Evaluation of Fermentation Conditions by *Candida tropicalis* for Xylitol Production from Sago Trunk Cortex

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Xylitol production from sago trunk cortex hydrolysate using *Candida tropicalis* was evaluated in shake flasks and a bioreactor. The fermentation and kinetic behaviours of this microorganism were investigated using sago trunk cortex hydrolysate and commercial xylose as substrate. Results obtained for sago trunk hydrolysate were close to the commercial xylose with xylitol yield of 0.82 gg^{-1} and productivity of 0.39 $gL^{-1}h^{-1}$. The maximum specific growth rate, μ_{max} for sago trunk cortex was higher (0.24 h^{-1}) compared to commercial xylose (0.17 h^{-1}). The bioreactor study showed an increase of about 6% (w/v) of xylitol concentration and 10% (v/v) of volumetric productivity when compared to the results obtained under the shake flasks, keeping xylitol yield above 0.8 g g^{-1}.

Keywords: Xylose; Xylitol; Sago trunk cortex; Candida tropicalis

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INTRODUCTION

Agricultural plant residues such as straw, bagasse, and trunk are disposed of after industrial, agricultural, and forestry activities. These materials represent an abundance of organic compounds and are composed of cellulose, hemicellulose, and lignin. These compounds can be a potential substrate if a process is available for their conversion into a value-added product (Foyle *et al*. 2007). Cellulosic material usually contains hexoses and hemicellulose that can easily be converted to glucose and xylose, respectively either by acid or enzymatic hydrolysis (Sun and Cheng 2002). Xylose can be used as carbon sources for the production of xylitol, a valuable alternative sweetener. Xylitol sweetness is similar to that of sucrose, but unlike sucrose it exhibits anticariogenic properties, which can promote oral health and prevent caries (Parajo *et al*. 1998). Owing to that advantage, xylitol has been used as a sweetener agent in a variety of food products, such as chewing gums, candies, and baked products (Winkelhausen *et al*. 2007). Bioconversion of xylitol is recognized as an alternative route to the chemical dehydrogenation process, which is claimed to be efficient and cost-effective (Nigam and Singh 1995; Parajo *et al*. 1998).

This study focused on the conversion of xylose obtained from hemicellulose of sago trunk cortex to xylitol. Malaysia is the highest sago producer in the world, exporting about 25,000 to 40,000 tonnes to Japan, Taiwan, Singapore, and other countries, and this value is increasing annually (Chew *et al*. 1999). Therefore, the production of sago wastes, such as sago trunk cortex (STC) will keep increasing proportionally along with the increase of sago starch production. Sago wastes, in the form of barks and husks, are

largely composed of cellulose and lignin, and therefore, both are wastes and pollutants. This study evaluated the production of xylitol in sago trunk hydrolysate on shake flasks culture, bench bioreactor, and the kinetic behaviour of the yeast *Candida tropicalis*.

MATERIALS AND METHODS

Pretreatment

Sago barks were purchased from local cultivated sago in Melaka, Malaysia. The cortex (the outer layer) was removed from the barks and the pith (the inner portion), which was then chopped to smaller pieces (25 x 150 mm). The particles used in the experiments varied in size from 5 mm to 10 mm.

Sago Trunk Cortex Hydrolysate and Detoxification

The sago trunk used in this study were pretreated with 8% sulphuric acid for 60 min of hydrolysis time, in an autoclave at 121°C at solid to liquid ratio of 1:10 in 250 mL flasks (Mohamad *et al*. 2011). The solid and liquid fraction was separated by using vacuum filtration and the remaining hydrolysate was neutralized using $CaCO_3$. The detoxification conditions were carried out with 2.5% concentration of activated charcoal at pH 5.5 and treated for 60 min at room temperature. The completely detoxified hydrolysates were recovered by using a vacuum filtration. All the experiments were conducted in duplicate.

Microorganism and Fermentations

The strain of *Candida tropicalis* was selected due to its ability to ferment xylose, and it has been shown to be a xylitol producer by researchers in the past (Soleimani *et al*. 2006; Winkelhausen and Kuzmanova 1998). Pure culture was donated by the Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, stored at 4 °C on Sabouraud dextrose agar and subcultured twice a month.

