Effect of Temperature on Tuber Starch (*Tacca leontopetaloides*) Pretreatment and Incubation for Glucose Production from *Ragi Tapai*

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Abstract

The aim of this work is to maximize the glucose production for the fermentation of *Tacca leontopetaloides* starch with *ragi tapai* by optimizing the temperature of the tuber starch processes. In this research work, four different types of *ragi tapai* from Sabah, Johor, Perlis and Kelantan were obtained and used to identify the best *ragi tapai* in the production of glucose. The different types of *ragi tapai* were first undergo identification and characterization process in order to determine selection of *ragi tapai*. This study was conducted to achieve the objective of the research: (1) To study the effect of pretreatment temperature towards the production of glucose by *ragi tapai*. (2) To study the optimum incubation temperature of the *ragi tapai* in the glucose production from tuber starch. A series of fermentation process of tuber starch were performed by varying the pretreatment temperature at 30°C – 70°C at fixed incubation temperature of 30°C and incubation temperature at 30°C – 50°C at fixed pretreatment temperature of 70°C with constant 5% (w/v) of starch slurry and 10% (w/v) of *ragi tapai* concentration for 180 minutes. DNSA test were performed via spectrophotometer at 540nm to quantify the production of glucose. In the fermentation at various pretreatment temperature, the lowest glucose yield obtained was at 30°C (7.333 g/L) and the highest glucose yield was at 70°C (46.760 g/L). Meanwhile in the fermentation at various incubation temperature, the lowest glucose yield obtained was at 30°C (7.333 g/L) and the highest was at 50°C (62.764 g/L). When the temperature increases, the glucose yield increases. From the results studied shows that *Tacca leontopetaloides* starch is a promising feedstock to produce glucose.

Keywords: Tacca leontopetaloides starch, Ragi tapai, Glucose production, Temperature of pretreatment, Temperature of incubation.

1. Introduction

Glucose has been produced commercially which acts as a renewable resource especially in production process of the biofuels. In the production process of the biofuel, glucose were used as the raw material provide the world the alternative energy for crude oil. Biofuel such as bioethanol has the potential to be used as a promise fuel as it is more volatile than water, flammable, burns with a light blue flame and has good fuel properties for spark ignition internal combustion engines. The bioethanol can be produced from low cost raw material which contain high starch content such as corn, potato and tuber starch which can be found abundant in Malaysia. This pathway is considered to be the best option because it promotes to safe the nature by reducing the consumption of petroleum and less pollution to the environment.

Polynesian arrowroot or scientifically known as *Tacca leontopetaloides* is a species of wild perennial herb plant belongs to the family of Dioscoreaceae which distributed naturally from western Africa through southern Asia to northern Australia [1]. The *T. leontopetaloides* is a plant of low elevations in the moist tropics which is commonly found in the area near to the sea and below elevations level of 200 metres. The ideal for the plant to grow best is in a fertile area, which favours in a condition of humus-rich soil in the shade of trees. According to [2], this plant is a perennial herb with a tuberous rhizome. The tuber root of the plant is rich in starch source which can be eaten raw or roasted, or can be extracted for other purposes.

The inhabitants of Low Islands in the Pacific Island use this underground tuber starch of *T. leontopetaloides* as an important food source. Additional to the importance of the tuber starch, it is also be used to stiffen fabrics in some of the islands. In other region of Polynesian islands on the other hand, the application of the bitter raw tubers are generally used as traditional medicine or remedy to treat stomach ailments such as diarrhea and dysentery [3]. In order to extract the starch from the tubers, the tubers are first need to be peeled and grated, the resultant pulp were washed with water for several times, finally in a sieve or cloth. It is important to make sure that the tubers were washed thoroughly to remove the layer of wax that coat the tubers which is said to be poisonous. There are many advantages of using this tuber starch as the material source such as low price material, abundant and easily to obtain and renewable resource.

In this work, *ragi tapai* is used to convert the starch material into glucose. *Ragi tapai* or or *ragi tape* contain microorganisms such as fungi, yeast and bacteria which is commonly used in the production of tapai. Referring to Gadjar [4], Tapai is a popular traditional fermented food in Asia, especially in Southeast Asia which uses cassava, rice or glutinous rice as the substrate that be fermented with *ragi tapai* or yeast. Occasionally tubers of the sweet potato are also fermented into tapai. Tapai has a sweet-acidic taste and mild alcoholic flavour [5]. These traditional delicacies have various name in different countries. It is known as *loongpang* in Thailand, *marcha* or *murcha* in India, *ragi* in Indonesia, *bubod* in the Philippines, *Chinese yeast* in Taiwan, *nuruk* in Korea, *banh men* in Vietnam, *koji* in Japan and *ragi tapai* in Malaysia [6].

