



CELL GROWTH KINETICS OF ASPERGILLUS ORYZAE IN INDUSTRIAL NATURAL RUBBER EFFLUENT SERUM

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ABSTRACT

A dynamic relationship exists between environmental conditions and the growth pattern of filamentous fungi. Growth kinetics such as the relationship between specific growth rate and the concentration of a substrate is one of the basic tools in microbiology. In such cases, a direct monitoring of the cell morphology and biomass distribution in the culture medium is potential. Hence the present work attempt to study the nutrient uptake and cell growth kinetics of a non-pathogenic fungus *Aspergillus oryzae* possessing the ability of bioremediation in the wasteful industrial rubber effluent. Four different models of Monod, Contois, Verhulst, and Tessier were used to investigate the cell growth kinetics in batch submerged fermentation process carried out in shake flasks. The compatibility of the experimental data fitted with Contois, Verhulst and Tessier models with the regression values are 0.65, 0.80, 0.21 and 0.84 respectively. Although Verhulst and Contois are the most suitable kinetic models to describe substrate utilization and cell growth behavior of filamentous fungi in submerged culture, the Tessier model was found best fitted with the experimental data. In the case of Monod, the maximum specific growth rate, μ_m and the half saturation constant, K_s were determined as 2.3 day⁻¹ and 4.84 g/l respectively. For Verhulst, the maximum specific growth rate, μ_m and maximum biomass, X_m in terms of cell dry weight were determined as 0.9 day⁻¹ and -1.5 g/L respectively. For Contois, μ_m was 3.4 day⁻¹ and K_s was obtained as 4.04 g/L. However, in Tessier model, μ_m was determined to be 1.1 day⁻¹ while the K_s was 54.05 g/l.

Keywords: skim latex serum, skim latex, hevea brasiliensis, unstructured models.

INTRODUCTION

Skim latex serum (SLS) is a by-product of rubber processing effluent. The waste serum is derived from the natural rubber latex of *Hevea brasiliensis* tree. The serum comprises of numerous non-rubber material such as minerals, carbohydrates, proteins, lipids, microorganisms and water (Aimi Izyana & Zairossani, 2011). These non-rubber materials contained in the serum is believed to be the source of microbial growth stimulator. Previous attempt on treating this waste via bioremediation with *Aspergillus oryzae* was a promising compared to the other fungi and hence drives an advanced investigation through the present work. Since the rate of bioremediation process is proportional to the amount of active microbial biomass used (Norris, 1993), this study focuses on the growth kinetic of *Aspergillus oryzae* for its maximum production of fungal biomass in SLS.

Fungal kinetics may significantly vary with the changes in culture conditions. Utilization of nutrient by *Aspergillus oryzae* during batch fermentation results in the increment of the microbial mass in respect to time. Determination of cell mass concentration during microbial mass can be done by either direct method through cellular dry weight of the biomass or indirect method through measurement of product formation (Shuler and Kargi, 2002). Till date, there is no study conducted specifically focusing on the growth kinetics of *Aspergillus oryzae* in SLS. The evaluation of growth kinetic on *Aspergillus oryzae* by direct method is very limited and most researchers focused on the indirect method (Sandhya *et al.* 2005; Chutmanop *et al.* 2008; Yin *et al.* 2013). However,

the evaluations by indirect method did not provide details for the growth kinetics, such as the specific growth rates, product yield, rate of product formation and rate of biomass formation. Therefore, the present work focused on the growth kinetic evaluation by using the direct method.

Growth kinetics of filamentous microorganisms has been studied by various researches and there are a few models exist that can be used to portray the microbial growth and substrate utilization during the fermentation such as Blackman, Tessier, Moser and Contois model (Shuler and Kargi, 2002). All these models were actually a mathematical expression generated to explain the behavior of a given system generate (Okpokwasili and Nkweke, 2005) such as microbial cell growth in liquid medium. In this study, the growth kinetics of *Aspergillus oryzae* in SLS media was evaluated by using Monod, Contois, Verhulst and Tessier Model. These four models were chosen because of its common and simple basic microbial kinetic model used to describe the growth of fungus (Alqhtani, 2013).

