

PRODUCTION OF AMYLOLYTIC ENZYMES FROM RAGI TAPAI – EFFECT OF MOISTURE CONTENT

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Abstract— Starch processing are common process in beverages, food, pharmaceutical and textiles industries as well as in bioethanol production. These days, bioconversion of starch using starch acting enzymes are preferred over the conventional method of using chemical catalyst. Therefore, the demand for starch acting enzymes (amylolytic enzymes) has rose tremendously for the past years and expected to increase in the next decade. The objectives of this present study is to produce amylolytic enzymes from Ragi Tapai and to determine the optimum moisture content for the production of amylolytic enzymes. Amylase enzymes was produced through solid state fermentation using Tacca starch as carbon source. The fermentation took place for 96 hours at room temperature (37°C) and samples were taken every 24 hour. The moisture content was varied from 83% to 96%. The result shows that the maximum amylase activity was 0.9314 U/mL.min at 72 hour incubation time and 90% moisture content. The effect of carbon feedstock on amylase activity was also conducted to compare between Tacca starch and soluble starch. It result shows that enzyme activity on Tacca starch was higher than soluble starch.

Keywords— *Ragi, Tapai, amylolytic, enzymes, Tacca leontopetaloides.*

I. INTRODUCTION

Amylolytic enzymes (amylase) is a hydrolyzing enzymes that has the ability to hydrolyze starch into simple sugar. It is highly significant and valuable enzymes in biotechnology, constituting 25% of the total world enzymes (Souza, *et al.*, 2010). It can be obtained from wide range of living system such as animals, plants and microorganisms. Commercially, enzymes used are produced by microorganisms due to its lower manufacturing cost, consistency, and required less time and space for operation (Ong, 2015). According to Ibrahim (2008), the yield for enzymes production from microorganisms are higher than plant and animal sources. They can be controlled physiologically and phsico-chemically, easier to be extracted and making the downstream processes less difficult with low processing cost. With the use of microorganisms, process optimization and modification are easier to be conducted. Several known amylase-producing microorgarnisms are *Saccharomycopsis sp.*, *Pseudomonas sp.*, *Streptomyces sp.*, *Lactobacillus*, *Bacillus spp.*, *Proteus*, *Escherinchia*, etc.

Amylolyic enzymes has widen application in industrial processes, most commonly, in starch processing industry. The International starch market index projected the growth rate of

starch application in the world is 2-3% annually (International

Starch Institute, 2018). Along with the increasing starch utilization in industries, demand for industrial enzymes are also expected to increase exponentially in the next decade. Currently, Malaysia is greatly relying on imported enzymes for starch processing industry. This has amount up to US\$ 3.5 million annually for more than 1 million kg of crude enzymes consumption (Ibrahim, 2008). In that sense, the cost for starch processing in Malaysia will increase due to the increasing enzymes' demand. If industrial enzyme can be produced within the country, it will greatly reduce the reliant on export and decrease starch processing cost significantly. Therefore, research on amylolytic enzymes production are necessary as the beginning of Malaysia's enzymes industry.

Malaysia is a country known for its richness in its natural resources. Among them, there are many sourcecs of raw material for amylolytic enzyme's production such as Ragi tapai (Figure 1.1). Ragi tapai is a dry food starter, made up from various ingredient, that contain meixed cultures of yeast, bacteria and molds (Hesseltine, *et al.*, 1990). Ragi are used to ferment glutinous rice or cassava into tapai, which enjoyed widely in Southeast Asia and parts of East Asia. The individual and combined role of these mixtured culture during fermentation process, determine the quality of Tapai produced (Barus, *et al.*, 2013). There are several amylolytic microorganisms that has been identified in Ragi. According to Hesseltine, *et al.* (1990), ragi consist of molds such as *Mucor*, *Rhizopus*, *Aspergillus*, *Amylomyces* and about 30 different bacterian including *Bacillus spp.* Some yeast that has also been identified are *Sacchromyces cerevisiae*, *Candida krusei*, *C. pelliculosa*, *C. utilis*, *C. magnoliae*, *C. glabrata*, *C. sphaerica*, *Cryptococcus laurentii* *Rhodotorula mucilaginosa*, and *R. glutinis* (Chiang, *et al.*, 2006).



