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Multi-Response Optimization for the Production of Antifungal Compound and Decomposition of Municipal Solid Waste decomposition by *Streptomyces* Sp. GMR22

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Abstract: Organic waste from the traditional market can be converted into value-added products via microbial fermentation. This study aims to evaluate the use of the proximate composition of traditional market waste to optimize the decomposition process and targeted output of decomposition products (in this case, antifungal compound) based on multi-responses analysis. A proximate combination of heterogeneous solid organic waste is a new approach to process organic waste using solid-state fermentation. It allows us to combine varied organic waste materials based on their nutritional value without adding a synthetic chemical compound. The proximate composition of traditional waste was adjusted with the addition of crop residue. Then response surface methodology (RSM) was used to determine the effect of the proximate composition on the production of an antifungal compound by *Streptomyces* sp. GMR22. RSM analysis showed the proximate composition of 4.55 g ash, 5.50 g protein, 1.61 g fat, and 41.00 g carbohydrate, which significantly correlate to the highest production of crude extract (0.014 g extract/g substrate) having a fungal inhibition zone of 19.8 mm. Antifungal activity and the growth of *Streptomyces* sp. GMR22 were optimized together via desirability function. Intended for the fermentation process, a substrate with proximate composition of 6.6 g of ash, 3.1 g of protein, 1.5 g of fat, and 41 g of carbohydrates will produce a zone of activity of 1.6 mm and 9×10^8 cells/g with an overall desired value of 0.8628. At the end of the fermentation process, value approximation and high-resolution mass spectrometry (HRMS) analysis were conducted to confirm the decomposition process and antifungal compound production. Proximate compositions show decreased carbohydrate content while increasing protein and ash content, which indicates the decomposition process. HRMS data shows that 405 compounds were detected; 157 compounds following *m/z* cloud database, with two dominant compounds (furanone derivative and oleamide), indicated as an antifungal. Multi-response optimization using response surface methodology with desirability function can be used to determine the composition of waste and optimize multi-response (antifungal activity and *Streptomyces* sp. GMR22 growth) to meet the optimum value of each response.

Keywords: solid-state fermentation, response surface methodology, Streptomyces.

鏈黴菌生產抗真菌化合物和分解城市固體廢物的多響應優化。转基因产品 22

摘要：來自傳統市場的有機廢物可以通過微生物發酵轉化為增值產品。本研究旨在基於多響應分析評估使用傳統市場廢物的近似成分來優化分解過程和分解產物（在本例中為抗真菌化合物）的目標輸出。異質固體有機廢物的近似組合是一種使用固態發酵處理有機廢物的新方法。它使我們能夠根據其營養價值組合各種有機廢料，而無需添加合成化合物。傳統廢物的近似組成通過添加作物殘留物進行了調整。然後使用響應面方法來確定近似組合物對鏈

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黴菌產生抗真菌化合物的影響。转基因产品 22。響應面法分析顯示 4.55 g 灰分、5.50 g 蛋白質、1.61 g 脂肪和 41.00 g 碳水化合物的近似組成，這與具有 19.8 毫米真菌抑制區的最高粗提取物產量（0.014 g 提取物/g 底物）顯著相關.抗真菌活性和鏈黴菌的生長。转基因产品 22 通過合意性函數一起優化。用於發酵過程，具有近似組成 6.6 g 灰分、3.1 g 蛋白質、1.5 g 脂肪和 41 g 碳水化合物的底物將產生 1.6 毫米和 9×108 個細胞/g 的活性區，具有總體期望值為 0.8628。在發酵過程結束時，進行數值近似和高分辨率質譜分析以確認分解過程和抗真菌化合物的產生。近似組成顯示碳水化合物含量降低，而蛋白質和灰分含量增加，這表明分解過程。人力资源管理系统數據顯示檢測到 405 種化合物； m/z 雲數據庫中的 157 種化合物，具有兩種主要化合物（呋喃酮衍生物和油酰胺），表明為抗真菌劑。使用具有合意性函數的響應面方法的多響應優化可用於確定廢物的組成並優化多響應（抗真菌活性和鏈黴菌屬转基因产品 22 生長）以滿足每個響應的最佳值。

关键词：固态发酵, 響應面方法，鏈黴菌。

1. Introduction

Traditional markets in Indonesia are the second-largest stream of municipal solid waste after household waste. However, since source separation and waste processing are not commonly practiced, most of the waste generated from traditional markets is burned for waste disposal. Without proper treatment, traditional market waste has little economic value, thus reducing interest for the community or private sector in handling these types of waste [1]. Traditional market waste can be used to produce valuable bioactive compounds through biochemical conversion by SSF.