A single colony of *Candida tropicalis* was subcultured in a 500 mL Erlenmeyer flask containing 23 g L^{-1} D-xylose, 10 g L^{-1} yeast extract, 15 g L^{-1} KH_2PO_4, 3 g L^{-1} $(NH_4)_2HPO_4$, and 1 g L^{-1} $MgSO_4 \cdot 7H_2O$ (Walther *et al*. 2001). The flasks were incubated at 30 °C for 18 to 24 h to obtain an initial cell concentration with OD600nm of 0.4 to 0.6 and subsequently inoculated into the production medium at 10% (v/v). The batch cultivations were conducted in shake flasks and bioreactor. The shake flask fermentations were conducted in 500 mL Erlenmeyer flasks and contained 200 mL of sago trunk hydrolysate medium as carbon sources at 34 ºC on a rotary shaker at 250 rpm for 72 h at pH 4.

Fermentation in the bioreactor was carried out using a 3 L stirred tank bioreactor (Fermentec Resources Sdn. Bhd., Malaysia) containing 1.5 L fermentation medium of sago trunk hydrolysate with agitation, aeration, temperature, pH, and dissolved oxygen control. A single six-blade turbine impeller mounted on the agitator shaft above the air sparger was used for agitation at 250 rpm and air flow at 0.4 Ll/min The initial pH was adjusted to 4 and was not controlled during fermentation. The low pH value is the optimum condition for *Candida tropicalis* as an acidogenic type of yeast (Sampaio *et al*. 2006; Ke *et al*. 2009). The fermentation broth was sampled every 6 h to monitor the xylose and xylitol concentration.

Xylitol production was controlled by the fermentation conducted with pure xylose as a carbon source. The medium containing 23 g L^{-1} D-xylose, 10 g L^{-1} yeast extract, 15 g L^{-1} KH_2PO_4, 3 g L^{-1} $(NH_4)_2HPO_4$, and 1 g L^{-1} $MgSO_4 \cdot 7H_2O$. The experiments were conducted with the same conditions as the fermentation, with hydrolysate as the production medium. The experimental values were the means of two independent samples.

Analysis

Samples from the production medium were centrifuged at 10 000 x g for 10 min. The decanted supernatant was separated and used for sugar concentration analysis. Xylose, glucose, and xylitol were analysed using High Performance Liquid Chromatography (HPLC, Class VP (Shimadzu, Japan) with refractive index detector and the Inertsil NH_2 5 µm column (GLSciences, USA). The mobile phase used was 75% acetonitrile and 25% water with a flow rate of 0.5 mL/min and an oven temperature of 30 °C. The cell concentration was estimated by measuring the absorbance at 600 nm using the UV-VIS spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA). The dry cell weight was determined by drying in an oven at 95 °C until a constant weight was achieved.

Growth Kinetics for Fermentation Process

The rates of xylose consumption, xylitol production, and yeast population growth can be predicted from a kinetic model. During yeast growth in fermentation medium, new cells are formed catalytically from substrate with specific growth rate, µ (h^{-1}) and this can be expressed as,

$$r_x = \mu X \quad (1)$$

or

$$\frac{dX}{dt} = \mu X \quad (2)$$

where r_x = rate of cell growth, μ is the specific growth rate (h^{-1}), x is the cell concentration (g L^{-1}), and t is time.

Monod Equation

A relationship between the specific growth rate, μ, and the limiting substrate concentration, S, was proposed by Monod (1979). The Monod equation states that,

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (3)$$

where S is the concentration of the limiting substrate (g L^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), and K_s is the saturation constant (g L^{-1}).

Lineweaver-Burk Method

The cell growth saturation coefficient, K_s and μ_{max} can be estimated by rearranging the Monod equation in Lineweaver-Burk form, by taking reciprocals of both sides of the equality sign,

$$1/\mu = K_s/\mu_{max} S + 1/\mu_{max} \quad (4)$$

where the slope of a plot of $1/\mu$ versus $1/S$ will allow K_s to be evaluated, while the intercept is $1/\mu_{max}$

RESULTS AND DISCUSSION

Composition of Sago Trunk Cortex and Hydrolysate

Analysis of the sago trunk cortex composition was carried out in the previous study (Mohamad *et al*. 2011). The sago trunk was composed of 44.13% cellulose, 21.09% hemicellulose, 23.30% lignin, and 1.53% ash. Meanwhile, the sago trunk cortex hydrolysate was composed of 24.1 gL^{-1} xylose, 2.5 gL^{-1} glucose, 2.53 gL^{-1} furfural, and 2.15 gL^{-1} phenolic compounds. The detoxification treatment has minimal effect on sugar recovery, with only 4% of sugar loss of xylose and glucose (Mustapa Kamal *et al.* 2011).

