TIGER MILK MUSHROOM CULTIVATION BY USING SUBMERGED CULTURE TECHNIQUE

N. A.RAHMAN[1], F. DAUD[2], M. S.KALIL[1], S.AHMAD[1]

[1]Department of Chemical & Process Engineering
Faculty of Engineering & Built Environment, Universiti Kebangsaan Malaysia
43650 UKM Bangi, Selangor, Malaysia.
[2]School Of Biosciences & Biotechnology, Faculty Of Science & Technology, Universiti Kebangsaan Malaysia
43650 UKM Bangi, Selangor,
MALAYSIA
e-mail; liza@eng.ukm.my

ABSTRACT ~ The aim of this project is to study the growth of tiger milk mushroom or *Lignosus rhinocerus* by using submerged culture technique. The strains used in this study are *L. rhinocerus* AOI and *L. rhinocerus* 7-19 but for growth kinetic only strain of *L. rhinocerus* 7-19 is studied by using Monod model. Based on the Monod model, parameters such as specific growth rate, μ and Monod coefficient, Ks can be determined. Value for μ_{max} is 0.54 hr^{-1} and value for Ks is 0.29 g/L. Doubling time, t_d is 22 minutes and 25 seconds, and biomass yield coefficient, $Y_{X/S}$ is 0.25. The optimum growth conditions for *L. rhinocerus* 7-19 are studied by using 6 types of carbon sources and 6 types of nitrogen sources. The data show that the highest growth is obtained by using lactose, followed by xylose, maltose, glucose, sucrose and fructose. Lactose shows the highest growth rate because of high percentage of carbon content. Besides, the biomass growth also high for xylose, due to xilose is a type of wood sugar and factor of mushroom habitat. The study of nitrogen sources show that the usage of ammonium salts such as NH_4Cl, NH_4NO_3 and $(NH_4)2HPO_4$ can hinder the growth of biomass. The usage of other nitrogen sources such as meat peptone, yeast extract and peptone can increase the growth of biomass. Thus, the presence of carbon source and nitrogen source are crucial to biomass growth for both strains.

Keywords: Biomass, *β-glucan*, *Lignosus rhinocerus*; Monod Model; Nitrogen Sources

1 INTRODUCTION

Mushrooms have long been valued as tasty and nutritious food by different societies around the world [1]. Rates of protein mushrooms can vary from 10-40% of dry weight. Mushrooms contain all the essential amino acids, but the quantity of amino acids containing sulfur and metionin sistin is quite limited [2]. Polysaccharide known as *β-D-glucan* is water-soluble, with a chain of heterosaccharide xylose, mannose, galactose, and uronic acid or *β-D-glucan*-protein complex of proteoglycans [4]. Antitumor polysaccharides can be isolated from spores, mycelium or dehydrated submerged liquid culture.

Tiger milk mushroom (*Lignosus rhinocerus*) is a local mushroom used in a traditional medicine. The active ingredient in tiger milk mushroom is *β-glucan* [5]. Each mushroom drug containing *β-glucans* but *β-glucan* content in the tiger milk mushroom is higher than mushrooms type known as Ling Zhi or Ganoderma [3]. Fig. 1 shows the β-glucan in the form of powder extracted from tubers tiger milk mushroom. *B-glucan* powder is ground and dried by freeze drying method [3]. Most of the research carried out for tiger milk mushroom

focusing on nutrition [6,7,8] and methods of cultivation.