The type of microorganism used during SSF determines the fermentation product. Therefore, in addition to its ability to grow in a solid medium, the selection of microorganisms is also based on the ability of the microorganism to generate enzymes or secondary metabolites. SSF produces metabolites used as raw materials in feed and bio-based industries [2, 3]. Moreover, SSF has been applied to produce valuable bioactive compounds using various substrates [4, 5].

Actinomycetes, such as *Streptomyces,* can be used in SSF of traditional market waste due to their ability to produce antibiotic compounds that can be used as biocontrol agents. *Streptomyces blastmyceticus* strain 12-6 exhibited strong antifungal activity against *C. acutatum, C. coccodes, C. gloeosporioides, F. oxysporum,* and *T. roseum* [6]. *Streptomyces roseoflavus* NKZ-259 displayed high antagonistic activity against six fungal pathogens and reduced the incidence of the gray tomato mold by 66.67% [7]. Also, *Streptomyces* strain KX852460 has antifungal activity against *Rhizoctonia solani* AG-3 KX85246, the causal agent of target spot disease in tobacco leaf. With that being said, SSF may be applied to produce the antifungal compound for plant protection using traditional market waste as substrates [8, 9].

The main component of the traditional market waste is vegetable waste, which is suitable as a substrate for SSF by *Streptomyces*. However, the exact composition of traditional market waste was varied from time to time. For the optimal production of antifungal compounds, optimum substrate composition and fermentation conditions for *Streptomyces* and its secondary metabolite production are required. Statistical and design methods are required to calculate multiple variables with the minimum sample amount. Response surface methodology (RSM) analysis can be applied to determine the optimum model for substrate composition. RSM has been used to optimize cultivation media to produce secondary [10].

The desirability function (DF) approach generated from RSM is an efficient method to determine optimum performance levels for one or more responses simultaneously. The two RSMs were then optimized with multiple response prediction and optimization. This procedure utilizes the desirability function. The method of desirability function is one of the most popular for multi-response problems [11].

In this study, traditional market wastes were used as a substrate for SSF. The proximate composition was adjusted with the addition of crop residue to enhance the antifungal compound production by *Streptomyces* sp. GMR22. The antifungal activity against *Fussarium oxysporum* was evaluated. RSM analysis was applied to determine the effect of the proximate composition on crude metabolite production and antifungal activity.

2. Methodology

2.1. Microorganisms

Streptomyces sp. GMR22 was obtained from the

Laboratory of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada. The fungal pathogen *Fusarium oxysporum* was obtained from the Laboratory of Plant Diseases, Faculty of Agriculture, Universitas Gadjah Mada.

2.2. Inoculum Preparation

Streptomyces sp. GMR22 bacteria were grown in submerged culture in 500 ml flasks containing 100 ml of tryptic soy broth medium. The flasks were inoculated with 1 ml of isolates culture and incubated at room temperature for 8 days, shaking at 150 rpm.

2.3. Solid-State Fermentation

SSF was performed on a 500 ml Erlenmeyer with 42.5–54 g of waste. Nutrient optimization and fermentation conditions on antibiotic production were studied by adding crop residue, which has a proximate stable test result in traditional market waste according to the proximate composition. Before inoculation and incubation were carried out, the waste was sterilized in the autoclave with a temperature of 121°C for 15 minutes. Each Erlenmeyer was then inoculated with 10 ml of spore suspension and incubated at 37°C for 8 days.

2.4. Extraction of Crude Metabolites

Crude metabolites were extracted at eight days of fermentation through immersion using methanol. The extract was evaporated to get the crude residue. Antifungal activity was determined by the diffusion method. The crude residue was dissolved in 15 µL of dimethylsulfoxide (DMSO) and applied on a paper disc [12]. The paper disc was then placed on the surface of the potato dextrose agar media, which has been overgrown with the *Fussarium oxysporum*. The plate was incubated at 30°C for 18–24 hours.