In the work of [7], the *ragi tapai* or *ragi tape* which contain thermophilic bacteria such as *Bacillus licheniformis* and recombinant strains of *Escherichia coli* are capable to produce enzyme called alpha amylase. Furthermore, the *ragi tapai* also contain other microorganisms such as *Aspergillus niger* and *Rhizopus* species which have the capabilities to produce enzyme glucoamylase. Both of these enzymes are used in the conversion of starch into glucose.

The yield of glucose can significantly affected by the pretreatment and fermentation temperature, which to some extent, increases with the increase in temperature [7]. In the pretreatment process, the selection of suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agricultural industrial materials for microbial growth and product formation [8]. The pretreatment of starch is commonly related to the process of gelatinization of the starch. Gelatinization in one of the most important observed physicochemical and functional properties of starch because the completeness in starch gelatinization will influence the conversion of starch into fermentable sugars in the subsequent process. Gelatinization process is the process associated with amorphous swelling and disintegration of crystalline domains of the starch which can be affected by the pretreatment temperature [9]. Since gelatinization process is a temperature-dependent, the starch is not completely gelatinized at a given temperature even when it was held at that temperature for an infinite time [10].

2. Materials and Mehtods

2.1. Materials

The raw material used in this study is a powder starch of *Tacca leontopetaloides* as received. The processed powdered starch are used in the hydrolysis experiment as the substrate for the conversion into simple sugar. In this experiment, the cultures used were commercialised *ragi tapai* starter obtained from local market from Johor, Sabah, Perlis and Kelantan. The ragi starter was in a form of dry flattened circular cakes shape obtained in a small plastic bag packaging. Also, 10 g/L of yeast extract and 10 g/L of peptone were used to prepare YEP broth.

The Table 1 shows the proximate composition of *Tacca leontopetaloides*. From Table 1, the composition of the total carbohydrate recorded the highest concentration among other components. The total carbohydrate contained in the *Tacca leontopetaloides* is 78.19% while the other components is less than 10% in concentration.

Table 1: Proximate Composition of *Tacca leontopetaloides* [11].

Parameter	**Concentration (%)**
Moisture content	8.66 ± 0.01
Crude protein	6.79 ± 0.02
Crude fibre	5.44 ± 0.03
Crude fat	0.51 ± 0.04
Ash	0.41 ± 0.04
Total carbohydrate	78.19 ± 0.05

2.2. Sample Preparation

All apparatus were sterilized at 121°C for 15 – 20 minutes in the autoclave machine before used.10ml of YEP broth was poured into empty universal bottles by using a measuring cylinder. 1g of *ragi* powder was weighed and added into the universal bottle containing YEP broth. The bottle was shake to allow well mix of the *ragi* powder in the YEP broth. The same steps were performed with the other three *ragi tapai* starter. The universal bottles contain *ragi* samples were labelled Johor, Kelantan, Perlis and Sabah. The sample at time zero was diluted 10X and the optical density (OD) reading was measured at 540nm by using spectrophotometer. The ragi cultures were placed in the incubator shaker at 37°C and 200rpm for 24 hours.

2.3. Observation of Microorganism

After 24 hours of incubation, the sample was taken to measure the OD reading to check any growth of the microorganism present in the increament of OD reading. Then, the each of the samples were subcultured via spreading technique on nutrient agar for growth observation. The nutrient agars were placed in the incubator for 24 hours at 37°C. A gram staining method were performed to the single colony of the strain before examined under the microscope.

2.4. Selection of *Ragi Tapai*

Starch slurry of 5% (w/v) was prepared by dissolving 5g of *Tacca leontopetaloides* starch in 100ml of preheated distilled water in a beaker. The temperature preheated distilled water was maintained at 60°C for 5 minutes before raise the temperature to 70°C for an hour. After obtaining the starch slurry with the appearance of translucent and gel-like consistency, the starch slurry was allowed to cool at room temperature until reach 37°C to 40°C. After that, 30ml of starch slurry was transferred into three conical flasks each by using a syringe. The three conical flasks indicate of replication for each of *the ragi tapai* powder. Then, 10% (w/v) of *ragi tapai* was prepared by dissolving 3g of *ragi tapai* powder in 30ml of 1% peptone in a conical flask and then be placed in the incubator shaker for 30 minutes at 37°C and 200rpm. Next, 3ml of 10% *ragi tapai* culture suspension was pipetted into 30ml of starch slurry in a conical flask. All of the conical flasks were placed in the incubator shaker with a fixed condition of 30°C and 50rpm. Samples were taken to determine the glucose production from each flask after 20 minutes, 40 minutes and 60 minutes of incubation. The highest glucose yield from all of the four types of *ragi tapai* was selected for further experimental parameter.