Unstructured Models for Cells Growth Kinetics

The microbial growth kinetics is expressed in terms of specific growth rate (μ) and saturation constant for substrate (K_s). The unstructured kinetic model tested were Monod, Contois, Verhulst and Tessier models for cell growth as given in the following equations. Which used to describe this sigmoidal relationship between specific growth rate (μ) and substrate (S):



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$$\text{Monod}, \mu = \frac{\mu_{\max} S}{K_s + S} \quad (1)$$

$$\text{Contois}, \mu = \frac{\mu_{\max} S}{K_s X + S} \quad (2)$$

$$\text{Verhulst}, \mu = \mu_{\max} \left(1 - \frac{X}{X_{\max}}\right) \quad (3)$$

$$\text{Tessier}, \mu = \mu_{\max} (1 - e^{-K_s S}) \quad (4)$$

where μ is the specific growth rate (day^{-1}), μ_{\max} is the maximum specific growth rate (day^{-1}), S is the concentration of the limiting substrate (g/l), and K_s is the saturation constant, equal to the substrate concentration at one-half the maximum specific growth rate (g/l).

The specific growth rate (μ_{net}) of *Aspergillus oryzae* in particular SLS concentration was determined during exponential phase by utilizing equation (5) given below:

$$\mu_{\text{net}} = \frac{1}{X} \frac{dX}{dt} \quad (5)$$

where X is cell mass concentration (g/l), t is time (h), and μ_{net} is net specific growth rate (h^{-1}).

The time required to double the microbial mass is given by equation (6) as follow:

$$\tau_d = \frac{\ln 2}{\mu_{\text{net}}} \quad (6)$$

where τ_d is the doubling time of cell mass.

To better describe growth kinetics, growth yield ($Y_{x/s}$) in the fermentation was defined as follow:

$$Y_{x/s} \equiv -\frac{\Delta X}{\Delta S} \quad (7)$$

The yield parameter $Y_{x/s}$ represents the cell mass produced from a unit mass of substrate.

MATERIALS AND METHOD

Sample Collection and Media Preparation

The skim latex waste was collected from a latex concentrate rubber factory in Kedah (Malaysia). The skim latex rubber particles were coagulated with 5% (v/v) sulfuric acid and further centrifuged to remove the coagulated latex which results in yellowish serum solution known as the skim latex serum (SLS). This serum was then pooled into a batch volume and stored at 4 °C prior for further use.

Subculture and Inoculum Preparation

Aspergillus oryzae (ATCC 10124), *Aspergillus niger* (ATCC 9642), *Phanerochaete chrysosporium* (ATCC 24725), and *Rhizopus oligosporus* (locally isolated) were maintained on 3.9% (w/v) of potato

dextrose agar (PDA) and sub-cultured for three days at 30 °C in an incubator. Fungal inoculum was prepared by washing the growing culture on four PDA plates with 25 ml of sterile distilled water. The spore suspensions were rubbed prior to filter using filter paper Whatman™ no.1 into a 250 mL flask. The spores were counted with a hemocytometer to maintain the concentration of 2.8×10^7 cells/mL.

Screening of Fungal Growth in Skim Latex Serum (SLS) as Sole Media

A 50% (v/v) concentration of SLS was prepared by diluting the SLS with distilled water. All experiments were conducted in triplicate by incubating at 30 °C and 150 rpm with 5% (v/v) inoculum for 4 days.

Preparation of Skim Latex Serum (SLS) as Sole Media for Cell Growth Kinetics Evaluation

A series concentration of SLS [10-90% (v/v)] were prepared by diluting the SLS with distilled water. All experiments were conducted in triplicate by incubating at 30 °C and 150 rpm with 5% (v/v) inoculum for 4 days.

Fungal Biomass Yield Determination

Fungal biomass analysis was monitored through the direct method of dry weight (Bratbak and Dundas, 1984) where the biomass was filtered and dried in an oven at 50-60 °C for 24 hours before cooling in desiccator and weighed. The filtrate was recovered for reducing sugar analysis.