2.5. Box-Behnken Design and Response Surface Analysis

RSM was analyzed using Minitab software. The design of the experiment used was Box Behken Design with four factors on three levels. The factor variables are X1: ash content (2.5–8 g), X2: protein content (1.5–5.5 g), X3: fat content (0.5–2.5 g), and X4: carbohydrate content (35–41 g) to determine the response Y: inhibition zone. After the response was determined for each experiment, each response was placed into a second-order polynomial model expressed by the formula:

$$Y = B_0 + \sum B_i x_i + \sum B_{ii} x_i x_j + \sum B_{ij} x_i x_j \tag{1}$$

where Y is the predicted response (inhibition zone), B_0 is a constant coefficient, B_i is the primary coefficient, B_{ii} is the quadratic coefficient, and x_i is the independent variable.

2.6. Multi-Response Using Desirability Function

Multi-response surface optimization using the desirability function was analyzed using Minitab software. The individual response was taken from previous research [13]. The better scheme was used to maximize the target, and the objective is max $y_i(x)$:

$$d_i\left(y_i\,(x)\right) = \begin{cases} 0 & y_i(x) \le y_i^{min} \\ \frac{y_i(x) - y_i^{min}}{y_i^{max} - y_i^{min}} & y_i^{min} \le y_i(x) \le y_i^{max} \\ 1 & y_i(x) \ge y_i^{max} \end{cases} \tag{2}$$

where $y_i(x)$ is the desirability value and y_i^{min} and y_i^{max} are the experimental values, the lowest and highest values, respectively. Individual desirability values are combined into the overall desire function (D) by calculating the geometric mean:

$$D = \left(\prod_{i=1}^{n} d_i^{w_i}\right)^{1/\sum w_i} \tag{3}$$

where w_i is the relative weight of the response, which reflects differences in significance response.

2.7. High-Resolution Mass Spectrometry (HRMS) Analysis

The organic extracts of *Streptomyces* sp. GMR22 were analyzed by Thermo Scientific TM Dionex TM Ultimate 3000 RSLC nano Ultra-High-Performance Liquid Chromatography (UHPLC) coupled with the Thermo Scientific TM Q Exactive TM High-Resolution Mass Spectrometer. A Hypersil Gold aQ analytical column (50 mm x 1 mm x 1.9 µ m) with a flow rate of 10 µ L/min was used. Solvent A consisted of water and 0.1% formic acid. In comparison, solvent B consisted of acetonitrile and 0.1% formic acid with the total run time of 30 min for full MS at 70,000 Full Width at Half Maximum (FWHM) Resolution Data Dependent MS2 at 17,500 FWHM Heated Electrospray Ionization (H-ESI) [14]. Positive compounds were identified using Thermo Scientific™ Compound Discoverer Software with *m/z* cloud database [15].

3. Results and Discussion

The optimization of *Streptomyces* sp. GMR22 growth on traditional market waste had been studied in the previous research [23]. In this research, we studied the effect of substrates on the production of metabolites that have antifungal activity (hereafter "antifungal compound") and its optimization. The inhibition zone as a response was then studied, and the result generated a second-order polynomial equation which contains a predicted response (inhibition zone) Y, independent variables (ash, protein, fat, and carbohydrates) X1, X2, X3, and X4, and the quadratic and linear interaction X12, X13, X14, X23, X24.

The interaction between proximate composition components is demonstrated by the binomial coefficient of X12, X22, X32, and X42 which overall shows a high significance of the response (Table 1). Linearly, only X1 (ash) has a significant effect on the production of the antifungal compound. At the same time, the interaction relationship was shown by the significant coefficients on X1X2, X1X4, and X3X4 components with a P-value of 0.05 and an F value of 4. A conclusion can be drawn from these data that ash either linearly or quadratically affects antibiotic production significantly. In contrast, the components of protein, fat, and carbohydrates do not have a linear effect. At the same time, the two-way interactions that affect antibiotic production occurred between ash and protein, ash and carbohydrates, and fat and carbohydrates.

Table 1 Analysis of variance (ANOVA) of the regression equation from Box-Behnken design

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	16	949.589	59.349	52.10	0.000
Blocks	2	5.807	2.903	2.55	0.127
Linear	4	231.406	57.851	50.79	0.000
X1	1	221.442	221.442	194.40	0.000
X2	1	0.755	0.755	0.66	0.435
X3	1	0.021	0.021	0.02	0.894
X4	1	0.324	0.324	0.28	0.606
Square	4	600.343	150.086	131.76	0.000
X1 * X1	1	481.333	481.333	422.56	0.000
X2 * X2	1	8.841	8.841	7.76	0.019
X3 * X3	1	96.901	96.901	85.07	0.000
X4 * X4	1	1.333	1.333	1.17	0.305
2-Way Interaction	6	142.275	23.713	20.82	0.000
X1 * X2	1	46.923	46.923	41.19	0.000
X1 * X3	1	0.002	0.002	0.00	0.964
X1 * X4	1	90.250	90.250	79.23	0.000
X2 * X3	1	0.010	0.010	0.01	0.927
X2 * X4	1	0.250	0.250	0.22	0.649
X3 * X4	1	4.840	4.840	4.25	0.066
Error	10	11.391	1.139		
Total	26	960.980			