2.5. Effect of Pretreatment and Incubation Temperature

In this experiment, to prepare a starch slurry of 5% (w/v), first 100ml of distilled water was measured by using a measuring cylinder and poured into a beaker. The beaker contain 100ml of distilled water was placed on the hot plate with a magnetic stirrer. The beaker was labelled as 30°C with a constant stirring of magnetic stirrer at 300rpm until the temperature maintain at 30°C (no preheat) for 5 minutes. Then, 5g of *Tacca leontopetaloides* starch powder were poured into the beaker and let it mix for 1 hour with a maintained temperature of 30°C. After that, the starch slurry was allowed to cool at room condition. Next, 30ml of the starch slurry were transferred into three 100ml conical flasks each, labelled 30°C (1), 30°C (2) and 30°C (3) by using a syringe. The three conical flasks indicate three trials for the respective temperature. Then, the starch slurry were let to cool down at room temperature condition. Next, 3ml of 10% *ragi tapai* culture suspension were pipetted into each of the flasks. After that, the flasks were placed in the incubator shaker at 30°C and 50rpm of agitation. 2ml of samples were pipetted into falcon tubes to determine the glucose production from each flask after 60 minutes, 120 minutes and 180 minutes of incubation. The experiment was repeated with different pretreatment temperature of 40°C, 50°C, 60°C and 70°C. The amount of glucose production was measured and compared from all of the pretreatment temperature of the starch slurries. The highest glucose yield from starch slurry indicates the optimal pretreatment temperature.

The same method was performed to determine the effect of incubation temperature towards the glucose production from the starch slurry. In this experiment, the incubation temperature was varies from 30°C, 35°C, 40°C, 45°C and 50°C at a constant pretreatment temperature of 70°C. The same steps were performed as in the first part of the experiment which to determine the pretreatment temperature, the glucose production was measured and compared from all of the incubation temperature and the highest glucose yield from the starch slurry indicates the optimal incubation temperature.

2.6. Analysis Method

In this research, a process of determination of glucose via DNS method is performed to analyse the production of glucose from starch. In the same time, the standard calibration curve of known glucose concentration was made to identify the production of glucose in the conical flasks exerted to a different temperature condition. The 3,5-dinitrosalicylic acid solution will react with glucose to form 3-amino-5-nitrosalicylic acid which appear in orange colour. In the preparation of standard calibration curve of known glucose concentration, 3% (w/v) of glucose concentration will be analysed via DNS method. The samples were centrifuged at 10,000rpm for 5 minutes. A series of dilution was performed to the glucose solution before measuring the OD reading by using a spectrophotometer at 540nm. The data obtained were used to plot the standard curve of the absorbance versus glucose concentration graph.

Then, the same steps of analysis method were performed to the samples of the experiment. The samples of experiment include the samples of varies pretretment temperature (30°C, 40°C, 50°C, 60°C and 70°C) and incubation temperature (30°C, 35°C, 40°C, 45°C and 50°C). The readings of the absorbance were record and the concentration of the glucose can be determined by using the prepared standard curve of known glucose solution.

3. Results and Discussion

In the process of simultaneous saccharification and fermentation (SSF), there are several factors need to be consider to achieve the highest yield of glucose production [8]. One of the crucial factor need to be consider in the SSF is the temperature of the process. In this experiment, two most important factors were studied which is the pretreatment and incubation temperature of the SSF. It is important to study the factor of temperature in SSF as it carefully regulated during hydrolysis and fermentation as it has vital impact on the overall conversion efficiency and product yield [12]. From the work of Lamsal [13] and Plumier [14], separate saccharification and fermentation, it stated that during the conventional hydrolysis of starch the optimum temperature for liquefaction is between 85 - 105°C. While the saccharification is usually done at 50 - 60°C [15].