Figure 1.1. Ragi Tapai. (<https://www.expatgo.com/my/2015/02/02/make-rice-wine/>. Copyright 2018 by ExpatGo.)

Starch is the key to enzymes production. It is commonly use as

fermentation media or carbon source. In a study conducted by Divakaran, *et al.* (2011), shows that, among the four substrate (starch, rice powder, wheat powder and ragi powder), starch gave the highest production of enzymes. Starch come from different sources such as maize, potato starch, corn starch, cassava, tacca, etc. Researches have conducted to determine optimum starch media for the production of amylase enzyme. However, studies on Tacca as carbon source for amylolytic enzyme has not been conducted yet. *Tacca leontopetaloides* is a wild herb belongs to the Taccaceae family, and commonly known as arrowwood, Likir, Lukeh, Kacunda or Kecondong. Based on the physicochemical study of Tacca starch by Kunle, *et al.* (2003), comparing to maize and potato starch, the amylose content of Tacca starch is higher than maize but lower than potato. It has smaller granule size relative to maize and potato starches and mainly polyhedral with edges. Its solubility and swelling power is relatively higher than the other starches, making it better disintegrants in drug formulations. Thus this study was conducted simultaneously to shows the potential of Tacca starch as a carbon source for amylolytic enzymes production. The aim of this study is to produce amylolytic enzymes from ragi tapai and to determine the effect of moisture in fermentation media (Tacca starch) for the production of amylolytic enzymes from ragi tapai.

II. METHODOLOGY

A. Materials

The culture used was commercialized Ragi tapai bought from Tesco Shah Alam and the Tacca flour used in this experiment was extracted from Tacca tuber. The Ragi and Tacca was placed in a sealed container and stored in a cabinet at room temperature.

B. Isolation, screening and determination of amylolytic enzymes

Amylolytic microbe were isolated from Ragi tapai similar to the method used by Hasan, *et al.* (2017). In brief, 1 g of Ragi was weighted and suspended in 9 mL of sterile distilled water. After a serial dilution (10^{-1} to 10^{-6}) of this suspension, 50 μ L of each diluted suspension was spread on starch agar plates (4% soluble starch, 2% nutrient agar). Then the plates were kept for incubation for 24 h at 37°C. Pure colonies appeared on incubated plates were further sub-cultures in the same media.

The microbial isolates were subjected to plate assay for screening of amylolytic strains by starch hydrolysis test (Vaidya & Pragma, 2015). The starch agar plates containing microbial isolates were flooded with Gram's iodine (1% Iodine, 2% KI) solution for 1 min and then discarded. The basis of the detection and screening are based on the presence of clear zone (no blue color) around a colony. The clear zone indicated its starch hydrolyzing ability whereas blue color around a colony indicated an inability to degrade starch Hasan, et al. (2017).

C. Production of Amylolytic Enzymes

Fermentation media (1 g Tacca flour, 10mL of distilled water) was inoculated with 1 g of Ragi tapai and place in a dark place at room temperature for 96 hrs. Samples of culture medium was taken every 24 hrs. At the end of the fermentation period, the samples was centrifuged at 10000 rpm for 15 min to obtain the crude amylase extract.

D. Effect of Moisture Content

To study the effect of different moisture content of fermentation media on amylase production, Ragi tapai was inoculated in the media having different moisture and incubated at room temperature for 96 hrs. The moisture was adjusted by adding 5, 10, 15, 20 and 25 mL of distilled water, (83, 90, 93, 95 and 96% moisture content respectively) to fermentation media (1 g Tacca flour).