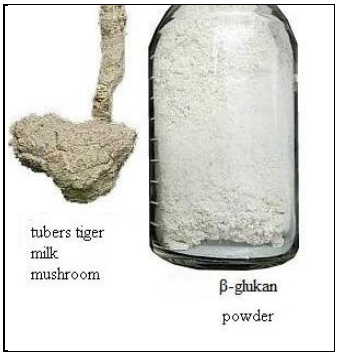


Fig. 1. β-glucan in the form of powder extracted from tubers tiger milk mushroom

According to [3] Wong 2011, most of conventional mushroom cultivation period is a time consuming. As a solution, submerged culture techniques are used to accelerate the production period of tiger milk mushrooms biomass. Several clinical studies have shown that compounds from mushrooms can prevent tumor growth [9]. Mold can produce penicillin, lovastatin, ciclosporin, griseofulvin, cephalosporin, ergometrine, and methylergometrine. Research has resulted in the discovery of psilocybin mushrooms, and dietilamida lisergat acid. Mushrooms produce a variety of unique chemicals and poisons such as paclitaxel drug α-amanitin that is dangerous [10, 11]. The main problem may be encountered if the tiger milk mushroom to be commercialized is the cost of producing high values of *β-glucan* and the process of extraction and purification of the product requires advanced technology. In addition, the 'halal' status of tiger milk mushroom growth medium is not guaranteed.

The aim of this project is to study the growth of tiger milk mushroom or *Lignosus rhinocerus* by using submerged culture technique and mycelium growth on different media. This objective is achieved by doing the comparison study at different culture medium and the optimum growth conditions.

2. MATERIALS AND METHOD

The strains used in this study are *L. rhinocerus* AOI and *L. rhinocerus* 7-19. Growth kinetic of *L. rhinocerus* 7-19 is studied by using Monod model. Both strains were cultured in the sterile potato dextrose agar (PDA) medium in petri dishes. Mycelium culture needs to be done by using three petri dishes (PDA) for each strain. Incubation period is 7 days at 25°C. Then, the stock cultures should be stored in conventional refrigerator at 4°C [12].

Tiger milk mushroom cultivation for both strains performed on nutrient agar medium and medium to PDA (Potato Dextrose order). Stock culture to a small cut in squares and put on a new agar medium. Added to petri dishes were incubated at 37°C. Observations made after seven days.

This method is used to study the growth of mycelium in the form of biomass. This growth study using six Erlenmeyer flask with a volume of 500 mL in a 250 mL liquid medium. Culture medium used was medium MCM (Mushroom Complete Medium). Table 1 shows the types and quantities of materials used [13] .

Table 1. Medium MCM (*Mushroom Complete Medium*)

material	Quantity (g/L)
Glucose	20
Meat Peptone	2
Yeast extract	2
Dikalium hydrogen phosphate K_2HPO_4	1
Magnesium sulfate heptahidrat $MgSO_4.7H_2O$	0.5
Potassium dihydrogen phosphate KH_2PO_4	0.46

Tiger milk mushroom strains that were studied using strain 7-19. After MCM medium prepared in Erlenmeyer flask, it were sealed with cotton and covered with aluminum basket.

Flask should be gently agitated to be in a homogeneous medium. Then the flasks were autoclave at 121°C and the pressure is 15 psi for 15 minutes followed by, cooling of the flasks before the mushroom culture included. Flask weight must be recorded.

Afterward, the experiment was conducted in a laminar flow chamber. Tiger milk mushroom culture on PDA agar medium used as inoculum. Myceliums on agar plates cultured for a week have a uniform growth.

Next the PDA that containing mycelium will be cut square (1 cm x 1 cm) cubes of five cuts for each culture so that the cut will be assigned to each flask except flask number 6. Then the flask closed with cotton and aluminum basket. Flask weight must be recorded to determine the weight of the culture that has been inserted. Then, the flask should be placed in incubator rotating with a speed of 150 rpm. Each flask represents a certain culture period. The first flask (3 days), the second flask (6 days), the third flask (9 days), the fourth flask (13 days), the fifth flask (16 days) and the six flask (16 days).

