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Response Surface Methodology based Optimization of Microbial Amylase Production using Banana Peels as Carbon Source

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Abstract

Amylase is an enzyme that catalyzes the hydrolysis of polysaccharides such as starch into small units include disaccharides and monosaccharides such as glucose. It is found diversely in different sources, including animals, plants, vegetables, fruits as well as microbes. Amylases of microbial origin are favorable due to many advantages. Besides, microbial enzymes production is more economical compared to other sources. Optimization of enzyme production is quite challenging, especially when it is conducted conventionally due to the many parameters involved. Hence, applying Response Surface Methodology facilitates the design of the experiment and optimizes the production effectively. In this study, three independent variables, namely (A) Temperature, (B) pH, and (C) Banana peels concentration, were selected for the optimization of the amylase production. The result of the study indicated that the run-6 has the highest activity of amylase at 4.10 U/mL, with the optimum temperature at 60°C, pH 6 and 25% (w/v) of banana peels concentration. Further optimization of the amylase production, including recombinant gene expression, different expression hosts, and purification of the crude amylase, are highly recommended.

Keywords: *Amylase, Response Surface Methodology, Banana Peels.*

INTRODUCTION

Amylases were one of the first enzymes to be produced commercially by microorganisms. The use of microorganisms in amylase production is economical as microbes are easy to be manipulated to obtain enzymes of desired characteristics (Nagarajan, Deborah Paripuranam & Umamaheshwari, 2010). Genus Bacillus is known as a good producer of amylase for various applications. Bacillus is widely used for the production of amylase, and these bacteria need a rich source of nutritional medium to grow. Different fruit and vegetable peels are usually considered as waste providing a rich source of starch and nutrients for bacteria (Paul & Sumathy, 2013).

Amylases are amylolytic enzymes that have the ability to degrade starch or glycogen into valuable products, thus representing an important biocatalyst in carbohydrate metabolism (Shobhana et al., 2013). Amylases are identified by the differing in the glycoside bond they attack and are classified into three major classes; α, β, and γ amylase. However, only α-amylase is the most useful

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amylase type in industrial applications, and it is also one of the major classes of amylase that have been identified in microorganisms other than glucoamylase (Paul & Sumathy, 2013). Amylases are used in industry due to advantages such as cost-effectiveness, consistency, less time and space required for production, and ease of process modification and optimization. An increase in the numbers of competitive industries and the application of technology in amylase production leads to improved properties of the product, such as raw starch degrading amylases (Paul & Sumathy, 2013). During the saccharification or liquefaction of starch, amylase is used for wrap sizing of textile fibers, clarification of haze formed in beer or fruits juices, and for pre-treatment of animal feed to improve the digestibility (Shaishta Kokab et al., 2003). However, the cost of production is expensive, and a search for the most cost-effective fermentation strategy does not guarantee obtaining expected results.

Most products produced are being made using enzymes at over 500 industries (Johannes & Zhao, 2006; Kumar & Singh, 2013). Amylase is commonly used in industry due to its increased demand in present-day biotechnology and accounts for about 30% of the world's enzyme production (Swetha Sivaramakrishnan et al., 2006). Besides, Adrio and Demain (2014) reported that amylase has a wide range of applications, including paper industries, in the production of textiles and detergents, in chemical and food and beverages, biofuels, animal feed, sugar syrup, bakery, and pharmaceutical. In industrial sectors, amylase production usually meets process demands, improves the process of fermentation, and has successfully replaced the chemical hydrolysis of starch in starch-processing industries (Vengadaramana, 2013).