E. Estimation of Total Protein

This method for total protein was described by Caprette (2015). The standard curve for protein concentration was constructed using different concentration of Bovine Serum Albumin (BSA). One mL of each dilution of standard, unknown sample and blank solution were prepared in separate test tubes. In each test tube, 0.90 mL reagent A (0.2% $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$, 10% Na_2CO_3 , 50% 1N NaOH) was added and mixed. The mixed solutions were incubated in a 50°C water bath for 10 minutes, then cool to room temperature. Then 0.1 mL reagent B (2% $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$, 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10% 1N NaOH) was added to each tube and mixed. The mixture was incubated for 10 min at room temperature. Three mL of diluted Folin-Ciocalteu reagent was added rapidly to each tube and incubated for 10 min in the 50°C and cooled to room temperature. Absorbance of the solution was measured at 650 nm in 1 cm cuvettes. The absorbance was plotted for different BSA concentration. Then the unknown protein concentration of sample was determined using constructed standard curve obtained.

F. Reducing Sugar Analysis

Amylase activity was determined using reducing sugar analysis (Vaidya & Pragma, 2015). Mixture of 1.5 mL of 1% Tacca starch and 2 mL of 0.1M phosphate buffer (pH 6.5) was prepared and about 0.5 mL of crude enzymes were added to react the mixture. Reaction mixtures were incubated for 15 minutes at room temperature (37°C). The reaction was stopped by addition of 1 mL of DNS reagent in boiling water bath for 10 minutes. The amount of reducing sugar released was determined by measuring the absorbance at 540 nm using UV-Vis spectrophotometer. If the reading of absorbance is high, necessary dilution should be made. One unit of amylase activity defined as the amount of enzyme required for releasing 1 μ g of reducing sugar (maltose) per minute under assay conditions. The activity of enzymes was calculated by using the following Equation 1.1 (Hasan *et. al.*, 2017):

$$\text{Amylase activity, } U \text{ mL}^{-1} \text{ min}^{-1} = \frac{\text{Amylose released } (\mu\text{g}) \times \text{Total volume of reactive media (mL)} \times \text{Dilution factor (DP)}}{\text{Molecular weight of maltose} \times \text{enzyme used (mL)} \times \text{Time of incubation (min)}} \quad (1.1)$$

G. Effect of different fermentation substrate

To study the potential of tacca starch as a fermentation substrate for amylolytic enzymes production, the enzymes (at optimum condition) was subjected to DNS test using 1% conventional

soluble starch. The result was compared with the result obtained earlier.

III. RESULTS AND DISCUSSION

A. Isolation, screening and determination of amylolytic enzymes

Potential amylolytic microbe was isolated from Ragi tapai using enrichment techniques. Suspension of Ragi tapai was spread on starch agar plate and incubated. After inoculation, individual colonies were isolated and further sub-cultured on starch agar plate to obtain pure culture. The colonies were selected based on their morphological differences. These pure cultures were subjected to plate assay by flooding them with Gram's iodine to identified potential amylolytic enzymes producer. Figure 1.2 shows isolated microbial strains from Ragi tapai. A total of three microbial strains named as S1, S2 and S3 were isolated. Among which two strains (S1 and S2) shows clear zone. The principal of this assay are based on the reaction between iodine and starch. Iodine will react with starch to form a dark blue starch-iodine complex. Clear zone (non-blue colored) indicate the absence of starch within the region. According to Sharma *et al.*, (2015), appearance of clear zone shows that the colonies has the ability to hydrolyze the starch within that region. Thus, the positive result from these 2 strains indicate the presence of amylolytic microbe in Ragi tapai. In agreement with the statement by Calmette (1892), Ragi tapai consist of starch-degrading microbes (amylolytic microbes) that capable of breaking-down carbohydrates and proteins in a fermentation substrate (grains, legumes and roots). This statement further supported by Hesseltine *et al* (1988), stating that Ragi tapai contains mixture of several wild yeast, molds and 30 different bacteria. Among them, the most commonly identified amylolytic microbial strains in Ragi are *Bacillus spp* (Barus *et al*, 2013; Joo *et al*, 2007; Barus & Wijaya, 2011), *Sacchromyces cerevisiae* (Chaing *et al*, 2006) and *Saccharomycopsis fibuligera* (Hesseltine *et al.*, 1990). Since, strains of amylolytic microbes has been identified in Ragi tapai, thus, intensify the potential of it as the source for amylolytic enzymes production.