2.1. Effect of Carbon Sources

The influence of carbon source on the yield of biomass cultivation is done by using six different types of carbon sources such as glucose, fructose, maltose, lactose, sucrose, and xylose. Mycelium growth medium was the same as the basic medium except for material MCM glucose (20 g/L) changed with different carbon sources (20 g/L). Similar method of preparing the medium with submerged culture techniques as described above. Medium preparation is repeated using other carbon sources.

2.2. Effect of Nitrogen Sources

Effect of nitrogen source on the yield of biomass cultivation is done by using six different types of nitrogen sources for example yeast extract, peptone, meat peptone, diammonium hydrogen phosphate, ammonium nitrate and ammonium chloride. Mycelium growth medium was the same as the basic medium MCM except meat peptone (2 g/L) and yeast extract (2 g/L) changed with different nitrogen source (4 g/L). Similar method of preparing the medium with submerged culture techniques as described above. Medium preparation is repeated using other nitrogen sources.

2.3. METHOD OF ANALYSIS

2.3.1. The growth of mycelium of the worm biomass measurement

Growth medium in the flask should be filtered using Whatman filter paper grade 1 (11μm). Weight of filter paper before filtration performed should be recorded. When there is no medium that dripped down from the funnel, wet weight biomass was weighed and recorded. Then, the biomass was dried at 37°C until dry. Mycelium dry weight biomass was weighed and recorded

2.3.2. Analysis of Glucose Concentration

Glucose concentration was examined using dinitrosalisilik acid (DNS). This method is a modified method of Miller's method [14].

Reducing sugars were tested by inserting 2 mL of DNS reagent and 1 mL sample into a test tube. Test tube should be heated to the boiling water for 5 minutes until sample colour changed to dark red. Then, the test tube is allowed to cool and 10 mL of distilled water added into the test tube. The test tube should be shaken with a shaker test tube so that a homogeneous solution is obtained.

Solution added to cuvvete and read using UV-spectrophotometer at 600 nm wavelength. Liquid medium glucose concentration is determined by the method of Miller [14] where after the biomass is filtered, the filtrate taken to test DNS. The equation of a straight line from the graph of OD readings against a standard concentration of glucose used to determine the concentration of glucose medium.

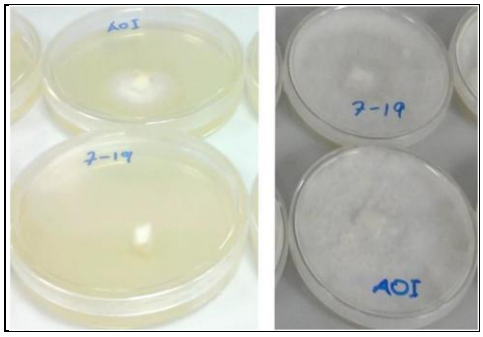
2.3.3. pH analysis

The filtered medium pH should be measured using a pH meter. There are two types of pH meters are used, Hannah and Fisher Scientific Accumet Instruments. Before reading the pH of samples taken, the pH meter rod should be soaked in distilled water first. These two values obtained are recorded and the average pH value is calculated.

3. RESULTS AND DISCUSSION

3.1. GROWTH IN SOLID MEDIUM

Both the tiger milk mushroom strains 7-19 and AOI were cultured in a two types of dish agar known as nutrient agar and PDA (Potato Dextrose order). Mushroom mycelium was cultured in the incubator for a week at 37°C. Fig. 2 shows the growth of mycelium on nutrient agar and PDA agar for both strains.



Nutrient Agar PDA Agar

Figure 2: Mycelium in the culture dish agar

Tiger milk mushroom mycelium is not suitable to live in nutrient agar medium but more suitable for PDA. Nutrient agar contains peptone 15 g/L, yeast extract 3 g/L, to 12 g/L, NaCl 6 g/L and glucose 1 g/L. PDA agar contains potato infusion of 4 g/L, dextrose 20 g/L and to 15 g/L.

The results show that mycelium is not suitable for living in nutrient agar medium because it did not have enough carbon sources for mycelium cell growth whereas for PDA it proved to be an ideal medium for mycelium growth because sufficient carbon source content.

