

Microbial Growth Kinetics in Isobutanol Production by *Saccharomyces Cerevisiae*

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Renewable sources of fuels are important for environmental sustainability. Biofuels are produced from renewable feedstocks through biological processes and can be found in the form of liquid, gas and solid. Isobutanol, a branched chain butanol isomer, is a type of biofuel that can be produced through the fermentation of biomass using microorganisms. In an aerobic fermentation, *Saccharomyces cerevisiae* (yeast) produced certain amount of isobutanol as by-product during the degradation of valine through the Ehrlich pathway. In the fermentation process, the determination of growth kinetics gives a better explanation regarding the relationship between specific growth rate and the concentration of a substrate. Kinetic coefficients are useful in providing information regarding the progression of either the chemical or biological reaction occurring in a system. In this study, the microbial growth kinetics, glucose utilisation and isobutanol productivity were investigated. The growth kinetics coefficients were determined by the Monod's model while the Ledeking-Piret's model were used in investigating the parameters involved in substrate's consumption and product's formation. The maximum specific growth rate, μ_{max} and the substrate saturation constant, K_s were determined from the slope and interception point (a plot between $1/\mu$ and $1/S$) during 48 h of fermentation period. The value of μ_{max} and K_s obtained were 0.74 h^{-1} and 57 g/L . The isobutanol yield ($Y_{P/S}$), biomass yield ($Y_{X/S}$) and the correlation between isobutanol production and yeast growth ($Y_{P/X}$) were found to be 4.79 mg/g , 0.1 g/g and 49.4 mg/g . The Monod's and Leudeking-Piret's models were found to be suitable to represent the experimental data of growth kinetics and product's formation kinetics in this study.

1. Introduction

Production of fuels from renewable sources is growing nowadays. The urge towards this idea is due to several factors related to environmental concerns (greenhouse effect, climate change and global warming) (Sanchez-Ramirez et al., 2015), fossil fuel price and energy security (Raganati et al., 2012). In 2008, approximately 88 % of the total energy used is derived from non-renewable fuel (Zhao et al., 2014). This situation has resulted in the deployment of greenhouse gasses as fossil fuel produces methane, nitrous oxide and carbon dioxide when burning in turn led to an increase in environmental temperature (Kumar and Gayen, 2011). The increase in fuel consumption and the political upheaval in the oil producing areas have led to the declining fossil fuel reserves (Hazrat et al., 2015). Nowadays, the source for the fossil fuel is decreasing since this fuel is produced from limited resources; therefore, there are steps to initiate the production of fuel from renewable sources. The number of vehicles and the industry are growing rapidly due to increasing population; this situation has caused the price of crude oil to increase thus directly affecting the global economic development and growth (Hazrat et al., 2015). There are several countries that have established their own policies and regulations in biofuel production (Melamu and von Blotnitz, 2011). Brazil is one of the countries involved where half of the energy supplied is derived from renewable sources (Pereira et al., 2015). According to Nagy and Hegedus (2015), the US countries have been mandated that the energy supply from renewable sources in 2020 to be up to 20 % with 10 % of renewable energy involved in transport sector. The energy production from renewable sources in the US countries is expected to increase in the future (Accardi et al., 2015). Biofuels from renewable sources can be categorised in three types; which are liquid, solid and gas (Dahman, 2012). There are several types of fuels that can be classified as biofuels, namely bioalcohol (Hacisalioglu,

2009), biodiesel, Fischer-Tropsch diesel, methane and hydrogen (Demirbas, 2008). At present, biofuels have become one of the most important sources of sustainable fuels, which have gradually received increasing attention from both the public as well as the scientific communities. According to Gonela and Zhang (2014), biofuel is encouraged as one of the source of transportation fuel due to its benefits as a renewable and environmentally friendly source of energy. The studies on environmental impacts found that biofuels are non-polluting. These fuels can be easily accessed and are available in the local area. Added benefits of biofuels are sustainability and reliability, thus suitable to be widely used in many areas (Demirbas, 2009).

Isobutanol, one of the butanol isomers, has been recognised for its potentiality as a fuel additive or substitute due to its attractive physical properties. Having favourable fuel characteristics such as a higher energy density, lower solubility in water and less volatility, isobutanol has become a superior fuel extender compared to bioethanol (Lee et al., 2012). As higher energy density gives a huge benefit in which the fuel consumption can be reduced while better mileage is obtained (Jin et al., 2011). Isobutanol produces less corrosion due to being less miscible in water and is suitable to be distributed using the existing pipelines. On the other hand, ethanol need to be transported via rail, barge or truck as it is completely soluble in water. The less volatility characteristic has made isobutanol a safer fuel to be used during high temperature and lower level of volatile organic compound is generated during handling (Durre, 2007).