Notes: P > 0.05 - insignificant difference; P < 0.05 - significant difference; X1 – ash; X2 – protein; X3 – fat; X4 - carbohydrates

In addition, the predictive rate generated from RSM antifungal model was also high, demonstrated by the R-value of 0.9821 (Fig. 1), indicating that the model could not explain 1.8% of the variations. This RSM model also shows that each proximate composition factor can be optimized with the highest desirability value of 1. This value was achieved from a combination of 4.55 g ash, 5.50 g protein, 1.61 g fat, and 41.00 g carbohydrates with the optimum inhibition zone value of 21.5184 mm, but the observed value was only 19.80 mm. The waste composition in the traditional markets changes every day. Meanwhile, the composition of the regulator has its proximate composition, so it is difficult to obtain the exact proximate composition as the optimal prediction so that the composition can be optimized according to the closest value.

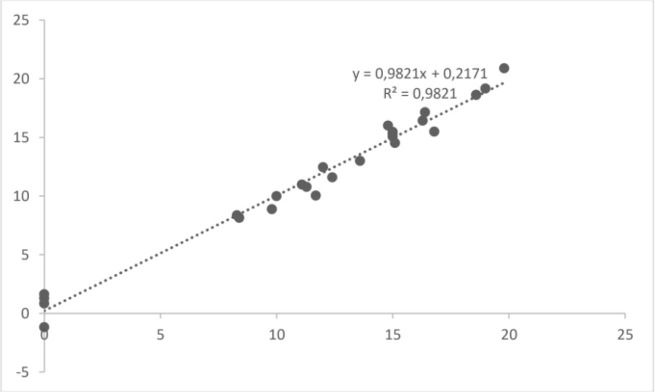


Fig. 1 Response surface methodology observed (x-axis) vs. predicted (y-axis) antifungal activity

In the previous study, carbohydrate and protein contents were the main factors in the growth of *Streptomyces* sp. GMR22 [13]. On the other hand, ash has a low significance, and fat has no significant effect. In contrast, interactions between factors that affect the antifungal compound production have ash as the most significant factor. The difference in the factors that affect the RSM growth of *Streptomyces* sp. GMR22 and the production of antifungal compounds is shown in the prediction patterns (Fig. 2).

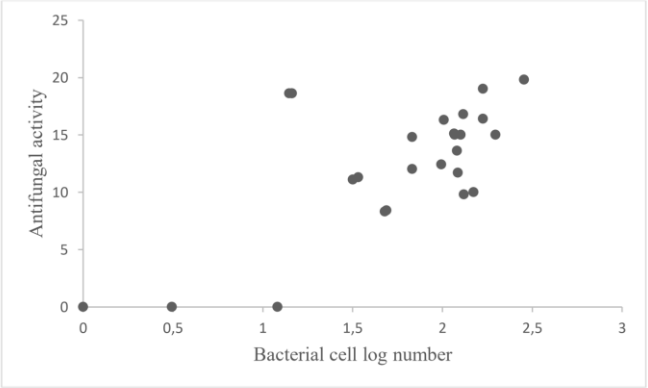


Fig. 2 Correlation between *Streptomyces* sp. GMR22 growth and antifungal activity under the experimental conditions generated by using response surface methodology

The production of antifungal compounds in *Streptomyces* is affected by many factors. Some factors have the same effect on bacterial growth, but others do not affect or even have the opposite effect. Based on its structure, carbohydrates and proteins are important factors in the production of antifungal compounds; these factors positively influence the growth of *Streptomyces*. In several studies, factors that affect the production of antifungal compounds contained in ash (P, K, Ca, S, Cu, Fe, Mn, Zn, Ni, Cr, Pb, and As) do not affect or contradict their effects on the growth of *Streptomyces* [16].

Metal is one of the most crucial factors in the production of antifungal compounds in several *Streptomyces* isolates. Washburn et al. [17] tested various *Streptomyces* and found that Mn, Zn, and Fe have a huge effect on antimicrobial compound production; several isolates produce antimicrobial compounds only in the presence of trace metals.