Mycelium cultured in a medium that will form hyphae, which seemed like a fine white thread. The fungal hyphae grow so uniformly across the surface. After a week of the culture, hyphae will start creeping on the side of a petri dish and hyphae will grow on the surface so uneven.

3.2. GROWTH IN A LIQUID MEDIUM

Tiger milk mushroom strain 7-19 was cultured in liquid Mushroom Complete medium (MCM).

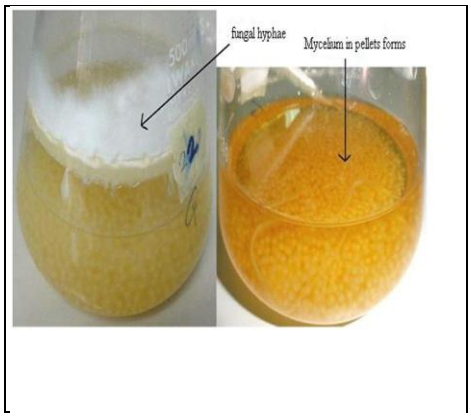


Fig. 3 : Appearance of the mycelium in liquid medium colony

Fig. 3 shows the appearance of colonies of mycelium in liquid medium. This result proved that the submerged culture technique can be used for developing Tiger milk mushroom strain 7-19.

Fig. 4 shows the mycelium cultured on a different day. Mycelium cultured in liquid medium will form colonies in the form of pellets or nodules.

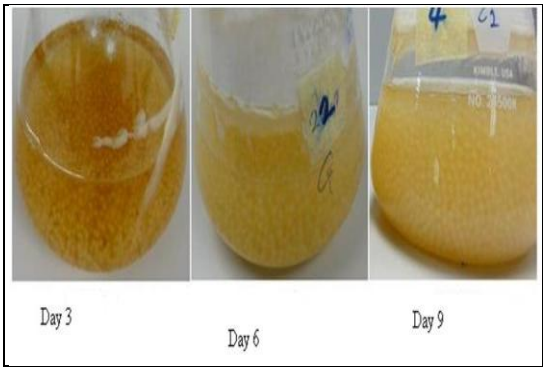


Fig. 4: Mycelium cultures on different days

After a certain period, fungal hyphae spread also grows on the side of the flask. Both strains of 7-19 and AOI do not differ significantly from mycelium. If the culture mediums have contamination, the liquid medium will become turbid and mycelium nodules cannot be seen clearly. MCM medium colour is brown. When the biomass increases, the medium colour changed to orange and became dull when biomass growth decreases.

3.2.1. Growth of Biomass in Submerged Culture

Growth profile

Growth of Tiger milk mushroom strain 7-19 was studied using submerged culture techniques. Each 500 mL Erlenmeyer flask used, representing different growth cycles. The parameters which are constant in this experiment such as the volume and type of medium, temperature, pH and agitation speed. In determining the tiger milk mushroom growth of cells in liquid medium, the resulting biomass must be filtered and dried. Optical Density (OD) reading techniques cannot be used because the resulting mycelium colonies in liquid medium in the form of pellets or nodules. Table 2 shows the weight gain of biomass within 16 days.

Table 2 : Weight of biomass produced.

Flask	Days	Wet Biomass (g)	Dry Biomass (g)	Percentage Dry Of Wet (%)
1	3	32.91	0.80	2.43
2	6	55.73	1.19	2.14
3	9	78.65	1.66	2.11
4	13	54.94	1.29	2.34
5	16	35.74	0.90	2.52

Fig. 5 shows dry weight of biomass until 16 days. Maximum growth of biomass is in day -9 and after this period, the weight of the biomass began to decline due to the lack of resources as a factor limiting glucose growth. Biomass growth requires adequate source of carbon and nitrogen nutrients.