S. cerevisiae (yeast) is able to naturally produce a small amount of isobutanol during fermentation process (Brat et al., 2012). Isobutanol is produced as the by-product during degradation of amino acid through the Ehrlich pathway (Kondo et al., 2012). The use of natural producer in isobutanol production gives the opportunity to avoid the heterologous pathway which is the expressions of alcohol synthesis genes in the production host. This process is complicated and requires substantial effort while the yield produced is quite low (Chen et al., 2011). The use of yeast as a production host gives several benefits as it is known to be tolerant towards alcohol. *S. cerevisiae* is able to grow in butanol concentration higher than 20 g/L (Knoshaug and Zhang, 2009). This microorganism is relatively robust, thus highly resistant to harsh conditions (Chen et al., 2011). Compared to bacteria, yeast is generally much safer as it is suitable to be used in food and feed applications. Currently, the isobutanol production by *S. cerevisiae* has been extensively studied. The low isobutanol production has led the researchers to find the alternatives including the expression of the isobutanol synthesis genes (Park et al., 2014) and by eliminating the competing pathways (Ida et al., 2015) for improving the isobutanol yield.

Microbial growth kinetics, such as the relationship between the specific growth rate of a microbial population and the substrate concentration, is an indispensable tool in all fields of microbiology, be it physiology, genetics, ecology, or biotechnology, and therefore it is an important part of the basic teaching of microbiology.. The kinetics model is important in providing the information related to biomass growth and product generation (Ariyanti and Hadiyanto, 2013). The relation between the specific growth rate (μ) of a population of microorganisms and the substrate concentration (S) can be expressed by theoretical models. The Monod model (Eq(1)) illustrates the influence of substrate concentration on cell growth rate:

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

Where μ can be defined as the specific growth rate (h^{-1}), μ_{\max} is the maximum specific growth rate (h^{-1}), S as the substrate concentration (g/L) and K_s is the substrate saturation constant (g/L). In order to predict the value of μ_{\max} and K_s , the Monod's equation can be linearised by reciprocating both sides of the equation. Based on Eq(2), a graph for $1/\mu$ and $1/S$ was plotted:

$$\frac{1}{\mu} = \frac{K_s}{\mu_{\max}} \frac{1}{S} + \frac{1}{\mu_{\max}} \quad (2)$$

The value of μ_{\max} and K_s can be obtained by plotting the graph of $1/\mu$ versus $1/S$ forming a straight line where the slope of tangent is equal to $\frac{K_s}{\mu_{\max}}$ and the y-axis intercept is $\frac{1}{\mu_{\max}}$. The growth yield, $Y_{x/s}$ in fermentation is defined as follows:

$$Y_{x/s} = \frac{\Delta X}{\Delta S} = \frac{X - X_0}{S_0 - S} \quad (3)$$

X_0 and S_0 are the initial inoculum concentration and the substrate concentration while X and S are the inoculum concentration and the substrate concentration at a certain time. Other coefficients can be determined using the following Eqs(4) and (5):

$$Y_{p/s} = \frac{\Delta P}{\Delta S} = \frac{P - P_0}{S_0 - S} \quad (4)$$

$$Y_{p/x} = \frac{\Delta P}{\Delta X} = \frac{P - P_0}{X - X_0} \quad (5)$$

where $Y_{p/s}$ is the ratio of the products to the substrate, ΔP is the change in product formation, ΔS is the change in substrate concentration, $Y_{p/x}$ is the ratio of the products to the biomass and ΔX is the change of value in biomass concentration.

The kinetics of microbial growth occurred during isobutanol production by *S. cerevisiae* using glucose as carbon source through fermentation has not been studied yet. The objective of this study is to investigate the kinetics of microbial growth and product formation involved in the production of isobutanol by utilising the available kinetics model.

2. Materials and Methods

2.1 Preparation of inoculum

A full loop of the yeast *S. cerevisiae* (baker's yeast, Mauri-pan) from agar slant was inoculated into a 50 mL flask with YPD medium consisting of glucose 20 g/L, peptone 10 g/L and yeast extract 20 g/L. The medium was incubated in an incubator shaker at 30 °C, 170 rpm for 20 h before being used as an inoculum for isobutanol fermentation.

2.2 Microbial fermentation

Fermentation was performed in a 500 mL Erlenmeyer flask containing 250 mL of synthetic medium consist of 140 g/L glucose, 8 g/L yeast extract, 8 g/L peptone, 3 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/L 2-ketoisovalerate and 0.5 g/L valine. Before starting the fermentation, the medium and glucose (autoclaved separately) were sterilised at 121 °C for 15 min. The samples were incubated in an incubator shaker (Sastec) at 30 °C, 177 rpm agitation speed with 5 vol% microorganisms for 48 h. During the fermentation process, the samples were collected every 12 h and centrifuged at 10,000 rpm for 10 min. The supernatant was stored at 4 °C for biomass, glucose and isobutanol estimation. All the experiments were conducted in triplicates.