Determination of Total Reducing Sugar

Total reducing sugar was measured according to (Miller, 1959) with glucose as a standard.

RESULTS

Screening of Potential Fungal Growth in (SLS) as Sole Media

Among all of the fungal biomass, *Aspergillus oryzae* gave the highest yield in 50% of SLS concentration as shown in Figure-1. Therefore, this fungi has been chosen as the potential fungi that could grow well in the wasteful SLS. Further study on the growth kinetics of this fungi in SLS was done in order to understand the mechanism of fungus growth and its biomass production through the time.

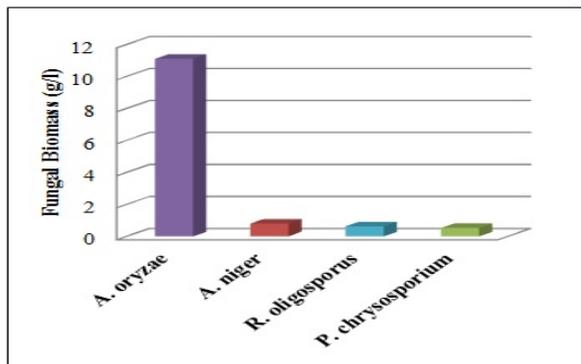


Figure-1. Fungal biomass yield in 50% (v/v) SLS. data are expressed as Mean±Standard Deviation (n=3).

Growth Pattern of Aspergillus Oryzae in Shake Flask Culture of SLS.

Figure-2 illustrates the growth pattern of Aspergillus oryzae in SLS media at 10%, 30%, 50%, 70% and 90% of SLS media concentration respectively.

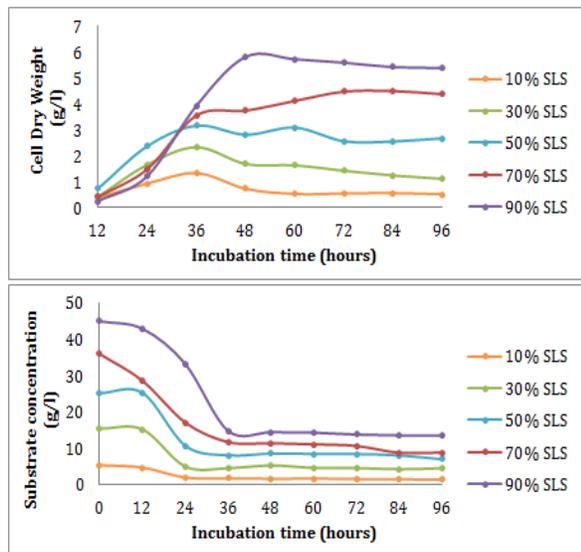


Figure-2. Growth pattern of A.oryzae in varied concentrations of SLS media.

The curves were plotted based on the cell dry weight of Aspergillus oryzae biomass at every 12 hour interval to 96 hour fermentation period. From the curves plotted, it was observed that the pattern of the growth curve is similar for all media concentrations. There is no visible lag phase observed from the curves in the first 12 hours of the fermentation. The exponential phase or also known as logarithmic phase was approached early at first 12 hours and extended over to 36 hours of fermentation for all three media concentration.

Table-1 represents a summary of the specific growth rate, doubling time, and biomass yield coefficient of Aspergillus oryzae in all five different concentrations of SLS. The specific growth rate for Aspergillus oryzae in

10%, 30%, 50%, 70% and 90% of SLS was 0.0516 h-1, 0.0719 h-1, 0.0610 h-1, 0.09 h-1 and 0.1158 h-1 respectively. Meanwhile the doubling time for Aspergillus oryzae in 10%, 30%, 50%, 70% and 90% of SLS was 13.44 h, 9.65 h, 11.37 h, 7.71 h and 5.99 h respectively.

The yield parameter Y_{xs} represents the cell mass produced from a unit mass of substrate. According to Table-1, this value was increased as the substrate concentration increased which suggest a lower biomass produced at higher SLS concentration. This yield coefficients are often varied with environmental conditions. But they are usually treated as constant (Levine, 2010).