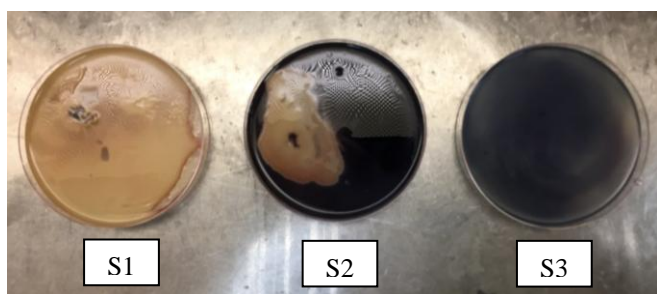


Figure 1.2. Isolated microbial colonies subjected to Gram's Iodine flooding

B. Production of Amylolytic Enzymes

The production of amylase was carried out via solid state fermentation and incubated for a period of 96 hour, in which sample was withdrawn for every 24 hour. Enzymes production was verified using Lowry test for protein determination and DNS test for reducing sugar analysis. The result for total protein determination was presented in Figure 1.3. The maximum protein concentration is 1.6378 mg/mL at 72 hour period. For reducing sugar analysis, result shown in Figure 1.4. Optimum enzymes production was also obtained at 72 hour period with maximum

amylase activity of 0.9314 U/mL.min. The same pattern can be observed in Figure 1.3 and 1.4, which shows increment with increasing fermentation period up to 72 hour. Then, it decline beyond 72 hour. The consistency between both results may due to the fact that enzymes is a protein. Enzyme production is based on the specific growth rate of microbial cell. Maximum enzyme production could be obtains only after certain incubation period which allows the culture to grow at a steady state. This study reveal that the optimum fermentation period for Ragi tapai strain is 72 hour. Chimata, *et al.*, (2010), reported similar kind of amylase enzymes production pattern for *Aspergillus niger* strain; enzyme production increases until a certain period of 72 hour and decline with further incubation. The maximum amylase activity obtained was 55 U/mL. Another similar observation was made by Anto, *et al.*, (2006), the maximum enzyme production by *Bacillus cereus* MTCC 1305 was at 72 hour incubation period, and the yield of enzymes decreased with further incubation. Hashemi *et al.*, (2011), also made similar kind of observation stating that the optimal incubation period for *Bacillus sp.* KR-8104 was 72 hour. At the point beyond optimum incubation period, microbial growth decline as nutrient become less available and waste product increases (Bailey, 2018). Overall, amylolytic enzymes can be produced from Ragi tapai with maximum incubation period of 72 hours.