Banana wastes are highly rich in carbohydrates and other basic nutrients that support microbial growth (Schiebar & Saldana, 2009). Hence, many suggested that banana peels could be utilized as a promising carbon source for the production of amylase (Paul & Sumathy, 2013). Amylases are one of the main well-known enzymes used in industry. It is an amylolytic enzyme that degrades starch or glycogen. Thus, amylase represents a group of catalytic proteins providing great importance in carbohydrate metabolism (Shobhana, Pooja, Komal & Sayali, 2013). Industrially, amylases are important in many applications, including foods, brewing, baking, textile, analytical chemistry, detergent, and pharmaceutical industries (Krishna et al., 2012). The demand for amylase production increases due to its advantageous characteristics thus has attracted various industries in developing enzymes with better properties.

As a waste, banana peel is commonly disposed of once being used or consumed. Approximately more than 10kg of banana is used in each local stall per day (personal communication), leaving massive of wastes being disposed of due to its non-functional value. In the process of these products, the banana peel accumulates in bulk leading to a serious problem to the environment (Krishna, Srivastava, Ramaswamy, Suprasanna & D'Souza, 2012). Hence, emphasizing the use of this waste as a cheap carbon source plays a significant potential in the application of agro-industrial wastes. Several efforts have been accomplished to reduce the negative impacts brought upon these wastes, at the same time utilizing them for the production of enzymes.

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The high production cost of enzymes has been one of the major barriers to their commercial production in many countries; alternatively, utilizing cheap carbon sources may reduce the cost of the production. In addition, banana peels are considered as one of the massive wastes being disposed of daily due to their non-functional value. Thus, banana fruits that are widely available in Malaysia can be used as a cheap carbon source due to the fact that banana peels are rich in starch composition, which makes them suitable as a medium for bacterial growth.

The RSM is a collection of mathematical and statistical techniques designed to analyze the effects of different independent variables in which a response of interest is influenced. Thus, the response can be optimized by simplifying the multi-parameters of variables simultaneously (Lee, Chung & Hung, 2004). The RSM emphasis designing, formulating, modeling, developing, and analyzing scientific data and products (Bradley, 2007). The optimization of enzyme production can be done by using the Response Surface Methodology (RSM) in order to seek the optimum conditions for a multivariable system (Shakti may Kar, Tapan Kumar Datta & Ramesh Chandra Ray, 2010). The RSM provides a mathematical model and statistical techniques designed to analyze the experimental data on the different effects of independent variables, as well as to provide an interaction prediction between the response and the variables. (Granato & Calado, 2014).

RESEARCH METHOD

Qualitative Enzyme Determination
The thermophilic bacteria culture sample was inoculated and streaked onto the plates containing Nutrient Agar (NA) added with 2% of starch. These plates were then placed in a ziplock bag of 60°C incubators for 24 h. On the next day, an iodine solution was flooded onto the bacterial plates and left for two h at room temperature.

Determination of Quantitative Enzyme
The quantitative enzyme assay was conducted according to the method described by Bernfeld (1955). An amount of 0.2 mL crude enzyme was added to a reaction mixture comprising 0.8 mL of 2 percent (v/v) soluble starch as a substrate (pH 7). The mixture was incubated in a waterbath at 60°C. After that, 2mL of DNS reagent was added to each test tube holding the combination, which was then boiled for 5 minutes in a boiling waterbath to stop the reaction before being allowed to cool at room temperature, then measured the absorbance at 540 nm.

Optimization of pH of the Enzyme
The optimal pH for thermophilic bacterial growth was identified by assaying amylase at various pH ranges from pH 4 to pH7. The test was conducted by aseptically inoculating a thermophilic bacterial sample into 100mL of sterile NB in a 250mL conical flask. Each of the conical flasks was adjusted to the appropriate pH and incubated at 60°C for 24 hours. After 24 h of incubation, the crude enzyme was harvested by centrifugation at 10,000 rpm for 10 min. The crude enzyme was then used to perform a quantitative enzyme assay.

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Effect of Banana Peels and Starch as Carbon Source in the Production Media

In order to determine the ability of banana peels waste served as a natural carbon source to effective produce enzyme amylase in comparison to commercial carbon source, in this case 2% (w/v) of starch was used which served as a control experiment. 20g of banana peels and 2% of starch were added separately in conical flask containing NB, adjusted to pH 6. After inoculation of thermophilic bacteria, the flasks were incubated at 60°C for 24 h. After 24 h, the contents were collected and proceed with the quantitative enzyme assay.