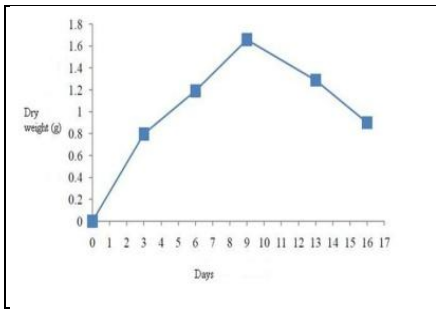


Fig.5: Graf of dry weight of biomass versus day of experiment

Table 3 shows the concentration of glucose in each flask representing a different culture period.

Table 3 Glucose concentration in each flask

Flask No.	Days	OD Reading	Glucose Concentration (g/L)
1	3	1.754	5.46
2	6	0.319	0.99
3	9	0.216	0.67
4	13	0.159	0.50
5	16	0.156	0.49

The profile of glucose concentration in each flask was further demonstrated as shown in Fig. 6.

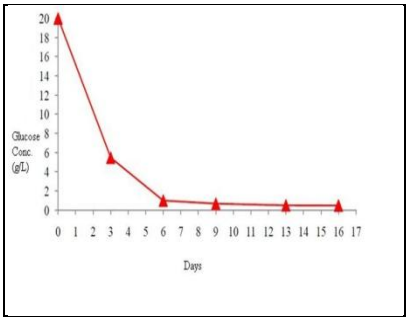


Fig. 6: Graf of glucose concentration versus day of experiment

Based on Fig.6, glucose concentration is decreased exponentially. In the day-6, the glucose concentration has reduced to 1 g/L. because the carbon source which is glucose used for biomass growth. This discovery similar with other researchers[1,2, 4] and theories[18].

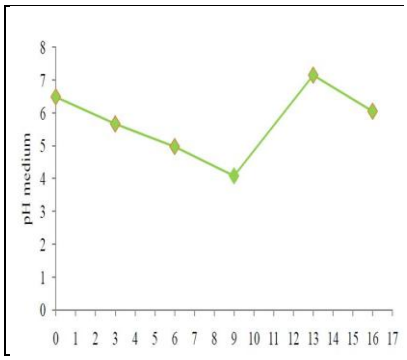


Fig. 7. pH Medium versus days

Fig. 7 illustrates that, the pH medium decreased to 4.08 on day-9 and increase until day-13 before it decreases again until day-16. Minimum pH value on day-9 is 4.08 due to the presence of organic acids. The pH value started to rise after day -9 due to the autolysis cells or amino acids [15].

3.2.2. Effect of Carbon and Nitrogen Sources on Growth

Tiger milk mushroom biomass growth studied using different carbon sources, namely glucose, fructose, maltose, lactose, sucrose and xylose. Table 4 shows the dry and wet weight biomass for each carbon sources. Only the biomass wet weight data taken in this experiment. Biomass dry weight data is calculated based on the average percent dry weight of the wet weight biomass in Table 2.

From Table 4, growth of tiger milk mushroom biomass was maximum–in lactose medium (0.70 g of biomass), followed by xylose, maltose, glucose, sucrose and fructose.

Table 4. Weight biomass of different carbon sources medium

Carbon source.	Wet weight of Biomass (g)	Dry weight of Biomass (g)
Glucose	21.75	0.50
Fructose	13.93	0.32
Maltose	22.61	0.52
Lactose	30.20	0.70
Sucrose	21.40	0.49
Xilose	25.41	0.59

Lactose, maltose, and sucrose are disaccharide sugars and have a higher percentage of carbon than the monosaccharide sugars such as glucose and fructose. High carbon content in lactose is one of the factors caused a higher biomass growth. Xylose is the second highest type of carbon sources in biomass production. This may be explained by habitat factors tiger milk mushroom usually grows in soil rich in humus and rotten wood. Wood sugar xylose is obtained by decomposing straw or wood fiber. The molecular structure of xylose may also facilitate the decomposition of nutrients by the cells of the tiger milk mushroom.

