A Simplified Model of *Aspergillus niger* Growth using *Nephrolepis biserrata* leaves for Exo-polygalacturonase Production

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Abstract. A simple model to describe the growth of *Aspergillus niger* during solid state fermentation using *Nephrolepis biserrata* leaves is a substrate for the production of exo-poligalacturonase for 192 hours. The rate of kinetics growth of *Aspergillus niger* and the use of glucose as substrate using a Model Monod has shown that the maximum utilization of glucose 0.32 g/L of *Aspergillus niger* biomass of 0.496 mg/gdsf of glucose uptake 5.96 g/L to 0.32 g/L at 24 hours up to 120 hours of incubation. Through a linear regression equation diperolehinilai μ_{max} = 0.043 per hour and Ks = 0.473 g/L for 120 hours of incubation. The values of kinetic parameters and the amount of biomass has a significant relationship with R^2 equal to 0.801 and exo-polygalacturonase optimum activity at 54.64 U/g. Data gathered for the kinetic parameters during evaluation of the production of exo-polygalacturonase in SSF by *Aspergillus niger*, a value $Y_{X/S}$ is 0.149 mg/g by using glucose from the medium. The values for the kinetic coefficient of exo-polygalacturonase income is $Y_{E/S}$ = 16.43 U / g, $Y_{E/X}$ = 114.4 U/mg and kinetic constants were qE = 4.79 U/(mg.h) and qS = 0.289 g/(mg.h).

Keyword: Monod Model, *Nephrolepis biserrata* leaves, kinetics growth of *Aspergillus niger*, exo-polygalacturonase production.

INTRODUCTION

The role of microorganisms in the Income Pectinase

Microorganisms play an important role in the production of pectinase for large-scale industrial use. Pectinase can be produced by solid state fermentation (SSF) or submerged fermentation (SmF). A variety of environmental factors for the fermentation process affects the production of pectinase. Among these factors are the concentration of nutrients, pH, temperature, humidity and the effect of inducer. In addition, carbon and nitrogen sources indicate an overall effect on the productivity of pectinase (Catarina *et al*., 2003).

Microorganisms which are a major source of industrial enzymes comprise 50% of fungi and yeast, 35% of the bacteria, while the remaining 15% are either from animals or plants. Filamentous fungi described as microorganisms can secrete a variety of enzymes involved in the breakdown and recycling of complex biopolymers from both plant and animal tissues. The enzymes secreted these fungi are mostly hydrolytic and plays an important role in the growth of fungi, carbon and nitrogen. Potential fungal enzyme production and secretion of the attention of many researchers to study the production of various types of enzymes using filamentous fungi on large-scale industrial processes (El-Enshasy *et al*., 2008).

Among the most practical filamentous fungus in solid fermentation process is like genera *Mucor, Ascomycetes, Aspergillus niger, Fusarium* and *Penicillium decumbens oxyporum* (Mamma *et al*., 2007). For bacteria are used is *Bacillus* sp. (Akcan *et al*., 2012; Sukumaram *et al*., 2005; Kumar *et al*., 2012), *Streptomyce*s sp. (Basha *et al*., 2009). As for the yeast used is *Saccharomyces* and *Candida* (Kumar *et al*., 2015). Microorganisms filament is widely used in fermentation sink (SMF) and fermentation solid state (SSF) for the production of pectinase were *Aspergillus niger* (Akhter *et al*., 2011; Dartora *et al*., 2002; Okafor *et al*., 2010;

Mamma *et al*. , 2007). However, pectinase production by *Aspergillus strain* found to be more effective in solid-state fermentation process compared with submerged fermentation process (Thangaratham *et al*., 2014).