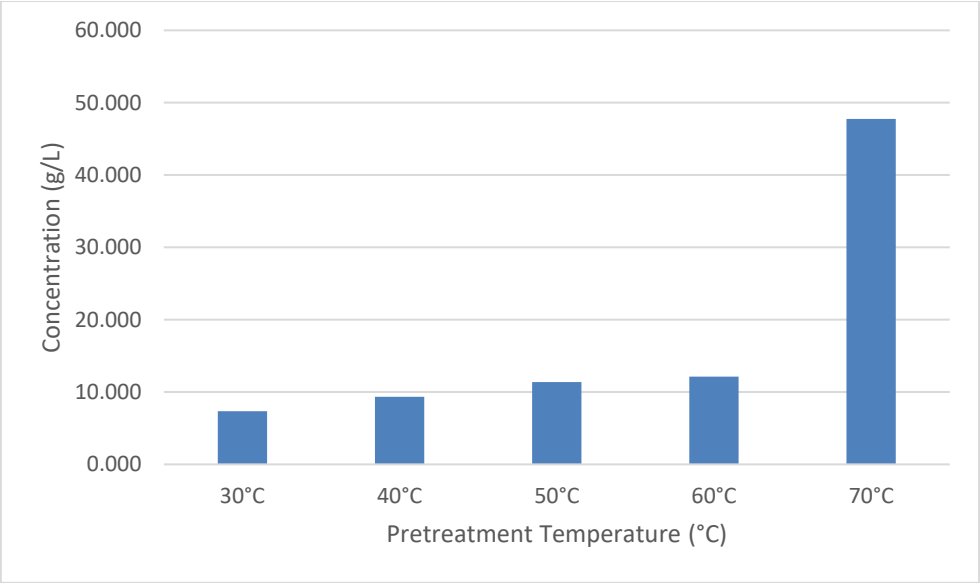

Figure 1: The glucose concentration after 180 minutes of incubation at 30ºC for five different pretreatment temperature of (30ºC, 40ºC, 50ºC, 60ºC and 70ºC) was calculated from the absorbance reading.

Table 1: The glucose concentration at various time of incubation for five different pretreatment temperature of (30ºC, 40ºC, 50ºC, 60ºC and 70ºC) was calculated from the absorbance reading.

Time (minute)	60	120	180
30ºC	1.626 g/L	2.707 g/L	7.333 g/L
40ºC	2.836 g/L	3.269 g/L	9.322 g/L
50ºC	2.966 g/L	4.177 g/L	11.397 g/L
60ºC	3.139 g/L	7.463 g/L	12.132 g/L
70ºC	19.396 g/L	38.680 g/L	46.760 g/L

In this experiment, the pretreatment temperature was varied from 30ºC, 40ºC, 50ºC, 60ºC and 70ºC at a constant incubation temperature of 30ºC, 5% (w/v) of starch slurry and 10% (w/v) of *ragi tapai* concentration. In the pretreatment process, the starch slurry was preheated and maintained at respective temperature for 1 hour before let to cool down and inoculation step. There is a significant different in the appearance of the slurries at various temperature. At the preatreatment temperature of 30ºC, 40ºC, 50ºC and 60ºC, the appearance of the slurries are nearly similar to each other. The starch powder was solubilized completely in the preheated distilled water but the gelatinization of the starch slurries are incomplete. At the end of pretreatment process, the starch slurries were still have white colour of the powder, less translucent and less viscous.

In contrast to earlier findings, the appearance of the starch slurry was completely different at 70ºC. The starch powder was solubilized completely in the preheated distilled water and the gelatinization is complete. At the end of the pretreatment process, no white colour of the powder left, the starch slurry was translucent and more viscous compared to the others. From Figure 1, the results obtained for pretreatment temperature (30ºC, 40ºC, 50ºC and 60ºC) indicates that the glucose concentration increased slightly as the pretreatment temperature increased. At 70ºC of pretreatment temperature, the conversion of glucose is the highest and drastically increased. This is might due to the optimum gelatinization temperature of the starch is at 70ºC which promote the completeness of gelatinization. A complete gelatinization of starch influence the glucose production in the fermentation process. Meanwhile at 30ºC of pretreatment temperature recorded the lowest conversion of glucose. From the observation at low pretreatment temperature, *ragi tapai* are capable to hydrolyze the ungelatinized starch but at very low rate. Thus,the glucose yield increases with the increases of pretreatment temperature. Referring to the previous study of Wangpor [16], the highest concentration of glucose was observed at 85ºC as the high temperature assist in the conversion of starch into fermentable sugar by promote swelling and reduce the crystallinity structure of the starch.