In this experiment, Erlenmeyer flask covered with aluminum foil and wrapped with parafilm. Thus, ventilation is limited. The resulting biomass is less than the culture flask using a cotton cover. Biomass cultivation is done during the week.

For the experiments using different nitrogen sources, only the observation of the day -7 was recorded. Type of nitrogen source used was peptone meat, ammonium chloride, ammonium nitrate, diammonium hydrogen phosphate, peptone and yeast extract. Observations of biomass growth were demonstrated in Table 5.

Table 5. Observations of biomass growth

Nitrogen source.	Observation
Meat Peptone	Biomass growth occur
Ammonium Chloride NH_4Cl	Biomass growth did not occur
Ammonium Nitrate NH_4NO_3	Biomass growth did not occur
Diammonium hydrogen phosphate $(NH_4)2HPO_4$	Biomass growth did not occur
Peptone	Biomass growth occur
Yeast Extract	Biomass growth occur

Based on the observations above, only meat peptone, peptone and yeast extract can be used as a nitrogen source medium for biomass growth occurs. No biomass growth occurs in ammonium salts such as ammonium chloride,

ammonium nitrate and diammonium hydrogen phosphate.

This can be explained by the presence of ammonia factor which is toxic to mushrooms. There is also a certain mushroom species that can withstand high levels of ammonia [16]. Experimental data prove the importance of nitrogen source for biomass growth of mold.

3.2.3. Changes in glucose concentration and pH on Biomass Growth

Fig. 8 illustrates changes in glucose concentration and dry weight biomass growth with time. Glucose concentrations decreased exponentially and the growth of biomass increased up to day-9 and then declined the next day.

Exponential growth phase began in the early period of culture up to day-9. Death phase begins after the day-9 due to the reduction of nutrients, carbon, nitrogen and glucose depletion of resources, and growth of biomass to reduce.

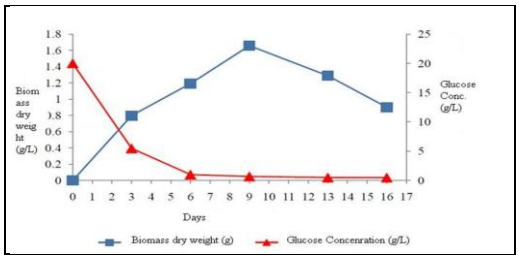


Fig. 8: Graph of biomass dry weight (g) and glucose concentration (g/L) against the number of days

Fig. 8 also indicates that the growth of fungus biomass was influenced by the glucose content in the liquid medium. Biomass growth requires a carbon source than glucose for cell growth. Glucose levels are very low on day 9 of biomass which resulting the growth began to decline.

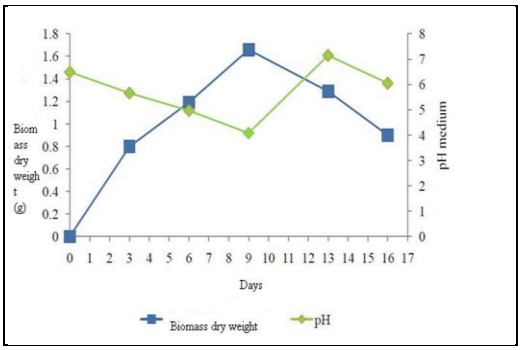


Fig. 8: Graph of dry weight biomass (g) and medium pH against the number of days

Fig. 9 shows the changes in medium pH and biomass growth on the number of days. Based on these graphs, the pH of the medium began to decrease when the biomass growth increased, and pH of the media began to rise when biomass growth decreased. On the first day to day -9, the growth of biomass is increasing with decreasing pH value due to the presence of organic acids such as oxalic acid [17].

