporin C (CPC) Production from the Fermentation Process

The amount of CPC production by media with the addition of TFH yielded more results than the control media (Figure 6) with the presentation of an increase of 85,39%. This increase in CPC production is possible due to the presence of amino acids which are the precursor of the CPC-forming which, among other things, has previously been identified with valine contained in TFH (Abraham and Newton 1961; Ryu et al. 2021).

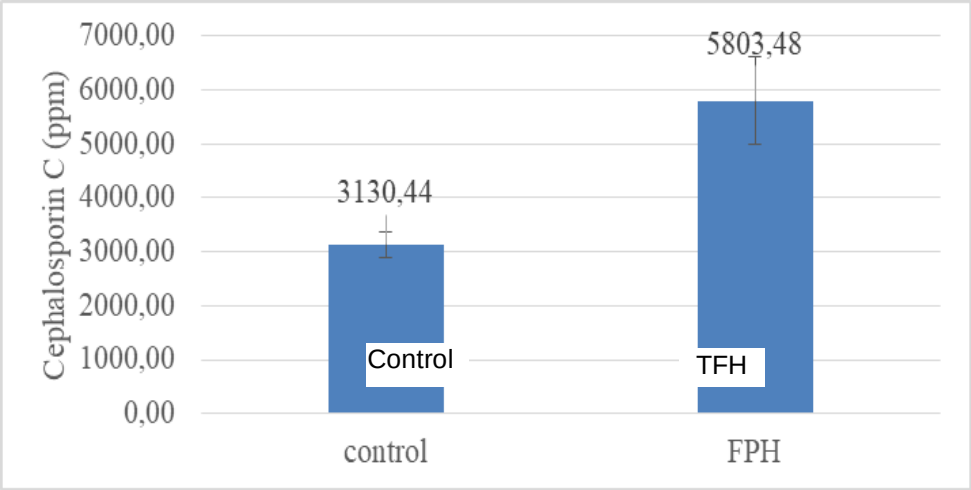

Figure 6. The CPC levels by the media with TFH and media without TFH (control) levels

CONCLUSION

This research concluded that despite the lower protein content and amino acid obtained in TFH, the addition of TFH as much as 5% v/v in the fermentation media of CPC has been shown to increase the productivity of *A. chrysogenum* in producing CPC up to 85,39% to the media without the addition of hydrolysate. While the FPH produced in this research has shown potential to increase the production of CPC, further improvement in the hydrolysis process, focusing on increasing the nitrogen content, is believed to be possible and could be a worthwhile area for future study.

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