The effectiveness of glucose production primarily relies on the adequate growth of the microbial cells of *the ragi tapai* in the broth that is often affected by the temperature [7]. The microorganisms require a suitable condition in order to achieve an optimal growth which affected by the incubation temperature. In the second part of the experiment, another parameter was studied at different incubation temperature from 30ºC, 35ºC, 40ºC, 45ºC and 50ºC at constant pretreatment temperature of 70ºC, 5% (w/v) of starch slurry and 10% (w/v) of *ragi tapai* concentration.

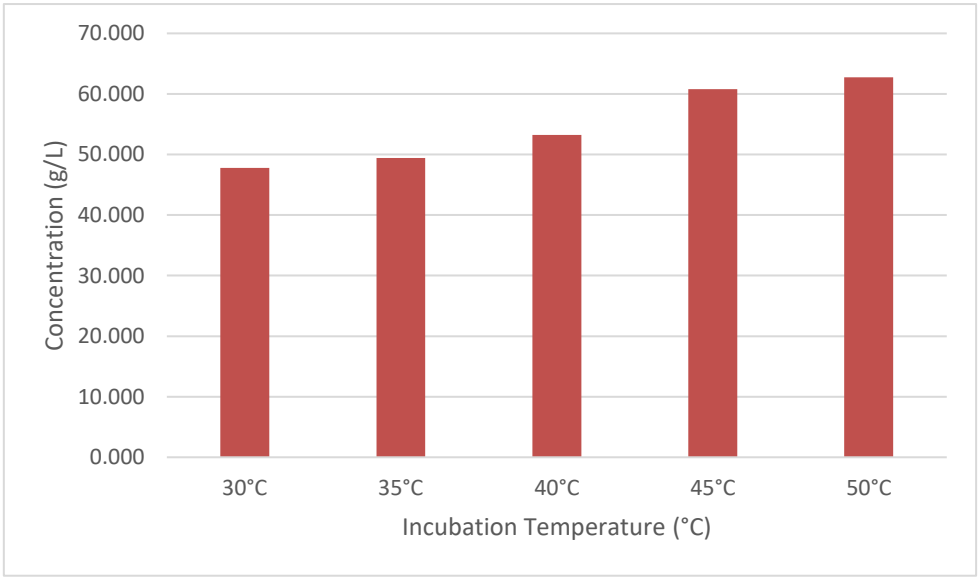

Figure 2: The glucose concentration after 180 minutes of incubation at a constant pretreatment temperature of 70ºC for five different incubation temperature of (30ºC, 35ºC, 40ºC, 45ºC and 50ºC) was calculated from the absorbance reading.

Table 2: The glucose concentration at various time of incubation for five different incubation temperature of (30ºC, 35ºC, 40ºC, 45ºC and 50ºC) was calculated from the absorbance reading

Time (minute)	60	120	180
30ºC	19.094 g/L	38.680 g/L	47.760 g/L
35ºC	20.261 g/L	40.756 g/L	49.447 g/L
40ºC	21.861 g/L	41.275 g/L	53.251 g/L
45ºC	28.909 g/L	45.339 g/L	60.775 g/L
50ºC	29.946 g/L	45.815 g/L	62.764 g/L

Figure 2 shows the lowest glucose concentration at incubation temperature of 30ºC and the highest glucose concentration at incubation temperature of 50ºC. The concentration of glucose production increases as the temperature of incubation increases. The optimum temperature for incubation in this experiment is 50ºC. Within the range of this study of Wangpor [16], the optimum temperature of saccharification step is 60ºC. In another research performed stated that the temperature process of fermentation starch hydrolysis is at 55ºC [17]. In general, fermentation is related to the enzyme activity by the microorganisms influenced by the temperature. If the incubation temperature increases, the enzyme amylase activity increases. Hence, rate of starch conversion into glucose increase produce in the increasing glucose yield. According to the previous research of Kunamneni [8], a high titer of amylase activity was obtained at the incubation temperature of 50ºC.

4. Conclusion

The effects of pretreatment and incubation temperature plays an important role to yield high production of glucose from starch. The optimum condition for pretreatment process of the starch are at 70ºC with constant incubation temperature of 30ºC, 5% (w/v) of starch slurry and 10% (w/v) of *ragi tapai* concentration. While the optimum condition for incubation process of the starch are at 50ºC with constant pretreatment temperature of 70ºC, 5% (w/v) of starch slurry and 10% (w/v) of *ragi tapai* concentration. The maximum yield of glucose achieved from the hydrolysis of starch was 62.764g/L. Suggestion for the future research, a more convenient and efficient analysis method can be used for glucose determination and quantification by using high performance liquid chromatography (HPLC).

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