Another observation from Fig. 9 was that on day 9 to day -16, decreasing biomass growth but increasing pH value due to the autolysis cells or amino acids [15], as there are sources of excess nitrogen. Ammonia will be produced and cause the increase in pH.

3.2.4. Ventilation effects on biomass growth

Mycelium requires oxygen for cell growth. Rate of oxygen in flask A and B was observed by covered both flasks with different medium such as flask A closed with aluminum foil and parafilem whereas flask B covered with aluminum foil and cotton.

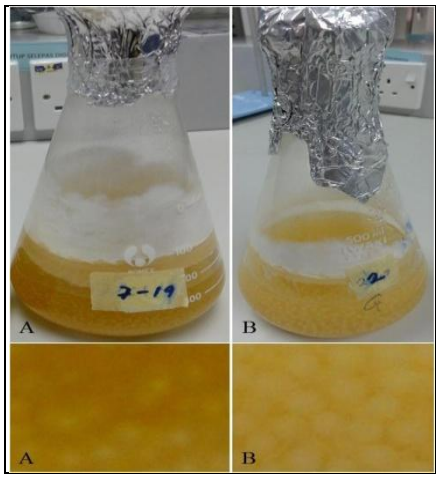


Fig. 10: Biomass cultivation
A) Flasks closed with aluminum foil and parafilem
(B) Flasks covered with aluminum foil and cotton.

Fig. 10 shows the tiger milk mushroom culture strain 7-19 with different methods. Culture time was for 6 days.

Ventilation in flask A was less than flask B. Therefore, gas exchange in flask A was slightly lower than the flask B. Ventilation affect the growth of mycelium as the gas transfer coefficient was inefficient based on the medium used to covered the flasks. Colony size and quantity of mycelium in flask B was larger than the flask A. In flask B medium colour fades due to low nutrient content than flask A.

Mycelium needs oxygen and nutrients to survive and produce carbon dioxide. Amount of oxygen become limiting factors to the growth of mycelium. This observations illustrates that type of material used for covering the flasks have a significance effect to the mycelium growth. Com binations of aluminum foil and cotton is more effective than the aluminum foil and parafilem. This could be explained as the structure of cotton comprises of more pores compare to parafilem.

3.3. GROWTH KINETICS

In an ideal culturing process, cell growth requires sugar substrate to produce cells. In theory, a graph of cell growth against time is divided into several parts, lag phase, exponential phase, decline phase, stationary phase and death phase.

Lag and stationary phase cannot be identified because of the short time between the two sample flask culture is quite long. Based on Fig. 11, the interval between samples is of three days, so the resulting shape of the graph does not resemble the theoretical graph of ideal cell growth.

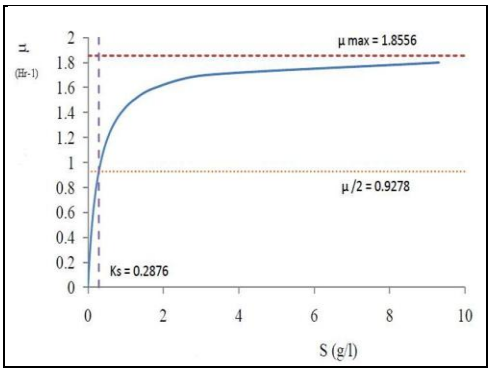


Fig. 11: Graph of the specific growth rate versus glucose concentration

Fig. 11 shows a graph of the specific growth rate versus glucose concentration based on the Monod model and the kinetic parameters were determined. The double time is the time required to double the quantity of biomass. Calculation of the double time is as follows.

$$t_{d=\frac{\ln 2}{\mu}} = \frac{\ln 2}{1.8556} = 0.3735\ hours$$
$$= 22.41\ minutes$$