Inoculum Preparation and Xylitol Fermentation in Shake Flasks and Bioreactor

The preparation of inoculums was done in the reference medium (pure xylose and nutrient) and sago trunk hydrolysate, without adding nutrient. The reason to use xylose derived from sago trunk hydrolysate without adding nutrient was to identify the potential of this xylose as a substrate for the growth of *Candida tropicalis*. In addition, the growth of *Candida tropicalis* and xylitol production from sago trunk hydrolysate was compared with the pure xylose medium. Notably, the use of pure xylose for *Candida tropicalis* growth in the reference medium is a limiting factor, since this substrate has a high cost compared to other carbon sources. Besides, its availability in the market is also limited (Silva *et al*. 2006). Hence, the use of sago trunk cortex hydrolysate for inoculum preparation can overcome this problem and could reduce fermentation costs.

The growth of *Candida tropicalis* in sago trunk cortex hydrolysate and reference medium was studied. The results showed that there were no significant differences between the two media (Figs. 1 and 2). The maximum xylitol concentration was obtained at 48 h for both media. However, the reference medium showed faster xylose consumption and earlier xylitol accumulation than the sago trunk cortex medium. This situation could be due to the presence of glucose in the sago trunk cortex hydrolysate, whereby the yeast consumed glucose rather than xylose. Xylitol started to accumulate once the glucose had been depleted at 20 h in the sago trunk hydrolysate medium. Canilha *et al*. (2008) found a similar situation with the cultivation of *Candida guilliermondii* in the wheat straw hydrolysate. Likewise, similar results were also found by Silva *et al*. (2006), whereby the researchers found the use of *Candida guilliermondii* was greater in the rice straw hemicellulose hydrolysate than in the reference medium with a maximum xylitol concentration of 51.5 gL^{-1} and 64% xylitol yield relative to the theoretical value. Moreover, recent studies of Wang *et al.* (2012) obtained a xylitol yield of 71% from corncob hydrolysate by using immobilized *Candida tropicalis.*

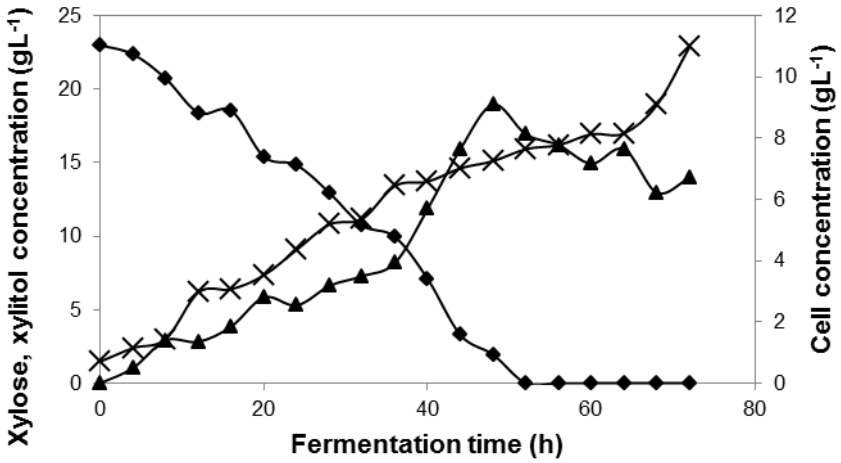

Fig. 1. Fermentation profiles of xylitol production on the shake flasks by *C. tropicalis*, in reference medium. Legends: (♦) xylose, (▲) xylitol, and (**x**) cells