Evaluation of Cell Growth Kinetics

The kinetic constants and parameters μ_{max} and K_s were determined based on the curve fitting method. of each model. The values of specific growth rate (μ) was calculated according to the cell dry weight as fungal biomass concentration (X) and reducing sugar concentration as limiting substrate concentration (S) during the exponential growth phase.

Monod Model

Monod model is an unstructured model which is a substrate concentration dependent. A linearized form of equation (1) was used to plot a linear graph as shown in Figure-2 and to determine the K_s and μ_m :

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{K_s}{\mu_m} \cdot \frac{X}{S} \tag{8}$$

Table-1. Summary of the specific growth rate, doubling time, and biomass yield coefficient of Aspergillus oryzae in all five different concentrations of SLS.

SLS (%)	μ_{net} (h ⁻¹)	τ_d (h)	Y_{xs} (g/g)
10	0.052	13.44	0.31
30	0.072	9.65	0.18
50	0.061	11.37	0.14
70	0.090	7.71	0.19
90	0.116	5.99	0.13

Investigation on curve fitting of cell growth with Monod model did show poor fitness (Figure-2). Based on the software analysis, μ_m and K_s of Monod model were evaluated as 2.3 d-1 and 4.84 g/l respectively (refer to Table-2). Also, in this case, R^2 was fitted on 0.653 that it does not seem so desirable. According to the results, Monod kinetic model is not seemed to be a suitable model to express the kinetic behavior of this strain.

Contois Kinetic Model

Contois kinetic model is an unstructured model. However, unlike Monod model, the Contois model is both substrate and cell concentration dependent (Alqahntani, 2013). A linearized form of equation (2) was used to plot a linear graph as shown in Figure-2 and to determine the K_s and μ_m :



$$\frac{1}{\mu} = \frac{X}{S} + \frac{K_s}{\mu_m} \cdot \frac{1}{\mu_m} \quad (9)$$

Investigation on curve fitting of cell growth with Contois model did show acceptable fitness (Figure-2). Based on the software analysis, μ_m and K_s of Contois model were evaluated as 3.4 d⁻¹ and 4.046 g/l respectively. Also, in this case, R² was fitted on 0.802 that it does seem desirable (Table-2).

Verhulst Kinetic Model

As can be seen in Figure 2, to evaluate the kinetic behavior fitness of *Aspergillus oryzae* with Verhulst model, a linear curve fitting method was used on the specific growth rate (μ) based on fungal biomass concentration (X) curve. A linearized form of equation (3) was used to plot a linear graph as shown in Figure-2 and to determine the K_s and μ_m :

$$\mu = \mu_m - \frac{\mu_m}{X_m} \cdot X \quad (10)$$

Results showed that the experimental data of the cell growth and substrate consumption in batch submerged culture did not have a good fitness with Verhulst model by the regression of 0.209. In this regard, the maximum specific growth rate (μ_m) and the maximum biomass concentration (X_m), was 0.9 d⁻¹ and -1.5 g/L respectively. (Table 2).

Tessier Kinetic Model

For Tessier model, a linearized form of equation (4) was used to plot a linear graph of limiting substrate concentration (S) versus $\ln \mu$ as shown in Figure-2 and to determine the K_s and μ_m :

$$\ln \mu = l \frac{1}{K_s} \cdot S + \ln \mu_m \quad (11)$$

Results as in Table-2 showed that the experimental data of the cell growth and substrate consumption in batch submerged culture did have a good fitness with Tessier model by the regression of 0.836. In this regard, the maximum specific growth rate (μ_m) and the half saturation constant (K_s), was 1.1 d⁻¹ and 54.05g/L respectively.

Table-2. A comparison of kinetic parameters of *Aspergillus oryzae* growth and substrate utilization with four different kinetic models.