The relationship between cell growth and substrate can be studied by mathematical methods. Monod equation relates changes in the concentration of substrate with a growth rate of cells [18]. Specific growth rate, μ is increase in cell mass per unit time. Theoretically, the increase in biomass (dx) at intervals of time (d_t) is proportional to the concentration of biomass (X). Specific growth rate calculation is as follows;

$$\frac{dX}{dt} \propto X \rightarrow \frac{dX}{dt} = \mu X$$
$$rearrange\ \mu = \frac{1}{X}\frac{dX}{dt}$$
$$By\ integration \int_0^i \mu dt = \int_0^i \frac{1}{X} dX$$

$$|\mu t|_0^i = |\ln X|_0^i$$
$$\mu(t_i - t_0) = \ln(X_i - X_0)$$

$$\mu = \frac{\ln \frac{X_i}{X_0}}{(t_i - t_0)} \quad (1)$$

Where μ = growth rate (hr^{-1})
X_i = final biomass (g/L)
X_o = initial biomass (g/L)
t_i = final time
t_o = initial time

Monod equation can be rearranged to the Lineweaver-Burk equation. Parameters in the equation can be found by sketching a graph of growth rate against glucose concentration by considering the exponential phase used in this rule. Initial cell concentration was 0.8 g/L. Table 6 shows the specific growth rate μ and the concentration of glucose, S.

Table 6. Specific Growth Rate

Hours.	*X* (g/L)	μ (hr^{-1})	*1/μ* (hr^{-}	*S* (g/L)	*1/S*
72	3.19	0.02	52.03	5.46	0.18
144	4.76	0.01	80.71	0.99	1.01
216	6.63	0.01	102.12	0.67	1.49

Table 6 shows that increase the time from 72 hours to 216 hours decrease the specific growth rate μ and the concentration of glucose, *S*. This findings follow the theories [18] and similar with other researchers [13,15].

Biomass yield coefficient, $Y_{X/S}$ is the ratio of the quantity of biomass produced from substrate used. In other words, $Y_{X/S}$ = concentration of cells produced (g/L) per glucose concentrations used (g/L). Table 7 shows biomass yield coefficient calculation.

Table 7. Biomass yield coefficient

Days	Cell Concentration (g/L)	Glucose Concentration (g/L)	Biomass yield coefficient
3	3.192	14.4929	0.22
6	4.764	18.9984	0.25
9	6.632	19.3218	0.34
13	5.152	19.5008	0.26
16	3.6	19.5102	0.18

From Table 7, it shows that biomass yield coefficient $Y_{X/S}$, increase gradually from day three until day nine but decrease afterwards. This result proves a relationship between concentrations of cells produced substrate used. This suggests that the proposed submerged culture technique could be used as an alternative method. Average biomass yield coefficient, $Y_{X/S}$ is 0.25.

4. CONCLUSIONS

Tiger milk mushroom strain 7-19 can be grown successfully by means of submerged culture in 250 mL of MCM medium. Biomass growth increased up to day-9 and started to decline afterwards. Mycelium culture was also successfully carried out using agar medium PDA. Tiger milk mushroom growth kinetics was studied by using the Monod model. Based on the growth model, parameters such as specific growth rate, μ and Monod coefficient, K_s can be determined. μ_{max} value is 0.54 $hour^{-1}$ and the value of K_s, is 0.29 g/L. Doubling time, t_d is 22.4 minutes, and the biomass yield coefficient, $Y_{X/S}$ is 0.25. Biomass growth experiments using different carbon and nitrogen sources also successfully performed. The maximum biomass concentration was obtained in culture with using lactose, followed by xylose, maltose, glucose, sucrose and fructose. For the nitrogen source, ammonium salts such as NH_4Cl, NH_4NO_3 and (NH_4) $2HPO_4$ inhibit the growth of cells biomass. The use of other nitrogen sources such as meat peptone; yeast extract and peptone also have affected the biomass growth. It can be concluded that the carbon and nitrogen sources are essential for growth of biomass.

5. ACKNOWLEDGMENT

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