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KINETIC EVALUATION OF ETHANOL-TOLERANT THERMOPHILE *Geobacillus thermoglucosidasius* M10EXG FOR ETHANOL PRODUCTION

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ABSTRACT

Thermophiles are challenging to be studied for ethanol production using agricultural waste containing lignocellulosic materials rich in hexose and pentose. These bacteria have many advantages such as utilizing a wide range of substrates, including pentose (C5) and hexose (C6). In ethanol production, it is important to use ethanol tolerant strain capable in converting lignocellulosic hydrolysate. This study was aimed to investigate the growth profile of ethanol-tolerant thermophile *Geobacillus thermoglucosidasius* M10EXG using a defined growth medium consisted of single carbon glucose (TGTV), xylose (TXTV), and a mixture of glucose and xylose (TGXTV), together with the effect of yeast extract addition to the media. The experiments were conducted at the School of Biotechnology and Biomolecular Sciences of The University of New South Wales, Australia on a shake flask fermentation at 60°C in duplicate experiment. Cultures were sampled every two hours and analised for their kinetic parameters including the maximum specific growth rate (μ_{max}), biomass yield (Yx/s), ethanol and by-product yields (acetate and L-lactate) (Yp/s), and the doubling time (T_d). Results showed that this strain was capable of growing on minimal medium containing glucose or xylose as a single carbon source. This strain utilized glucose and xylose simultaneously (co-fermentation), although there was glucose repression of xylose at relatively low glucose concentration (0.5% w/v), particularly when yeast extract (0.2% w/v) was added to the medium. The highest biomass yield was obtained at 0.5 g l^{-1} on glucose medium; the yield increased when yeast extract was added (at 0.59 g l^{-1}). The highest specific growth rate of 0.25 was obtained in the phase I growth when the strain was grown on a mixture of glucose and xylose (0.5% : 0.5% w/v) medium. Diauxic growth was shown on the mixture of glucose, xylose, and yeast extract. The strain produced low level of ethanol (0.1 g l^{-1}), as well as low level (0.2 g l^{-1}) of by-products (L-lactate and acetate) after 15 hours. The results suggests its potential application for fermenting lignocellulosic agricultural wastes for ethanol production.

[***Keywords***: *Geobacillus thermoglucosidasius*, thermophile, kinetic parameters, ethanol]

INTRODUCTION

Bioethanol is a renewable alternative energy reported to be environmentally-friendly compared to other energy sources. Numerous reports and reviews have been published on the production of ethanol by fermentation, and various strains of mesophilic bacteria, yeasts, and fungi have been reported applicable for the production of ethanol (Dien *et al*. 2003; Desai *et al*. 2004; Demain *et al*. 2005; Chinn *et al*. 2006; Keating *et al*. 2006; Stephanopoulos 2007). The natural ability for thermophiles to utilize a wide range of sugars such as pentoses at high temperature (Larsen *et al*. 1997) renders them as potential microorganisms for ethanol production from cheap lignocelluloses, i.e. cellulose, hemicellulose, and lignin (Olsson and Hahn-Hagerdal 1996). The thermophilic bacteria also have advantages for ethanol production due to lower contamination risk, cost saving in industrial scale, and the wide range of sugars utilization. Many thermophiles producing ethanol have been investigated (Dien *et al*. 2003; Desai *et al*. 2004; Demain *et al*. 2005; Chinn *et al*. 2006; Keating *et al*. 2006; Stephanopoulos 2007).

Cellulosic livestock from agricultural residues is an attractive alternative feedstock that can be fermented to ethanol after appropriate pretreatment without impacting the food and feed supply (Zaldivar *et al*. 2001; Wyman 2003). In contrast to corn starch, biomass of agricultural wastes contains significant amount of pentose sugars that are recalcitrant to be fermented by industrial ethanol producer such as *Saccharomyces cerevisiae*. The natural ability to use a wide range of sugars such as pentose and hexose for carbon sources of ethanologenic thermophiles, therefore would be beneficial for converting lignocellulosic hydrolysate to ethanol. Ethanol production using agricultural waste is potential to be implemented in Indonesia due to its abundant availability.

Kinetic study of newly isolated strain is important for further investigation. This includes capability for using carbon sources, bioprocess engineering for higher products, and for the subject of genetic manipulation. However, publications on the growth

profile of ethanologenic thermophiles are scarce and genetic manipulation on this organism is limited. Reports were mainly emphasized on isolation, biomass production, and thermostable enzyme production (Degryse *et al*. 1978; Sonnleitner *et al*. 1982; Hyun *et al*. 1983; Wiegel *et al*. 1985; Shcherbakova and Serdyuk 2000; Dermitas *et al*. 2003; Chinn *et al*. 2006).

The recently characterized thermophile such as *Geobacillus thermoglucosidasius* M10EXG, isolated from waste compost, is considered as a potential strain for ethanol production, because this strain is tolerant to 10% (v/v) ethanol (Fong *et al*. 2006). Study on the capability of the strain to utilise both glucose and xylose is essential as lignocelluloses comprise of hexose and pentose. The objectives of this study were to evaluate the growth profile and the capability of *G. thermoglucosidasius* M10EXG strain of using a single or mixture of carbon sources such as glucose (hexose) and xylose (pentose).

MATERIALS AND METHODS

The study was conducted at the School of Biotechnology and Biomolecular Sciences of The University of New South Wales (UNSW), Australia in 2006. The experiments were carried out in a shake flask fermentation in duplicate.