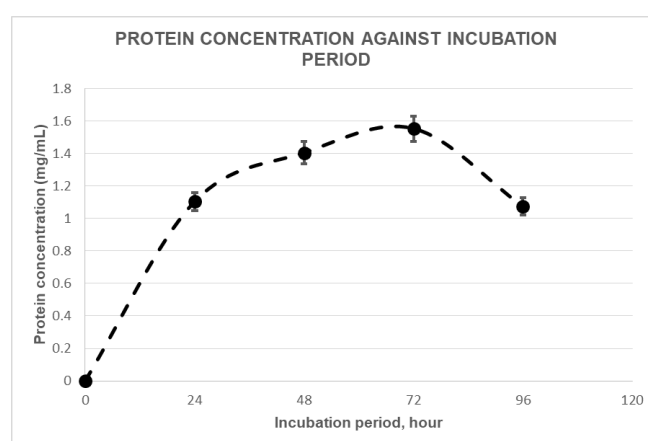


Figure 1.3. Protein concentration against incubation period

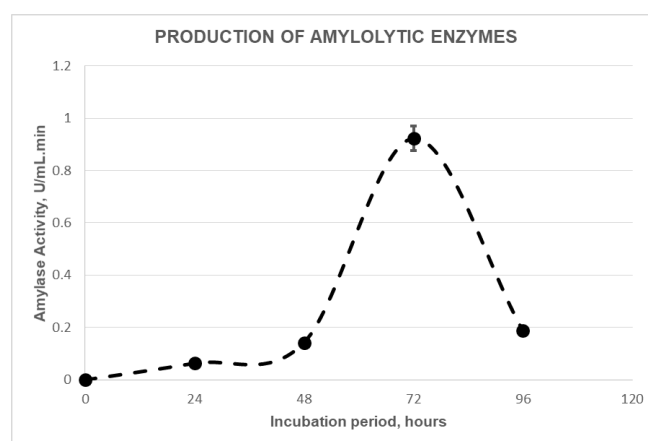


Figure 1.4. Amylase acitivity against incubation period

C. Effect of Moisture Content

Moisture content is an important factor that influences the growth and product yield of microbe in SSF. It affects both aeration and nutrients solubility and suitability to be utilized by microorganisms (Fadel, 2000). Moisture reported to facilitate

microorganisms for better utilization of substance by causing swelling of the substrate (El-Shishtawy, *et al.*, 2014). The result is presented in Figure 1.5 for both protein concentration and amylase activity at optimum cultivation period. The result shows that moisture level has an effect on the enzymes level produced by the microbe, as well as the total protein released. The highest protein concentration is 2.4410 mg/mL at 15 mL moisture level. However, the maximum amylase activity was observed at 90% moisture (10 mL) which is 0.9314 U/mL.min. Based on similar studies, it was reported that the optimum moisture levels for amylase production using *Bacillus sp.* (Baysal, *te al.*, 2003), *Penicillium expansum* (Erdal & Taskin, 2010), *Aspergillus oryzae* (Francis, *et al.*, 2002) and *Asoergillus sp.* (Ellaiah, *et al.*, 2002) were 30, 70, 70 and 80 %. According to Kunamneni, *et al.*, (2015), maximum amylase activity for *Thermomyces lanuginosus* can be observed at 90%. In a study conducted by El-Shishtawy *et al.*, (2014), maximum production of amylase, xylanase and pectinase were at 70, 80 and 40% moisture content respectively. The yield of amylase by *Clostridium thermosulfurogenes* increased when the solid to moisture ratio was increased up to 1:2.5. However, any further increment of this ratio resulted in the decreases of enzyme yields, which may due to clumping of solid particles that leads to the smaller interparticles space and decrease nutrient diffusion. In contrast, low moisture content leads to the decrease solubility of nutrients present in the substrate. Similarly discussed by Anto *et al.*, (2006), higher moisture content changes substrate particles structure, decrease porosity, promotes development of stickiness and lower oxygen transfer rate. On the other hand, lower moisture content reduce solubility of nutrient to the solid substrate and causes lower degree of swelling and higher water tension. The moisture level demand for enzymes production in SSF differ according to type of enzymes, microorganisms, as well as substrate used; in term of their particle size and configuration of the particles (Fadel, 2000). Adjusting the optimum initial moisture content of substrate during SSF is important because moisture content changes during fermentation as a result of evaporation and metabolic activities (Anto, *et al.*, 2006).