Aspergillus sp. is one of the main agents of decomposition and decay of lignocellulosic waste and has the ability to produce various types of enzymes. Glucosidase high level in *Aspergillus* sp. is important to complete the conversion of biomass into simple sugars lignosellulosa. In addition, *Aspergillus* sp. also known as bioremediation agents effective (Verma *et al*., 2011). In the food industry, the *Aspergillus niger* has been exploited mainly in the processing of food (bread and starch) and drinks (wine, juice, beer and distilling industry). In the industrial production of pectinase also between fungi *Aspergillus niger* is the most frequently used (Rashmi *et al*., 2008). The use of *Aspergillus niger* is generally recognized as safe (Generally Recognized as Safe) by the State Food and Drug Administration (FDA) US (Schuster *et al*., 2002).

Most of the kinetic model to describe the growth of microbes in bioprocessing is empirical and based on the Monod equation Monod or modifications which take account of microbial growth inhibition caused by the product or substrate concentration is high (Altiok, 2004). Specific growth rate achieved in the exponential phase is dependent on the concentration of nutrients. If all the nutrients needed for growth is present in excess, the specific growth rate will reach its maximum value (μmax). The empirical relationship between growth (μ) and the concentration of nutrients or substrate (S) can be expressed by Monod kinetics as shown in Equation 1.

$$\mu = \frac{\mu_{max} S}{Ks + S} \tag{1}$$

where μmax is the maximum growth rate achieved when S >> Ks and concentrations of all other essential nutrients is constant. Ks remained constant known as saturated or semi-constant velocity is equal to the concentration of the rate-limited when certain growth rate is equal to half of the maximum (Shuler and Kargi, 2002).

This equation has been used as a linear equation such as Equation 2.

$$\frac{1}{u} = \frac{1}{\mu_{max}} + \frac{Ks}{\mu_{max}} \frac{1}{S} \tag{2}$$

Plot 1/μ versus 1/S which is known as the Lineweaver-Burk plot, gives a straight line with a gradient Ks/μmax and Y axis intercept equal to 1/μmax (Okpokwasili and Nweke, 2005).

Next, the product is also defined as the weight of the product and substrate used in Leudeking Piret equation like Equation 3 and Equation 4.

$$Y_{E/S} = \frac{\Delta E}{\Delta S} = \frac{E - E_0}{S_0 - P} \tag{3}$$

which, $Y_{E/S}$ is the ratio of the enzyme to the substrate, ΔE is the change in the number of enzyme (E to E_0) and ΔS is the change of use of the substrate (S_0 to S) during incubation. Kinetics of enzyme-based enzyme activity (U/g), estimated the equation Leudeking and Piret (Aquilar, *et al*., 2001), is the coefficient estimated from a linear correlation between the concentration of enzyme activity and substrate utilization.

$$Y_{E/X} = \frac{\Delta E}{\Delta X} = \frac{E - E_0}{X_0 - X} \tag{4}$$

which, $Y_{E/S}$ is the ratio of the enzyme to the substrate, ΔE is the change in the number of enzyme (E to E_0) and ΔX is the change in the total biomass of *Aspergillus niger* (X_0 to X). $Y_{E/X}$ is the coefficient of correlation is estimated to linear line between the activities of exo-polygalacturonase and total biomass of *Aspergillus niger*. Specific enzyme production rate is qE has been defined as Equation 5.

$$q_E = \mu . Y_{E/X} \tag{5}$$

Next, a specific substrate utilization rate is qs was defined as Equation 6

$$q_S = \mu / Y_{X/S} \quad (6)$$

METHODS AND MATERIALS

Spores of *Aspergillus niger* ATCC120120 used in this study were collected from the stock culture collection of Bioprocess and Applied Biology Laboratory, Faculty of Chemical Engineering and Energy, Universiti Teknologi Malaysia. Cultured stocks for the long term is provided in the form of a bead of culture and frozen at -80 °C.