Effect of Different Banana Peels Concentrations on Enzyme Production

To determine the optimal concentration of banana peel waste for bacterial growth, different peel concentrations ranging from 5-35 percent (w/v) were examined. Banana peels were weighed per 100mL of NB in grams (5-35 g). Each conical flask containing the mixture was adjusted to pH 6 (the optimal pH determined in step 3), labeled, and autoclaved for 15 minutes at 121°C. Each conical flask containing varying banana peel concentrations received 500uL of inoculum. The bacterial culture was kept at 60°C for 24 hours. The samples were collected the next day for the quantitative enzyme test.

Optimization of Amylase Production Using RSM

In this study, for the optimization of amylase enzyme production, three independent variables were selected, namely, (A) Temperature, (B) pH, and (C) Banana peel concentration, which was determined and designed using a Response Surface Methodology (RSM) software; Design Expert 10. Each independent variable was designed using Central Composite Design (CCD) at differently designed levels, namely; Low level (-1) and High level (+1), with a total set of 2 blocks and 20-set run experiments adjusted to Face Centered Central Composite Design (FCCCD) with the axial (star) points set at 1.0 coded units from CCD choice. Once the selected range of factors was chosen and entered as low/high level of variables, the effects of different independent variables were analyzed, thus predicting the interest optimum variable responses (Lokeswari, 2010).

The optimal point in optimization of the amylase enzyme was predicted using a second-order model (quadratic model) equation which can be expressed in equation (eq.3). y is a dependent variable or also known as the optimum value of amylase enzyme (IU/mL/min). $\beta 0$ is an intercept, $\beta 1$, $\beta 2$, and $\beta 3$ are linear, quadratic, and interaction coefficients, while xABC are factors (independent variables) and lastly ε is the error. Statistical significance can be determined from the evaluation of ANOVA, including the variance and coefficient of determination value (R2).

FINDINGS AND DISCUSSION

Qualitative Enzyme Determination

The qualitative enzyme determination was successfully conducted. The formation of a clear zone around the bacterial colonies indicated the positive amylase production by the isolate, as indicated

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in Figure 1. The formation of the clearing zone has been explained due to the ability of the amylase enzyme to hydrolyze the substrate (2% w/w starch) into smaller molecules such as maltose or glucose. The appearance of blue color on the NA palate, on the other hand, was due to the reaction of the iodine solution when it was flooded on the surface of the NA plate, with the starch present in the NA. Therefore, the result of the experiment has confirmed the production of the thermostable amylase by the isolated bacteria.

Figure 1. The qualitative determination of thermostable amylase on NA plate supplemented with 2% of commercial starch.

pH Optimization of the Enzyme

The optimum pH for the production of thermostable amylase by the isolates was found at pH 6.00. Figure 2 shows that at this pH, the maximum amylase enzyme activity (1.4 U/ml) was recorded. Further raising or decreasing the pH resulted in reduced enzyme activity.

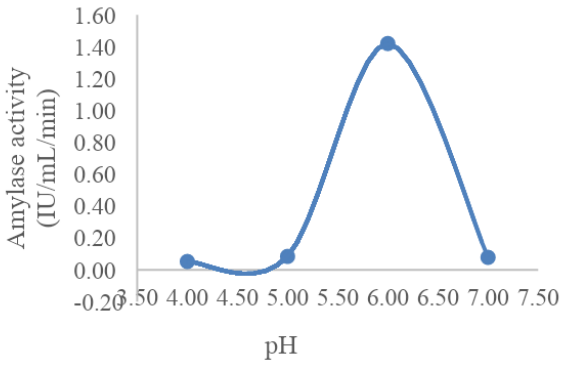

Figure 2. Optimum pH for thermostable amylase production by the isolate 3.