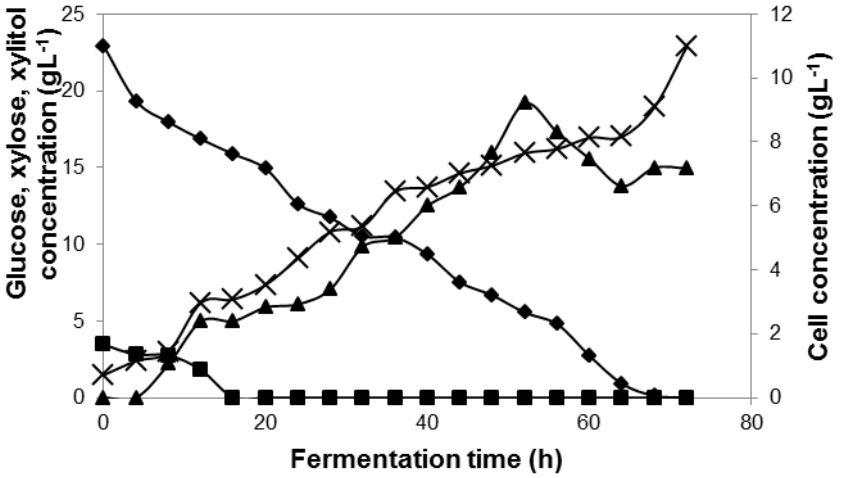

Fig. 2. Fermentation profiles of xylitol production on the shake flasks by *C. tropicalis*, in the shake flasks of sago trunk hydrolysate medium. Legends: (♦) xylose, (▲) xylitol, (**x**) cells, and (■) glucose

The inoculum grown in the previous sago trunk cortex hydrolysate was used in this experiment under the same conditions of temperature, pH, and agitation speed. The results obtained are shown in Fig. 3. Consumption of carbon source was faster with xylose depleting at about 54 h, and the maximum xylitol production was 20.938 g L^{-1}, achieved at the same time required for the shake flask, *i.e.* 48 h. Hence, the maximum xylitol concentration increased 6% by using a bioreactor. Applying the same conditions to *Candida guilliermondii*, Silva *et al*. (2006) attained the maximum xylitol concentration, which decreased by 14% in a bench bioreactor compared to aerated shake flasks.

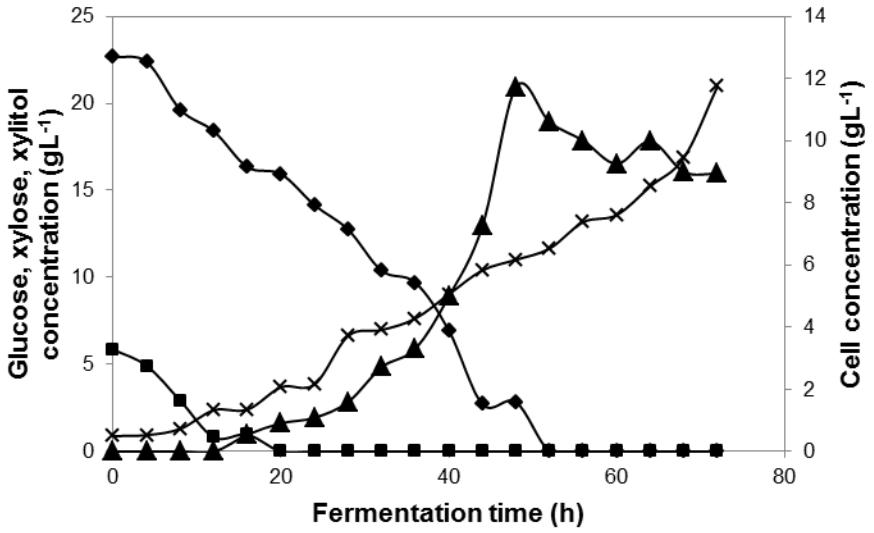

Fig. 3. Fermentation profiles of xylitol production by C. tropicalis, within 3-L stirred tank bioreactor. Legends: (♦) xylose, (▲) xylitol, (**x**) cells, and (■) glucose

The kinetic parameters obtained in the media are presented in Table 1. The results confirmed that the use of *Candida tropicalis* grown in the sago trunk hydrolysate (shake flasks and bioreactor) had no significant differences when compared to the reference medium. These results proved that the sago trunk hydrolysates implied an effective xylose source for the xylitol production.

Table 1. Kinetic Parameters of the Experiment Conducted under the Optimum Conditions with *Candida tropicalis* Grown in the Reference Medium and the Sago Trunk Hydrolysate

Parameters	Reference Medium	Sago Trunk Hydrolysate Medium	
		Shake Flasks	Bioreactor
Initial xylose concentration (g L^{-1})	23.00	22.90	22.68
P_m (g L^{-1})	19.77	18.95	20.93
Time to reach maximum xylitol concentration (h)	48	48	52
Q_P (g $L^{-1}h^{-1}$)	0.41	0.39	0.43
$Y_{P/S}$ (g g^{-1})	0.85	0.82	0.83
$Y_{X/S}$ (g g^{-1})	0.34	0.26	0.35
μ_{max} (h^{-1})	0.17	0.25	0.24
Ks	2.26	1.96	1.64

P_m, maximum xylitol production; $Y_{P/S}$, xylitol yield on the consumed xylose; $Y_{X/S}$, biomass yield on the consumed xylose Q_P, volumetric productivity; μ_{max}, specific growth rate, *Ks*, Monod cell growth saturation coefficient.