Solid State Fermentation

The *Nephrolepis biserrata* leaves of the substrate pretreated. Each of the leaves of the pretreatment weighed as much as 10 g/flask into a 500 ml Erlenmeyer flask and added together with basal medium ($(NH_4)SO_4$ 0.01% (w/v), $MgSO_4$. $7H_2O$ 0.05% (w/v), KH_2PO_4 0.05% (w/v), $FeSO_4.7H_2O$ 0.0005% (w/v), pectin of Nephrolepis biserrata leaves 1.0 % (w/v), yeast extract 0.1 % (w/v) and glucose 1.0 % (w/v). The moisture adjusted to 70% of humidity at pH 5.5 by the addition of medium. The substrate was sterilized using an autoclave at a temperature of 121°C for 20 minutes and stored at room temperature before it was inoculated. After cooling, the inoculation was carried out with 10% of inoculums and incubated at 28°C.

Enzyme Extraction

Fermented samples were collected every 24 h. In each sampling, 1 g of the sample was drawn out and mixed with 25 mL of citrate phosphate buffer (pH 4.0). The mixture was then placed on a vortex for 1 minute to ensure that enzyme or sugars on the surface were well-mixed with the buffer. Next, the suspension was centrifuged at 4000 rpm at 4°C for 20 minutes to separate the solid and liquid phase. The supernatant was used as a crude enzyme for various assays.

Analytical Procedures

1 g sample is removed and the fermentation is collected every 24 hours. For each sample, 1 g samples were removed then mixed with 10 mL of citrate phosphate buffer (pH 5.0). The mixture on a vortex for 1 minute to ensure all the enzyme or sugar on the surface of the media extracted into the buffer. Next, suspended material at a speed of 4,000 rpm, 4 ° C for 30 minutes to separate the solids (pellets) and liquid (supernatant or crude enzyme). The supernatant was used to research the content of exo- polygalacturonase enzymes and proteins. Determination of enzyme activity is based on the DNS method kit (Dinitro Salicylic Acid Reagent) with some modifications. This method seeks to determine the carbonyl group of reducing sugars (Miller, 1959). For exo-polygalacturonase activity can be identified by the color intensity by using a spectrophotometer at wavelengths of 540 nm (Kumar *et al*., 2010). One unit of enzyme exo-polygalacturonase is amount µM of reducing sugars (reducing sugar), which is measured in terms of glucose, produced as a result of the action of enzymes in the extract 1.0 ml of 1 min at 35° C ± 1° C (Ali *et al*., 2010).

Determination of Biomass and Growth Kinetic of *Aspergillus niger*

The determination of the biomass is carried out with *'Indirect Biomass Estimation'* based of Ramachandran *et al*., (2005). Fungal biomass estimates made to determine the N-acetyl-Glucosamine is produced by acid hydrolysis of chitin contained in the fungal cell wall. A total of 0.5 g of fermentation is mixed with concentrated sulfuric acid (2 ml) and put in a 500 ml conical flask and the mixture is kept for 24 hours at a temperature of ± 30° C. The mixture is diluted with distilled water to make a solution of 1 N and autoclaved (15 psi for 1 hour at 121° C) and then filtered through filter paper. This solution is then neutralized with 4 N NaOH up to 100 ml with distilled water. 1 ml of solution mixed with 1 ml reagent acetyl acetone and incubated in a boiling water bath for 20 minutes. After cooling, ethanol (6 ml) was added followed by the addition of 1 ml of Ehrlich reagent and incubated at 65° C for 10 minutes. After cooling, the optical density of the reaction mixture was read with a spectrophotometer at 530 nm. Results are expressed as mg glucosamine per gram of dried substrate fermentation

(mg/gdsf). The determination of the growth kinetics of *Aspergillus niger* and use of substrate (glucose) is carried out using variables and data that generate exo-polygalacturonase in optimum condition. Glucose is the total count of glucose from the medium (glucose, *Nephrolepis biserrata* leaves pretreated, *Nephrolepis biserrata* leaves pectin and nutrients) reducing sugar-based DNS method (Miller, 1959). The incubation time for this experiment is over 192 hours and every 24 hours samples were taken and analyzed. The rate of growth kinetics of *Aspergillus niger*, glucose utilization and *Aspergillus niger* biomass and exo-polygalacturonase activities . Determination of kinetic constants calculated using the growth model of Monod and the product is also defined as the weight of the product (enzyme) with the substrate is calculated by the method Leudeking-Piret. Constant and coefficient of kinetic parameters determined from this study are as μmax, Ks, $Y_{X/S}$, $Y_{E/S}$, $Y_{E/X}$, qS and qE.