Copper also shows similar effects. Many genes relating to copper homeostasis are also required for the development of antimicrobial compounds [18].

The difference between antimicrobial compound production and bacterial growth also needs to be considered in fulfilling the proximate composition in traditional market waste treatment. Bacterial growth is indirectly aligned with the decomposition rate of organic solid waste. At the same time, antifungal compounds are the final product that can increase the value of processed traditional market waste. RSM can be used to determine a new mathematical model that can predict the peak point between bacterial growth and antifungal compounds production.

The desire function was added to the RSM to simultaneously maximize the value of microbial growth and antifungal activity. Limits (y_i^{min} and y_i^{max}) were set for each parameter used to optimize two responses (Fig. 3). Under optimal waste content with the range of proximate composition parameters (ash content [2.5–8 g], protein content [1.5–5.5 g], fat content [0.5–2.5 g], and carbohydrate content [35–41 g]), the composite desirability is very high until close to 1.

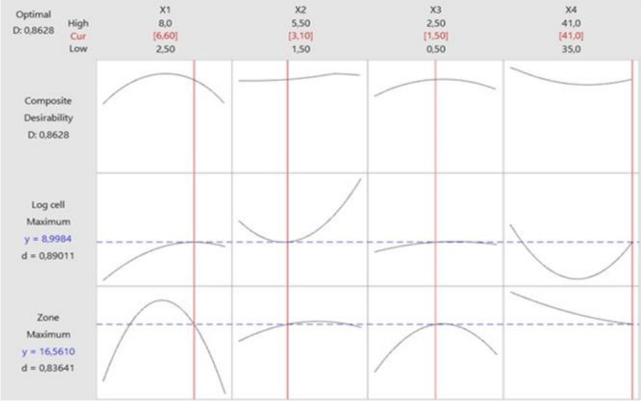


Fig. 3 Multiple response desirability functions of *Streptomyces* sp. GMR22 growth and inhibition zone generated with Minitab software. D represents the composite desirability value, d is the individual desirability value, X1 is ash, X2 is protein, X3 is fat, and X4 is carbohydrates. The black curved line is the value between low and high, and the straight red line is the optimal value

Desirability values that get closer to 1 indicate that the function has increased towards the desired target value and that the process was successfully optimized. Although the waste used has a minimal level that causes limited variation in optimization, the resulting desirability is quite high at 0.8%. This value indicates desirability was achieved. The results also bring up the required composition of waste and optimized with restrictions according to the value of waste and the predicted results obtained. The results were then compared with testing the actual results from the final sample of extracted waste treatment. The results obtained are not much different from the prediction of optimization results with an error level that can be tolerated.

After the fermentation process, proximate analysis was carried out to evaluate the decomposition process. The final concentration was 34.91% ash, 22.86% protein, 0.57% fat, and 41.66% carbohydrates, which demonstrated significant change compared to the proximate composition before the SSF process (Fig. 4). Carbohydrates show a significant decrease while protein and ash content highly increase. These changes indicate the fermentation process, and an increase in the amount of protein is possible because of the increase in the amount of microorganism biomass during the fermentation process [19]. The end of the fermentation process is possible due to a decrease in carbohydrates and increasing ash concentration.

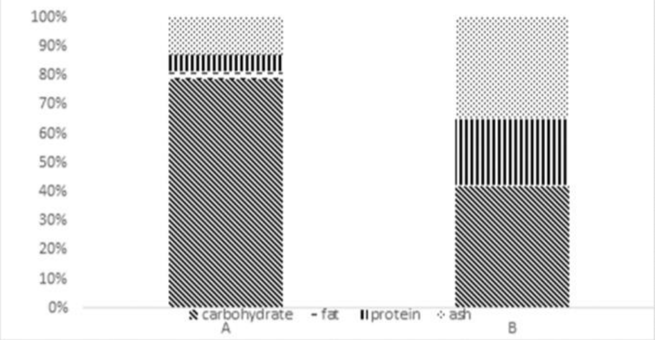
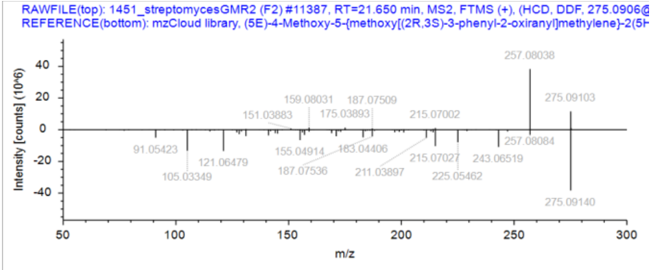