The results showed that there was an increase in the volumetric productivity when compared to the results obtained in the shake flasks. By performing the process using the bioreactor, the xylitol production of 20.938 g L^{-1} was attained at 48 h with a volumetric

productivity of 0.43 g $L^{-1}h^{-1}$ and xylitol yield of 0.83 g g^{-1}. These values were increased up to 10% from the reference medium in bioreactor.

Xylitol is produced when there is limited oxygen supply, and such conditions lead to an increase in xylitol productivity (Aguiar *et al.* 2002). In the bioreactor fermentations, the availability of oxygen was increased, leading to cell growth. The higher cell densities in the medium yielded limited oxygen availability in the vessel and this situation occurred were increased the xylitol accumulation as well as substrate consumption.

Growth Kinetics of *Candida tropicalis*

Growth kinetics of *Candida tropicalis* were determined by using pure xylose (reference medium) as the limiting factor. Specific growth rates of *Candida tropicalis* were determined according to Equation (1). The variation of specific growth rate, μ, with time is shown in Fig. 4 (A-C). From a very low value, it increases dramatically to a maximum value, μ_{max}. The same trend was observed with sago trunk hydrolysate in shake flasks and bioreactor.

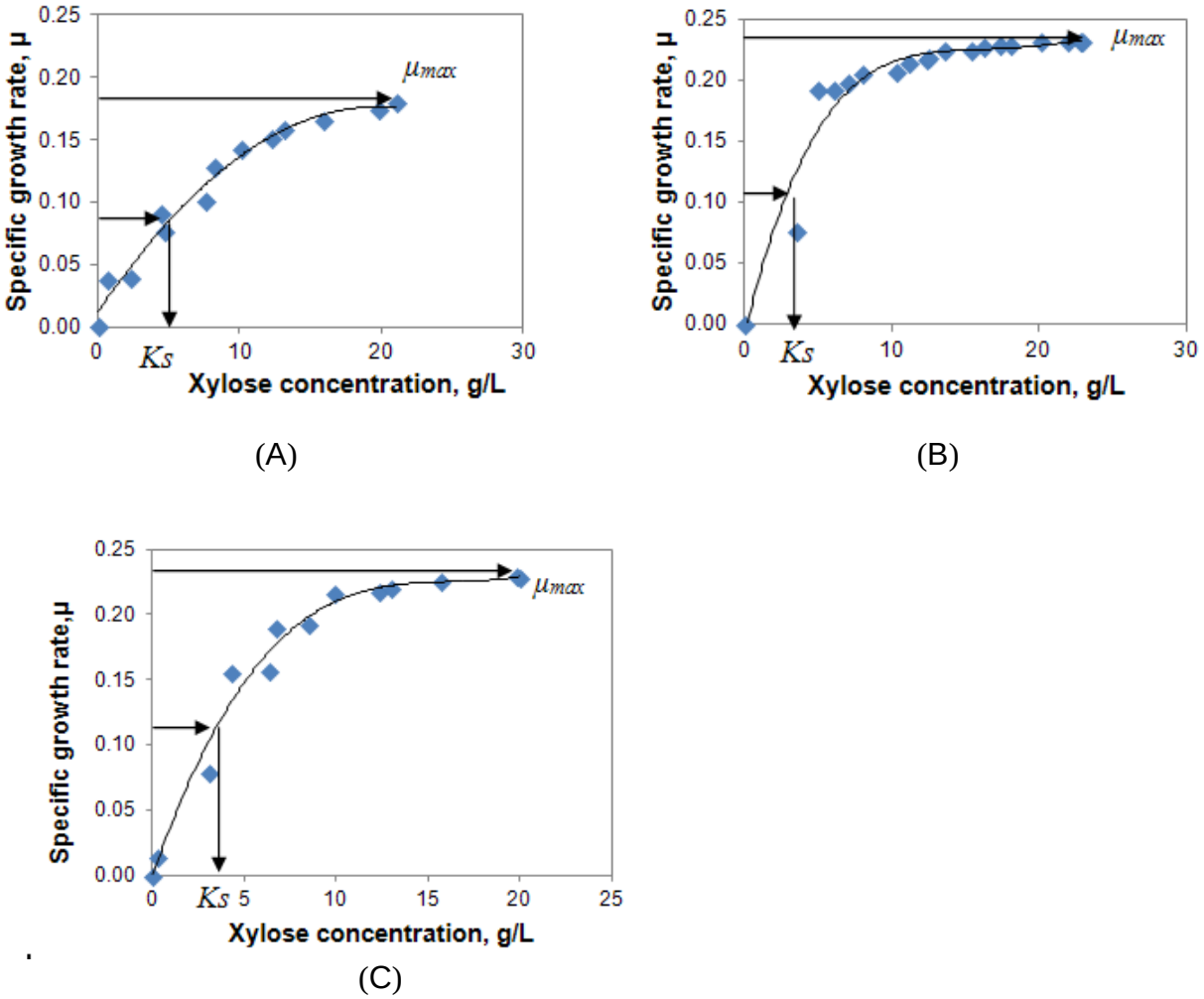

Fig. 4. Specific Growth Rate according to Monod equation (A) reference medium, (B) sago trunk hydrolysate in shake flasks, and (C) sago trunk hydrolysate in bioreactor

The saturation constant, K_s, and the specific growth rate, μ_{max} constant were determined using the Lineweaver-Burk method. This is shown in Fig. 5 (A-C). The values of the kinetic parameters μ_{max} and K_s characterize the organisms and growth substrate. If an organism has very high affinity for the limiting substrate (a low K_s value), then the growth rate will not be affected until the substrate concentration has declined to a very low level with a corresponding short decelerating phase for the culture. However, if the microorganism has a low affinity for the substrate (a high K_s value), then the growth rate will be deleteriously affected at a relatively high substrate level. Thus, the deceleration phase for the culture would be relatively long, and the yield of biomass will be reduced (Ahmad and Holland 1995). In this present study, K_s was found to be lower than the substrate concentration for reference medium (2.26 g L^{-1}).