RESULT AND DISCUSSION

Specific growth rate (μ) microorganisms are generally referred to the similarities that occur during the logarithmic growth phase. To determine the pattern of interaction between the use of glucose as the substrate with the analysis of the growth of *Aspergillus niger* glucose utilization and growth of microorganisms has been carried out during the production of the enzyme in this study. The *Aspergillus niger* growth pattern with an initial substrate concentration of 5.96 g/L under the optimum conditions of this study are shown in Figure 2

Figure 2 shows a time of adaptation to the environment metabolic *Aspergillus niger* found a very short time of incubation. This time is also the beginning of a phase of exponential (logarithmic phase) in which the initial glucose 5.96 g/L with the biomass 0.053 mg/gdsf reduced to 2.43 g/L with the biomass 0.18 mg/gdsf on 24 hour incubation time. After entering the 24 hour incubation time, the exponential phase very quickly in the use of glucose as an energy source for the growth of *Aspergillus niger* and up to 120 hours of incubation time, the use of glucose is at 0.32 g/L with the biomass at 0.50 m/gdsf.

In Figure 2, the utilization rate of glucose as substrate by *Aspergillus niger* showed glucose levels decreased significantly and indicates that the substrate has been used to the maximum. high or low concentrations of glucose residues affected by *Aspergillus niger* ability to convert the carbon source contained in the substrate into biomass and products (Sharma, 2012).

The *Aspergillus niger* optimal biomass production is dependent on the use of glucose that has been done in solid state fermentation. The concentration of glucose is used as substrate in these fermentations. Microorganism growth pattern is followed logistics model as shown in Figure 2, where the maximum utilization of glucose 0.32 g/L with the growth of *Aspergillus niger* biomass is up to 0496 mg/gdsf.