Model	Parameter Estimation	R-squared
(1) Monod	$K_s = 4.84 \text{ g/l}$ $\mu_m = 2.3 \text{ d}^{-1}$	0.653
(2) Contois	$K_s = 4.04 \text{ g/l}$ $\mu_m = 3.4 \text{ d}^{-1}$	0.802
(3) Verhulst	$\mu_m = 0.9 \text{ d}^{-1}$ $X_m = -1.502 \text{ g/l}$	0.209
(4) Tessier	$K_s = 54.05 \text{ g/l}$ $\mu_m = 1.1 \text{ d}^{-1}$	0.836

Tessier kinetic model is an alternative to Monod model. It is an unstructured model which is a substrate concentration dependent. However unlike Monod model, the transition to the saturating concentration is sharper than in Monod equation (Mrwebi, 2004). Table 2 summarized up all of the kinetic parameters of *Aspergillus oryzae* growth and substrate utilization among the four different kinetic models.

DISCUSSION

The main purpose of this work is to introduce non-pathogenic fungi in the wasteful SLS for waste utilization as well as remediation. Since the rate of bioremediation process is proportional to the amount of active microbial biomass used (Norris, 1993), this study focused on the cell growth kinetics for maximum production of fungal biomass in the wasteful SLS.

Based on the initial screening (Figure-1), the present work suggests that among the four non-pathogenic fungi tested, only *Aspergillus oryzae* strain exhibits the capability to grow independently in the 50% (v/v) diluted SLS without any growth supplement. The other three fungal strains were unable to grow which possibly due to the insufficient carbon source (sugars from liquid SLS) that could be vital for their growth. Although the important growth elements for fungi were inadequate, *Aspergillus oryzae* was able to grow in SLS which contains rubber polymer within the waste. This is in accordance with the work done by Maeda *et al.* (2005) who showed that *Aspergillus oryzae* could grow in biodegradable plastic culture condition as the sole carbon source and has the capability to degrade polymer through cutinase enzyme activity.

According to the growth pattern of *Aspergillus oryzae* in varied SLS concentrations as shown in Figure-2, there is no visible lag phase observed from the curves in the first 12 hours of the fermentation. Shuler and Kargi, (2002) stated that the short lag phase is caused by high concentration of some nutrient or growth factor needed by the microorganism. Thus, during the fermentation, a high concentration of vital growth nutrients for *Aspergillus oryzae* were available initially which contribute to the steepness of curve for the first 12 hours.

The exponential phase or also known as logarithmic phase was approached early at first 12 hours and extended over to 36 hours of fermentation for all three media concentration.

In exponential phase, the balanced growth occurred. Shuler and Kargi, (2002) claimed that the term of balanced growth indicated that all the growth rate of component of the cells is at the same rate. During the exponential phase, the growth rates of cells are independent of nutrient concentration due to hefty nutrient concentration in this phase.

From data in Table-1, it showed that as the SLS concentration increases, the specific growth rate also increases while the doubling time decreases. This result explains that as the nutrient or substrate concentration increased, the growth of *Aspergillus oryzae* was increased.



Hence, the time taken for the *Aspergillus oryzae* to replicate its biomass cell concentration into a double number is shorter since the doubling time was decreased. This is true since the specific growth rate is inversely related to the doubling time (Griffin, 1996) as shown in equation (6).

A classic kinetics for filamentous fungi including a lag and then, an exponential growth phase (Barclay, 1993; Garcia, 1997; Wang *et al.* 2011) as in Figure-2 and cubic model (Prosser, 1991) is best fitted for the growth kinetics of filamentous fungi.

Of all the different models that have been proposed, the Monod relationship is the one that has been most frequently used to describe microbial growth kinetics in culture systems. However, in this study, this model was not desirable to describe the growth kinetics of *Aspergillus oryzae* in SLS due to the low regression coefficient of 0.65.

The R2 (correlation coefficient) is frequently used to judge whether the model represents correctly the data, implying that, if the correlation coefficient is close to one, then the regression model is correct.