2.3 Analytical methods

A UV/visible spectrophotometer (Genesys 10S UV-Vis Spectrophotometer) was used to estimate the biomass concentration by optical density at an absorbance of 600 nm. The measured values were correlated and presented as dry weight cell concentration. The amount of glucose in the samples was also estimated by a UV/visible spectrophotometer at an absorbance of 540 nm using the dinitrosalicylic acid method (Miller, 1959). The concentration of isobutanol was determined by a gas chromatography equipped with a flame ionisation detector (GC-FID) and DB-WAX capillary column (50 m length, 0.32 mm inside diameter and 0.5 μm film thickness). The oven temperature ranging from 40 °C to 120 °C was raised at a rate of 15 °C/min and held at that particular temperature for 4 min. Then the temperature was raised up again with a gradient of 45 °C/min until 230 °C and held for 4 min. The injector temperature was 235 °C and detector temperature was 230 °C. Nitrogen was used as the carrier gas with 4.5 psi inlet pressure.

2.4 Growth and Product Kinetics

The kinetics of *S. cerevisiae* growth and glucose consumption was conducted using the optimum conditions for isobutanol production. The Monod's model was used to determine the growth kinetics while the product formation and glucose consumption were determined by the Leudeking-Piret's model. The constant and coefficient of kinetic parameters determined in this study were μ_{max} , K_s , $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$. The equations used to obtain the values of constants and coefficients are listed above.

3. Results and Discussion

The batch microbial growth kinetics in isobutanol production from glucose was studied in detail in order to determine the interaction between biomass growth and glucose consumption. The glucose concentration profiles along with biomass generation are shown in Figure 1. As can be seen, the exponential growth phase of yeast occurs after 6 h of fermentation in which the cells have already adjusted to the new environment (Ariyanti and Hadiyanto, 2013). Noticeably, the substrate component which was expressed in terms of glucose concentration was initially larger in the beginning of the fermentation which then started to reduce over time. The glucose concentration decreased drastically after 20 h of fermentation and produced 11.43 g/L biomass and 622 mg/L isobutanol titers (Figure 2). From the result, it was identified that *S. cerevisiae* was able to consume glucose rapidly. This ability has encouraged the use of *S. cerevisiae* in most industrial ethanol fermentation (Becker and Boles, 2003). According to Nor Azah et al. (2014), in the energy production process, the glucose consumption is crucial for the yeast's survival and the energy produced leads to the production of alcohol.

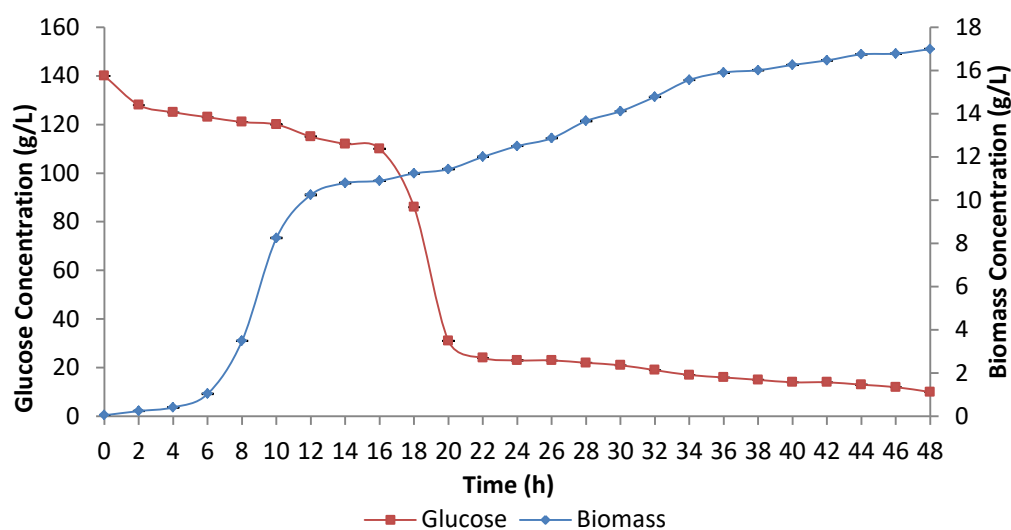


Figure 1: Interaction between biomass growth and glucose consumption during 48 h of fermentation for isobutanol production by *S. cerevisiae*