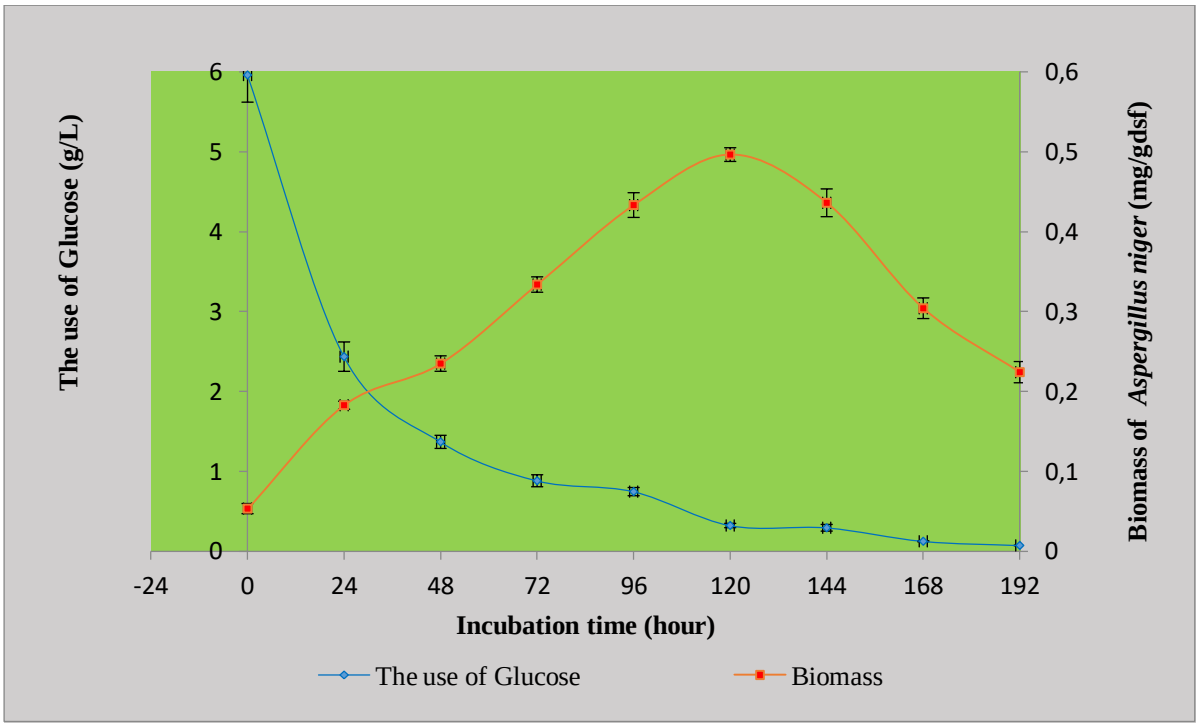

FIGURE 2. Relationship between *Aspergillus niger* biomass growth rate and glucose consumption in Solid State Fermentation on a 24 hour incubation time up to 192 hours

Umax and Ks value determination is made based on the curve 1/u versus 1/S as shown in Figure 3 from the uptake of glucose 5.96 g/L to 0.2 g/L at 24 hours and 120 hours of incubation. A linear regression equation obtained from the graph is equivalent to Y = 10.89 + 23.02x.

From this equation, the value obtained was 0.043 μmax per hour (maximum specific growth rate) and Ks was 0.473 g/L (saturation constant) from *Aspergillus niger*. The value of the growth is defined as the number of *Aspergillus niger* biomass produced per amount of substrate used, namely $Y_{X/S}$ is 0.149 mg/g. The activities of exo-polygalacturonase, μ per hour, the rate of *Aspergillus niger* biomass and the use of the substrate in the incubation time of 24 hours up to 192 hours. Figure 4 (a), (b) and (c) show the results obtained coefficients of linear equations or a straight line from the issue Leudeking Piret equation. The coefficient $Y_{X/S}$ is the coefficient estimated from the straight line correlation between the biomass of *Aspergillus niger* and the concentration of the substrate.