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Effect of Carbon Source from Banana Peels and Commercial in the Production Media
According to Happi et al. (2011) an average of 7.0-15.0 % of starch was reported in the banana peels, while Bezerra, Cruz Rodrigues, Amante, and Silva (2013) reported an average of 10.0 % of starch in the banana peels waste. In the present study, it is estimated about 100g of banana peels is equivalent to ≈10g of starch. Hence, 2g of the identical number of starches in 2% (w/v) of commercial starch and 20g banana peels were used in a comparison between commercial starch and banana peels starch (Table 1).

Table 1. Comparison between commercial starch and banana peels starch

Carbon source	Amount used in the experiment (g)	Starch content (g)	Amylase activity (IU/mL/min)
Commercial starch	2.00	2.00	1.49
Banana peel waste	20.00	2.00	2.02

The enzyme activity produced by the isolate cultivated in commercial starch-containing media and the isolate cultured in banana peel waste-containing media were successfully compared. Table 1 shows that 2g of commercial starch produced 1.49 U/mL of amylase activity, while 20g of banana peels produced 2.02 U/mL. Hence, the enzyme activity in media containing banana peels was found higher than that in media containing commercial starch. Thus, the result indicated banana peels had been preferable as a carbon source. Shobhana et al. (2013) also endorsed the use of banana peels as a less expensive carbon source for amylase production. Lokeswari (2010) reported that the media which contain banana agro-residual waste (banana peels) resulted in maximum productivity. Thus, banana peels produced a higher amylase production than any other agricultural waste such as citrus fruits, potatoes, wheat bran, etc. Chandrashekhar Unakal et al. (2012) also reported that production of enzyme by utilization of banana waste employed by fermenting organism gave a considerable interest can be given in using banana waste as an alternative source of amylase production, which is economical and profitable due to the inherent nature of the banana waste itself.

Effect of Different Banana Peels Concentrations on Enzyme Production
The content of banana peels was indicated as a percentage calculated in gram per 100mL volume of Nutrient Broth. As an alternative for commercial starch, the thermostable amylase-producing bacteria were cultivated on a culture including banana peel wastes. Banana peels were present in concentrations ranging from 5-35 percent (w/v). The result of the study as indicated (Figure 3) 25 % banana peels was found to produce the maximum activity of the amylase (2.49 U/mL).

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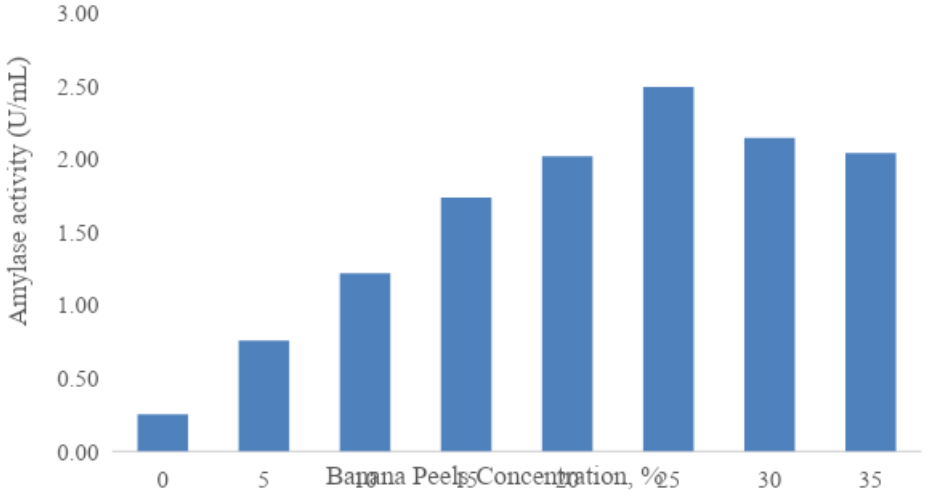

Figure 3: Effect of banana peels as alternative carbon source on the amylase production.