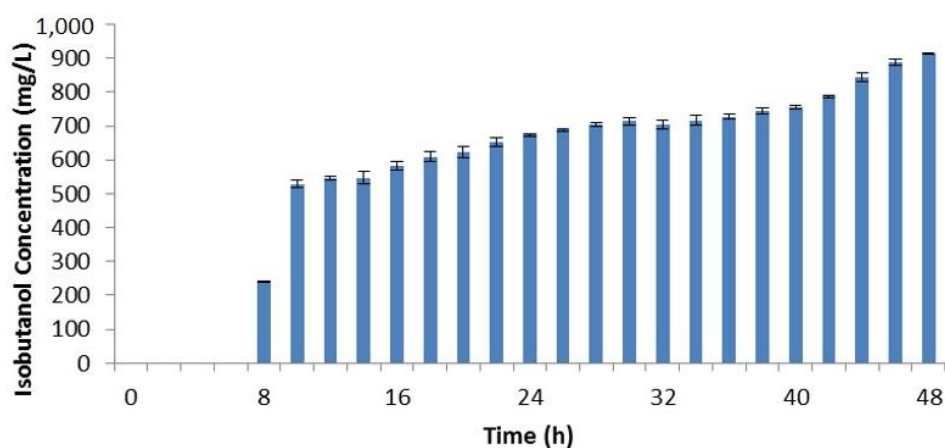


Figure 2: The production of isobutanol titers during 48 h of fermentation

Figure 2 shows the isobutanol concentration produced in 48 h of fermentation. *S. cerevisiae* starts producing isobutanol after 8 h of incubation. The isobutanol's yield increased proportionally with biomass growth and achieved the maximum isobutanol yield (914 mg/L) after 48 h of fermentation. The experimental data for biomass and glucose concentration during fermentation of isobutanol were used for the determination of kinetic parameters. Based on the Eq(2), the value of μ_{max} and K_s were obtained by plotting the graph of $1/\mu$ versus $1/S$ (Figure 3). The linear equation obtained was $y = 77.025x + 1.3443$ with the regression, $R^2 = 0.9369$. The estimation of μ_{max} and K_s were based on the y-intercept and slope of the regression line. The maximum specific growth rate (μ_{max}) and substrate saturation constant (K_s) were determined as 0.74 h^{-1} and 57 g/L .

The Leudeking-Piret model was used to predict several kinetic parameters. The biomass yield coefficient, $Y_{x/s}$ expressing the correlation of the amount of biomass produced and substrate consumed. The $Y_{x/s}$ obtained indicates that 0.1 g of *S. cerevisiae* produced per gram of glucose consumed. The $Y_{p/s}$ coefficient defines the relation between isobutanol production and glucose concentration consumed. The $Y_{p/s}$ was calculated based on Eq(4) and the result obtained shows that 4.79 mg isobutanol has been produced for one g of glucose consumed. In this study, the correlation between isobutanol production and biomass *S. cerevisiae*, $Y_{p/x}$ gave the value of 49.4 mg/g. This result indicates that one gram of biomass produced 49.4 mg isobutanol titers. The values for all kinetic parameters are shown in Table 1.

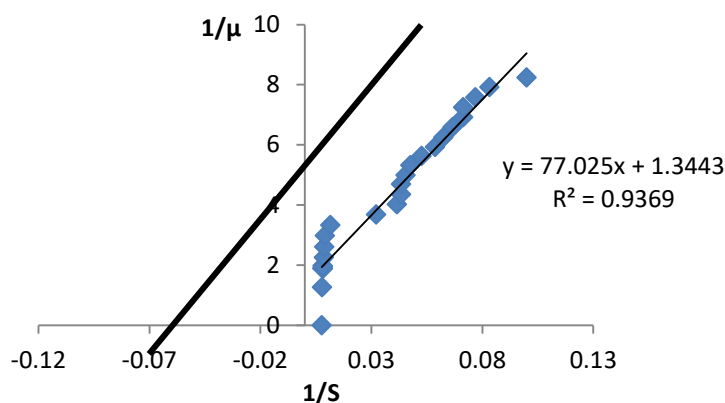


Figure 3: Determination of μ_{\max} and K_s for microbial growth during isobutanol fermentation

Table 1: Kinetic parameters during isobutanol production by *S. cerevisiae*

Parameter	Value
μ_{\max}	0.74 h ⁻¹
K_s	57 g/L
$Y_{X/S}$	0.1 g/g
$Y_{P/S}$	4.79 mg/g
$Y_{P/X}$	49.4 mg/g

4. Conclusion

Batch fermentation of *S. cerevisiae* in 500 mL Erlenmeyer flask produced 914 mg/L isobutanol and 16.9 g/L biomass after 48 h of fermentation. The kinetic parameters were successfully studied by fitting the experimental data with two models, Monod and Leudeking-Piret. It can be concluded that the relationship between biomass growth rate, glucose consumption and isobutanol production can be explained by the kinetic parameters obtained.

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