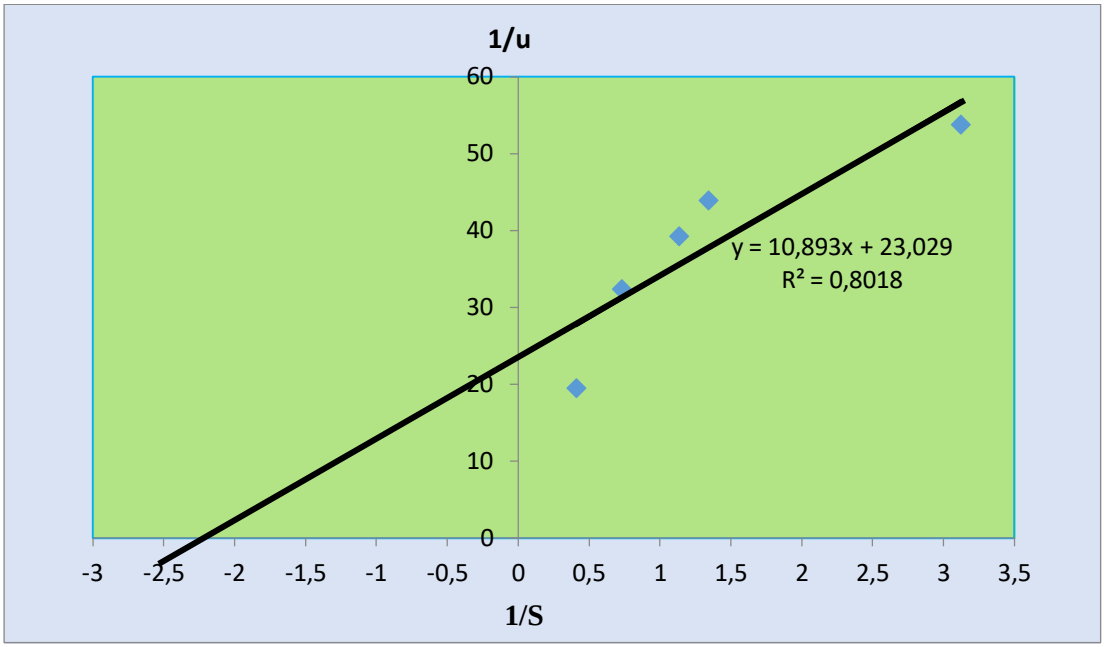

FIGURE 3. Curves linear μ_{max} and Ks for the growth of *Aspergillus niger* and substrate utilization

Coefficient $Y_{X/S}$ is defined as the total biomass of *Aspergillus niger* income of 0.149 milligrams per gram of substrate used. $Y_{E/S}$ of 16.43 U/L is the estimated coefficient of linear correlation between the activity and concentrations of exo-polygalacturonase glucose utilization. This shows that by 16.43 exo-activity unit of exo-polygalacturonase produced per gram of glucose consumed. In Figure 4 (c) shows a linear correlation between the activity of exo-polygalacturonase and total biomass of *Aspergillus niger* $Y_{E/X}$ of 111.4 U/mg. This value is defined as the total income of exo-polygalacturonase activity of 111.4 units per milligram amount of biomass produced by *Aspergillus niger*.

Leudeking-Piret model was also used to obtain the specific content of exo-polygalacturonase income, ie qE at 4.79 U/(mg.h) and the specific use of glucose as substrate, qS at 0.096 g/(mg.h). The values of kinetic parameters of exo-polygalacturonase shown in Table 1.