Considering 25g of 25% banana peels concentration produced the highest amylase activity, hence 25% of banana peels were the optimum concentration for the amylase enzyme production. A further increased in the concentration did not increase the amylase activity (Shobhana et al., 2013), in this case 25% as optimum concentration while increasing the banana peels concentration did not affect the growth of organism (Paul & Sumathy, 2013). Given that 25g of 25% banana peels produced the maximum amylase activity, therefore 25% banana peels was the optimal concentration for amylase enzyme synthesis. Further increased in concentration did not boost amylase activity according to Shobhana et al. (2013). From the experiment, with 25% increased of the banana peel concentration did not give any effect on the bacterial growth (Paul & Sumathy, 2013). The amylase activity in the negative control (growth media without banana peel wastes) showed the lowest enzyme activity (0.252 U/mL). The ideal concentration of banana peels, on the other hand, is dependent on the type of banana utilised, as well as the production conditions (temperature, pH, etc.). In the meantime, the amount of starch in a banana depends on how it is ripened (texture, colour, etc.). Green bananas are believed to have more starch than yellow bananas (Shobhana et al., 2013; Paul & Sumathy, 2013). The inoculum size was also found to be equally important factor for the production of amylase (Krishna et al., 2012).

5. Optimization of Process Parameters Using RSM

Once the predicted optimal variables were obtained from optimization using RSM design, the experiments were conducted according to the interest variables, hence quantitatively assayed at 540nm to determine the amylase activity or in this case being called response (Table 2). The results produced were in a form of evaluation, analysis of variation (ANOVA) to determine the

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regression model equation from this relationship, model graphs in variety of representations and post analysis. Using the RSM design called a Central Composite Design (CCD), chosen from the predicted equation fitting for a quadratic surface which was suitable and worked well for process optimization. The layout was designed respectively and measured according to the values recommended and designed by the CCD. According to Table 2, run-6 has the highest activity of amylase at 4.10 U/mL, conducted experimentally at 60°C, pH 6 and 25% (w/v) of banana peels concentration. The lowest amylase activity recorded experimentally gave out at 1.87 U/mL as run-4 (15% substrate concentration; pH 5; 50°C). From the relationship of the designed process parameters and responses ("Experimental") obtained as amylase activity, further quadratic regression model was analysed in order to predict the "Predicted" responses tabulated in Table 2.

Table 2. Summary of response data using RSM design

Run	Block	Parameters			Response, Amylase activity (IU/mL/min)	
		Temp, °C (A)	pH (B)	Peels Conc., % (C)	Experimental	Predicted
1	Block 1	60.00	6.00	25	3.89	3.77
2	Block 1	70.00	7.00	35	3.40	3.30
3	Block 1	50.00	5.00	15	2.48	2.15
4	Block 1	50.00	7.00	15	1.87	1.78
5	Block 1	50.00	5.00	35	3.51	3.36
6	Block 1	60.00	6.00	25	4.10	3.77
7	Block 1	60.00	6.00	25	3.86	3.77
8	Block 1	70.00	5.00	15	2.01	1.90
9	Block 1	70.00	7.00	15	2.05	1.78
10	Block 1	70.00	5.00	35	3.51	3.17
11	Block 1	60.00	6.00	25	3.91	3.77
12	Block 1	50.00	7.00	35	3.57	3.25
13	Block 2	50.00	6.00	25	3.66	3.96
14	Block 2	70.00	6.00	25	3.64	3.86
15	Block 2	60.00	6.00	15	2.33	2.54
16	Block 2	60.00	6.00	25	3.80	3.77
17	Block 2	60.00	5.00	25	2.70	3.04
18	Block 2	60.00	6.00	25	3.71	3.77
19	Block 2	60.00	7.00	25	2.74	2.93
20	Block 2	60.00	6.00	35	3.58	3.90

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