Fig. 4 Proximate composition of the waste mixture before (A) and after (B) the SSF process; each marked bar is a percentage of each proximate composition value

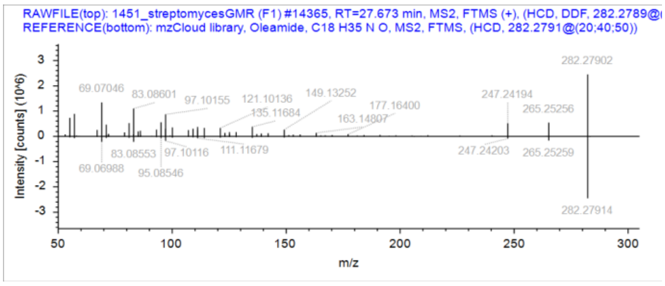
High-resolution mass spectrometry (HRMS) data shows that there are 404 metabolites detected from the methanol extract, whereas only 157 compounds match to *m/z* cloud database. Thirty-one compounds have more than 90% similarity but have different exact masses, and 217 compounds have less than 90% similarity. An HRMS database not found in the *m/z* cloud can be searched with the Natural Products Database with the same mass.

DMSO solvent was not detected in the extract, while the bioactive compounds with the highest or dominant peak were antifungal, antibacterial, and anti-tumor compounds. (5E)-4-methoxy-5-{methoxy[(2R,3S)-3-phenyl-2-oxiranyl] methylene}-2(5H)-furanone and oleamide are indicated to have an antifungal and antibacterial role.

Fig. 5 demonstrates *m/z* spectra of dominant active antifungal compound result from untargeted LC-HRMS where (A) shows (5E)-4-Methoxy-5-{methoxy[(2R,3S)-3-phenyl-2-oxiranyl]methylene}-2(5H)-furanone with the exact mass and (B) – oleamide with the exact mass.



A



B

Fig. 5 M/z spectra of dominant active antifungal compound result from untargeted LC-HRMS. (A) (5E)-4-Methoxy-5-{methoxy[(2R,3S)-3-phenyl-2-oxiranyl]methylene}-2(5H)-furanone with the exact mass of 275.09103 and (B) Oleamide with the exact mass of 282.27902

There are several hypotheses regarding the antifungal mechanism of furanone derivatives: one by facilitating the penetration of small compounds into cells by modifying the structure of the cell's wall, and the other is relating to the ability of furanone derivatives to interact directly with protein both in vitro and in vivo [20, 21]. Research conducted by Sharafudinov also shows that furanone derivatives can rapidly penetrate fungal biofilms [22]. Oleamide is a bioactive fatty acid ester with several beneficial effects, including antifungal and antibacterial activity [23].

The proximate composition that confirms the decomposition process and HRMS analysis, which confirm antifungal production, shows that multi-response optimization optimized waste decomposition, which is the target of waste treatment and production of the antifungal compound as a valuable product. Multi-response optimization using RSM with desirability function also resulted in balancing the process between decomposition and production of antifungal compounds. In the future, it can be applied to a variety of applications in waste treatment and the formation of a bioreactor with a variety of designs input that requires continuous optimization and prediction of waste treatment results to run a sustainable process.

4. Conclusion

This research aims to overcome the problem of traditional market waste, which is generally processed through composting. The heterogeneous and varied content of traditional market waste at different times or place makes it difficult to process them through SSF with certain target products. A proximate combination of heterogeneous solid organic waste is a new approach to overcome the undirected output of the fermentation process. This approach allows us to combine waste based on the nutritional content of each mixed waste and perform optimization with multi-response tools.

5. Limitations and Further Study

This research is limited to the use of traditional market waste in the province of Yogyakarta, Indonesia. Meanwhile, *Streptomyces* sp. GMR22 was used because of its adaptability and ability to produce antibiotic compounds that can inhibit the growth of *Fussarium oxysporum.* In addition, this research also did not calculate the economic aspects and the purity of the bioactive compounds produced.

Streptomyces sp. GMR22 can produce antifungal compounds effectively against *Fussarium oxysporum* under solid-state fermentation with traditional market waste as a substrate. Response surface methodology can optimize the composition of waste and determine strategies for effective waste management in terms of decomposition and end products for traditional market waste treatment. In the future, waste treatment in this research can be used for a wider range of organic waste and various kinds of microorganisms with various bioactive compounds.

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