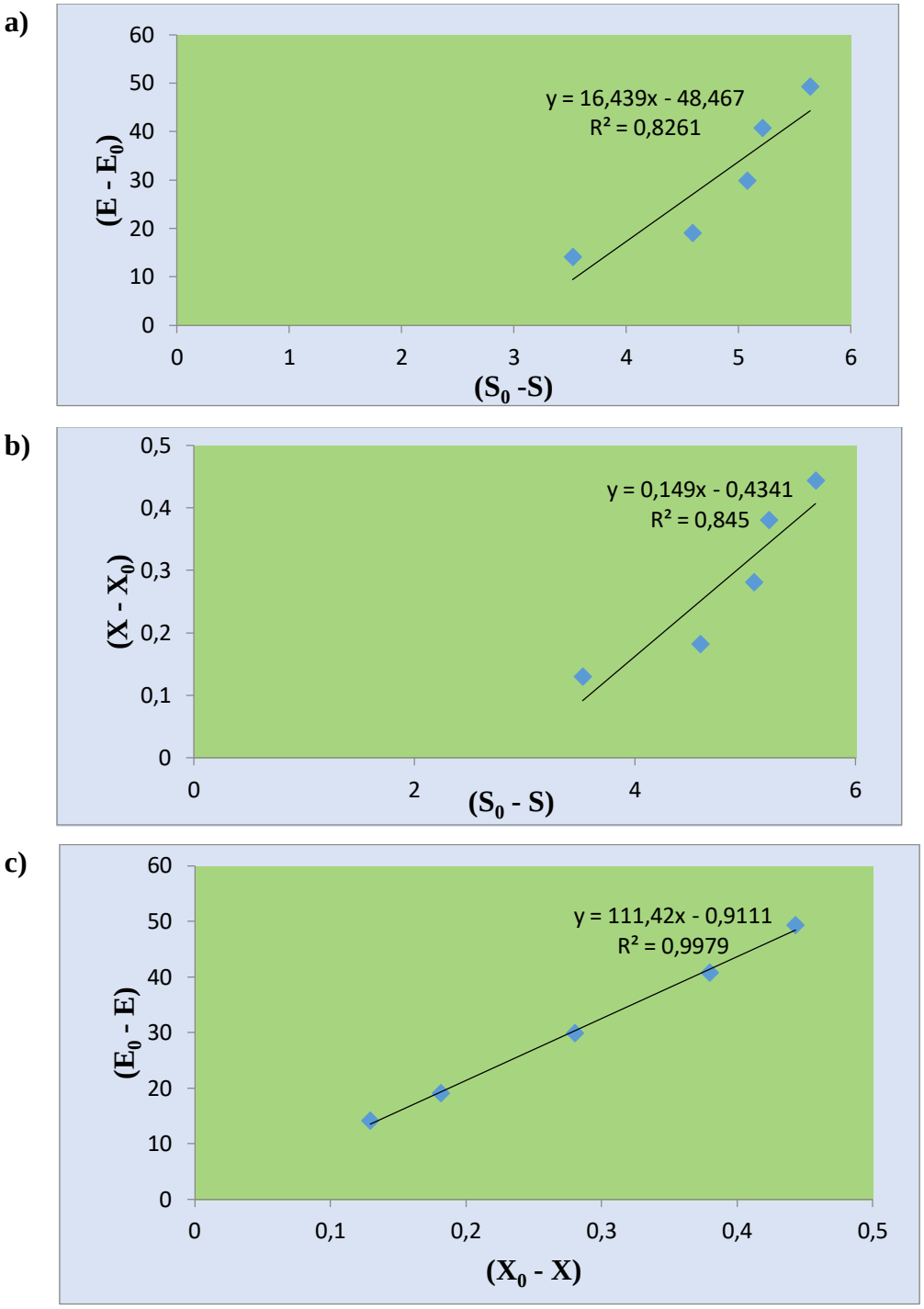

FIGURE 4. (a) Graph the relation ($E - E_0$) against ($S_0 - S$) coefficients $Y_{E/S}$., (b) Graph the relation ($X - X_0$) against ($S_0 - S$) determine the coefficient $Y_{X/S}$ and (c) relationship graph (($E - E_0$) against ($X_0 - X$) coefficients $Y_{E/X}$

TABLE 1. Value kinetic parameters of exo-polygalacturonase production by *Aspergillus niger.*

Parameters	Value of kinetics parameters
μ_{max}	0.043 per jam
Ks	0.473 g/L
$Y_{E/S}$	16.43 U/g
$Y_{X/S}$	0.149 mg/L
$Y_{E/X}$	111.4 U/mg
qE	4.79 U/(mg.*h*)
qS	0.289 g/(mg.*h*)

Monod model also clearly links the data findings (Rigon *et al*. 2009; Gerard *et a*l., 2006). The specific content of exo-polygalacturonase, ie qE at 4.79 U/(mg.h), showed that 4.79 units of exo-polygalacturonase activity per gram of biomass per hour *Aspergillus niger*. The specific rate of glucose as substrate, namely qS at 0.289 g/(mg.h), shows that the concentration of 0.289 grams of glucose as substrate used every milligram of *Aspergillus niger* biomass per hour.

CONCLUSION

Therefore, it was concluded that the relationship between the use of the substrate, the growth rate of *Aspergillus niger* and the enzyme exo-polygalacturonase can be deduced from kinetic parameters were obtained. When there is a good relationship between the amount of biomass and the production of enzymes, the kinetic parameters like μ, μmax, Ks and Yx/s showed a significant relationship as obtained in this research. The use of glucose as a substrate for the growth of *Aspergillus niger* with enzyme activity even in direct proportion, namely by increasing the amount of biomass has increased the enzyme activity**.**

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