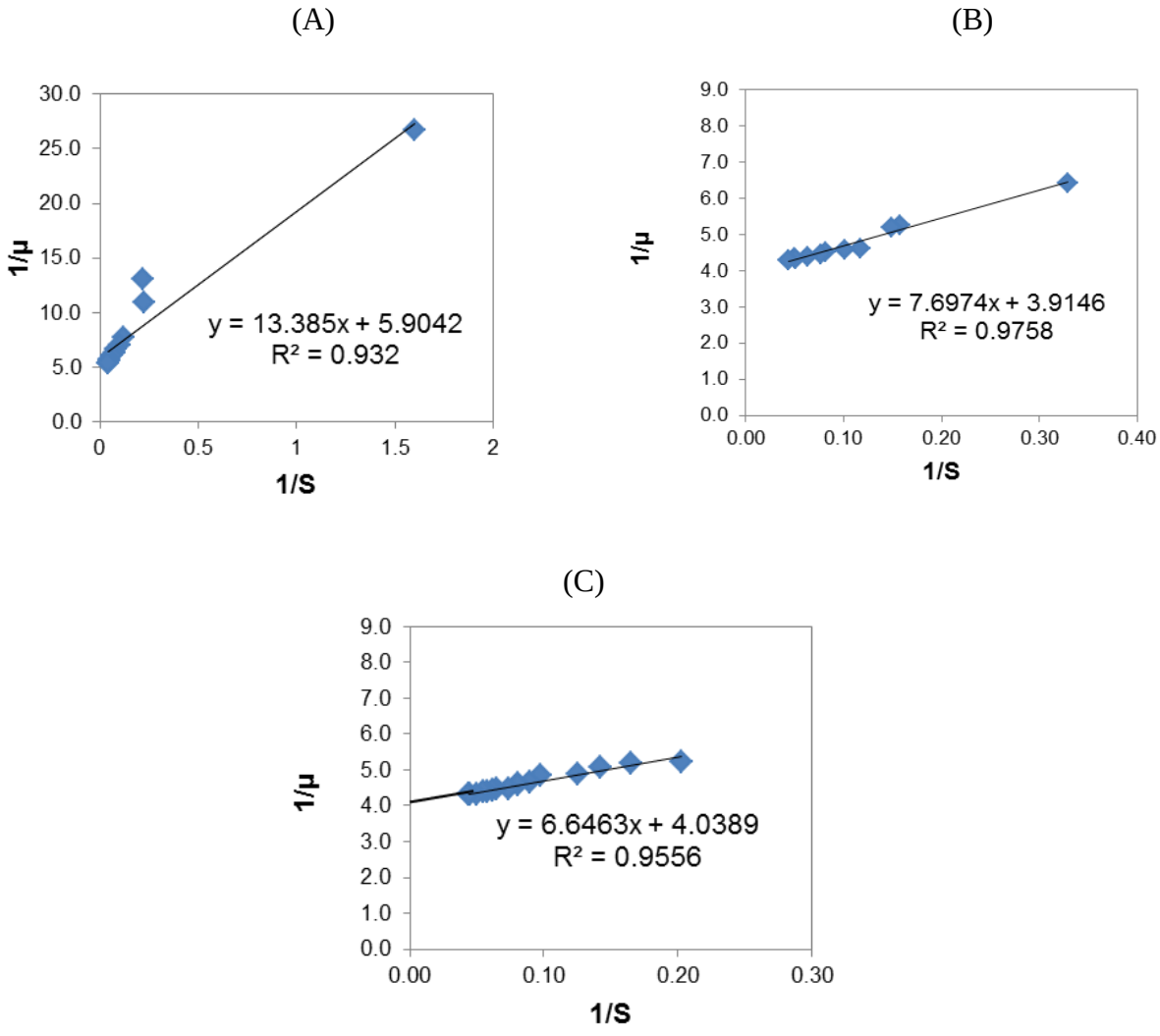

Fig. 5. Evaluation of growth constant by using the Lineweaver-Burk method (A) reference medium (B) sago trunk hydrolysate in shake flasks (C) sago trunk hydrolysate in bioreactor

The growth rate of *Candida tropicalis* was not affected until the decrease of xylose concentration. The growth kinetics study also indicated the decrease in saturation constant, K_s for growth in shake flasks (1.96) and bioreactor (1.64) for sago trunk

hydrolysate. This is due to the increase in the affinity that *Candida tropicalis* has for xylose obtained from sago trunk hydrolysate. The effects of μ_{max} and K_s value were identified for sago trunk hydrolysate medium in the shake flasks and bioreactor. The value of μ_{max} and K_s are presented in Table 1. The maximum specific growth rate, μ_{max}, for xylose obtained from sago trunk hydrolysate in shake flasks (0.25 h^{-1}) and bioreactor (0.24 h^{-1}) was slightly higher than specific growth rate in the reference medium. This situation could be due to the complex medium of sago trunk hydrolysate, which consists of other unknown substances that are consumed by the microorganisms. Aguiar *et al.* (2002) estimated a high value of maximum specific growth rate for *Candida guilliermondii* as high as 3.259 h^{-1} using D-xylose as the main substrate. This author explained that high estimated value of affinity constant counterbalanced the decrease in specific growth rate because of the low substrate concentration rather than high cell mass inhibition.

CONCLUSIONS

1. The production of xylitol from sago trunk hydrolysate was obtained in shake flask with xylitol yield (0.82 g g^{-1}) and productivity (0.39 g $L^{-1}h^{-1}$). A slightly higher value of xylitol yield (0.84 g g^{-1}) and productivity (0.44 g $L^{-1}h^{-1}$) were achieved in the bioreactor.
2. The sago trunk cortex hydrolysate appeared to be an alternative to the production of xylitol based on values of the selective fermentative parameters (YP/S = 0.85 g g^{-1}, QP = 0.39 g $L^{-1}h^{-1}$) that have been achieved without the necessity of adding nutrients to the media formulated by the detoxified hydrolysate.
3. Performance of the process in the bioreactor under optimum fermentation conditions allowed for an increase of about 6% in xylitol concentration and 10% in volumetric productivity when compared to the results obtained under the shake flasks, keeping xylitol yield above 0.8 g g^{-1}.
4. Specific growth rate was found to be higher when fermentation was done in the bioreactor compared to shake flasks. The controlling pH and temperature in the bioreactor may provide better aeration and mixing. This leads to better mass and energy transfer, resulting in faster and higher growth.

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