On the other hand, the kinetics parameters of Ks and μ_m of this model were comparable to Contois model. Contois model is one of the common models used to describe microbial cell growth and substrate uptake kinetics (Juska, 2011). In the Contois kinetics, a feature of this growth model is that cell-mass growth rate depends upon the concentrations of both substrate and cell mass with growth being inhibited at high concentrations of the cell mass.

The experimental data were well-fitted with R2 of 0.8 while the value of the half-saturation constant, Ks and the low maximum growth rate, μ_m were 4.04 g/l and 3.4 day⁻¹ respectively. A high μ_m indicates a rapid growth of *Aspergillus oryzae* in SLS while a high Ks indicates a low affinity of fungus for the substrate. This revealed that as the population density of biomass increases, there is an increasing obstruction to the substrate uptake and growth of any particular microbe. For example, at high biomass concentration, there is an inhibition on cell growth. In this case, it could be due to the formation of an inhibitor compound by the biomass or high biomass concentrations may give a very viscous medium that results in mass transfer problems.

In Verhulst model, the experimental data was poorly fitted with R2 value of 0.209 and the negative value of maximum biomass, X_m was unacceptable. Therefore, this model is not suitable to describe the growth kinetic of *Aspergillus oryzae* in SLS.

Among the four models that were taken into account, the Tessier model showed the best fit of the experimental data with R2 value of 0.836. The value of the half-saturation constant, Ks and the low maximum growth rate, μ_m were 54.05 g/l and 1.1 day⁻¹ respectively. A high μ_m and Ks indicates a rapid growth of biomass while a low affinity of fungus for the substrate. This Tessier model is useful to describe the substrate inhibition of *Aspergillus oryzae* in SLS. The extreme high Ks value proved that the reducing sugar was not the main nutrient for its growth due to its low affinity towards this substrate at its maximum growth rate. Therefore, there could be other element in SLS that could result in the growth inhibition.

Figure-3 illustrates up all four graphs of plotted experimental data for cell growth kinetic models which were related to data in Table-2.

CONCLUSIONS

In fungal bioremediation, the process rate is proportional to the amount of active microbial biomass. Therefore, this study is the first report on the substrate utilization and growth kinetics of *Aspergillus oryzae* in the industrial rubber effluent known as the skim latex serum (SLS). The experimental data on cell growth and substrate utilization was compared with the commonly used models such as Monod, Contois, Verhulst and Tessier and showed relatively acceptable fitting. The kinetic parameters and regression coefficient revealed that the Tessier model is the best suited model compared to the other three models. Therefore, Tessier model which is a substrate concentration dependent was evaluated as the best mathematical model to describe the growth kinetics of *Aspergillus oryzae* in the wasteful SLS. This model showed that there exist some kind of substrate inhibition towards the fungus growth and it was not due to the limited reducing sugar available in the SLS. This conclude that a further study on identifying the main nutrient available in the SLS that could contribute to the growth of *Aspergillus oryzae* is needed.

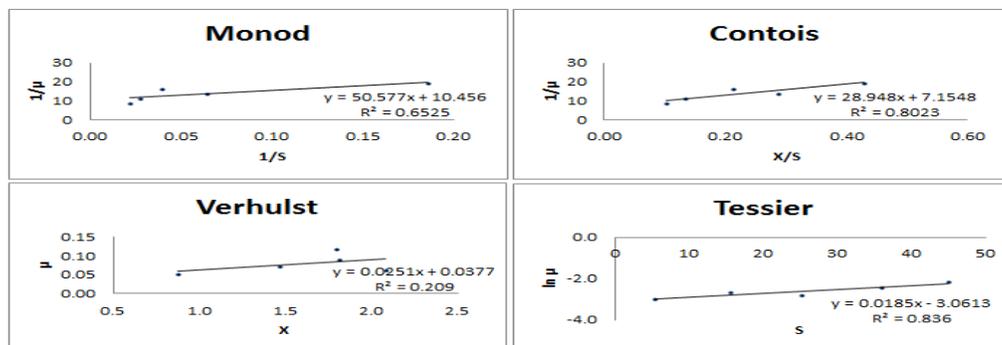


Figure-3. Graph of plotted experimental data for cell growth kinetic models.



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