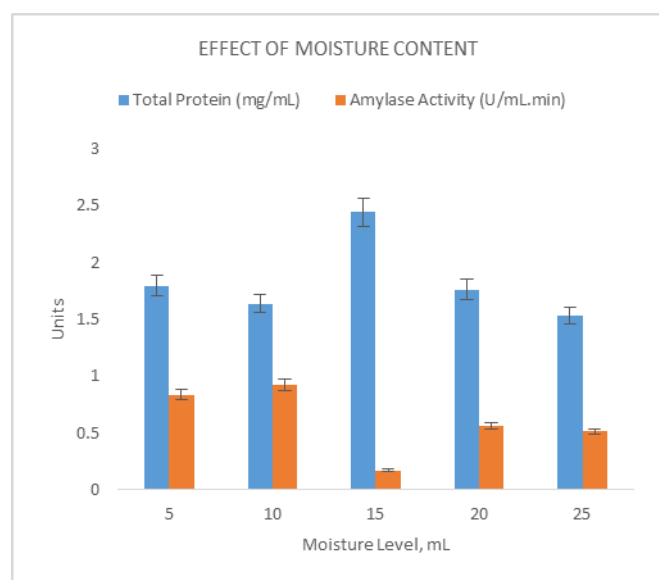


Figure 1.5. Effect of Moisture content on the production of amylolytic enzymes

D. Effect of different fermentation substrate

Microbial growth and enzymes production are greatly influenced by both environmental conditions and the nutrient available in the growth medium. The study on the effect of different carbon source was conducted on the production of amylolytic enzymes from Ragi tapai. Two different type of starch was used as substrate were Tacca starch and soluble starch. Figure 1.6 shows comparison of amylase activity for different fermentation substrate. The value for Tacca starch is 0.9314 U/mL.min and soluble starch is 0.0307 U/mL.min. The production of amylase using Tacca starch is 97% higher than soluble starch. It was reported earlier that, soluble starch was the best carbon supplement for amylase production in *Myceliaphthora thermophile*, D14 (Sadhukhan *et al.*, 1990), *Aspergillus fumigatus* (Goto, *et al.*, 1998) and *Thermomyces lanuginosus* (Kunamneni *et al.*, 2005). This is in agreement with a study by Farid (2011) which indicate that the amylase produced by *A. oryzae* LSI has a maximum activity using soluble starch as substrate at 55°C. However, Elegado and Fujio (1993) reported that wheat and cassava starches are the most favored substrates by all strains while potato and corn starches were the least. Another similar finding on starch hydrolysis using *A. terreus* amylase, indicated that wheat starch was the best substrate followed by rice starch, potato starch, parley flour, sago and topica starch (Ghose, *et al.*, 1990). Tacca starch granules from *Tacca leontopetaloides* were small (average particle size 3.5 μ m) relative to maize and potato starch (Kunle, *et al.*, 2003). Substrate with smaller particle gave higher enzyme activity due to an increased in surface area (Molony *et al.*, 1984) and high degree of solubility. Besides that, in SSF culture, particle size of substrate determines the void space which is occupied by oxygen (air). Since the rate of oxygen transfer affect microbial growth, the substrate should contain particles of suitable sizes to enhance mass transfer (Pandey, 1990). According to Omojola (2013), Tacca starch had relatively higher swelling power and solubility than other starches. This can be related to higher amylose contain of 28% which is above the normal range of 15-25%.

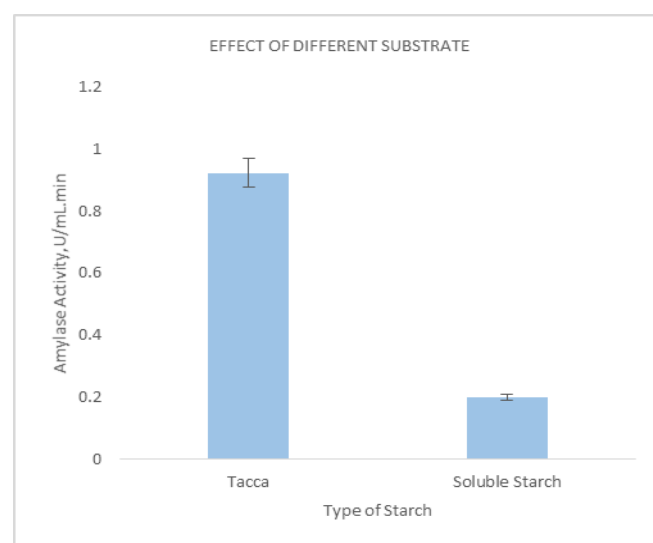


Figure 1.6. Effect of different substrate on amylase production

IV. CONCLUSION

Production of amylolytic enzymes from Ragi tapai was achieved under solid state fermentation using Tacca starch as fermentation substrate. The maximum amylase activity obtained

was 0.9314 U/mL.min corresponding to 1.6378 mg/mL protein concentration. The optimum production condition were 72 hour incubation period, 90% moisture level with Tacca starch used as carbon substrate.

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