Source of Isolate, Growth Condition, and Strain Maintenance

G. thermoglucosidasius M10EXG strain was isolated from the University Composting Unit. The strain was cultured on Luria Bertani (LB) agar medium at 60°C and kept at room temperature for short-term storage, and was suspended in 60% sterile glycerol and store at -70°C for a long-term storage. The strain was also deposited at the UNSW Culture Collection Unit as a freeze dried collection.

Carbon Sources

Glucose and xylose were used as carbon sources. However, yeast extract was also investigated for growth comparison study.

Defined media used were Thermus Minimal Media (TMM) with glucose as a single carbon source (TGTV), TMM with xylose as a single carbon source (TXTV), and TMM supplemented with glucose and xylose as carbon sources (TGXTV). TMM formula was based on Fong (2004) consisted of 920 ml Ten Salt Solution (TSS), 10 ml $FeSO_4.7H_2O$ (1mM dissolved in 0.4 Tricine), 10 ml K_2HPO_4 (0.132 M), 10 ml NH_4Cl (0.953 M), and 40 ml MOPS (1M, pH 8.2), and 10 ml RO water in 1 litre solution. Vitamin and trace element contents were as previously reported (Eguchi *et al*. 1996).

TGTV was made up of TMM enriched with filtered sterile 1% (w/v) glucose and 1 x vitamin and trace element solutions (1 ml vitamin or trace element per 1000 ml media). TXTV was prepared based on TMM supplemented with filtered sterile 1% (w/v) xylose, 1x vitamin and trace element solutions (1 ml vitamin or trace element per 1000 ml media). TGXTV consisted of TMM supplemented with filtered sterile 1% (w/v) of xylose and glucose, and 1x vitamin and trace element solutions (1 ml vitamin or trace element per 1000 ml media).

Culture Condition for Growth Profile Evaluation

Growth profiles of *G. thermoglucosidasius* M10EXG strain in utilizing different sugar sources were carried out in a batch culture experiment using TGTV, TXTV, and TGXTV media as previously described. The effects of yeast extract added to the media on the growth of the strain were conducted at 60°C with 200 rpm orbital shaking for 2 days. Inocula for shaking flask studies were prepared in 20 ml TGTV, TXTV or TGXTV, inoculated with a single colony from LB agar plate culture (Luria and Delbruck 1943), and grown overnight at 60°C, 200 rpm. Fermentations were carried out in 500 ml shaking flasks containing 250 ml of culture in duplicate at 60°C with 10% (v/v) inocula, 200 rpm. Bacterial growth was monitored by measuring the optical density at a wave length of 660 (OD_{660}) using an Ultrospec 2000 UV/visible spectrometer (Pharmacia, USA) of the liquid cultures at 660 nm. A correlation factor (0.94) for M10EXG (Fong 2004) was determined from calibration curve and was used to convert the absorbance value into the biomass concentration. All the experiment data were calculated from the duplicate experiments.

End Product Fermentation Analyses

Cultures were grown in 500 ml shake flasks containing 250 ml of culture medium at 60°C with 200 rpm orbital shaking under aerobic conditions. The fermentation products of the strain were determined and measured based on the end products, i.e. ethanol, L-lactate and

acetate analysed from 1 ml of samples taken from the culture. The samples were taken every 2 hours at duplicate. Samples were filtered through a 0.45 µm Minisart® filter (Sartorius, Germany) prior to High Performance Liquid Chromatography (HPLC) analysis. Analyses of fermentation end products were carried out using a HPLC with a CTO-10ASVP column oven (Shimadzu, Japan), fitted with an HPX-87H ion exchange column (Bio-Rad Laboratories, USA) operated at 60°C, and a SIL-1ADVP auto injector (Shimadzu Corporation, Japan). Injection volume was set at 5 µl. The Waters 510 HPLC pump and Waters 410 reflective index detector used were supplied by Millipore. Prior to injection, culture samples collected regularly during the fermentations were filtered with Minisart RC4 single-use non-sterile 0.45 µm filters (Sartorius, Germany). De-gassed 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 ml $minute^{-1}$.

Kinetic Growth Analyses

The kinetic growth of the strain such as the maximum specific growth rate (μ_{max}), doubling time (T_d), biomass yield ($Y_{x/s}$) and by-product (acetate and L-lactate) yields (Yp/s) were assessed based on the formulas of Stanbury and Whitaker (1984). The maximum specific growth rate (μ_{max}) was determined during the exponential growth (between interval of t_1 and t_2) using the following formula:

$$\mu_{max} = \frac{\ln x_2 - \ln x_1}{t_2 - t_1}$$

Where: x_1 and x_2 = concentration of biomass at t_1 and t_2

t_1 and t_2 = time 1 and time 2 during exponential growth

Doubling time (T_d) was determined during exponential growth using the following formula:

$$T_d = \frac{\log(2)}{\log(1 + \frac{r}{100})}$$, where r is growth rate constant

Biomass yield ($Y_{x/s}$) determined as amount of biomass produced/amount of substrate uptake was measured using:

$$= - \frac{(\frac{dx}{dt})}{(\frac{ds}{dt})} = \frac{x_f - x_i}{s_i - s_f}$$

where x and s are biomass and substrate and i and f are initial and final, respectively.

Ethanol, acetate, and L-lactate yield ($Y_{p/s}$) were measured using the following formula:

$$= \frac{p_f - p_i}{s_i - s_f}$$

where p and s are product and substrate, and i and f are initial and final, respectively.

Data Analyses

The data obtained from the experiments were analyzed using an Excel program.

RESULTS AND DISCUSSION

Growth of *G. thermoglucosidasius* M10EXG on a Single Carbon Source Medium

Growth of *G. thermoglucosidasius* M10EXG on glucose TGTV medium

Cellulose hydrolysis produces glucose which could be utilized by most commonly used microbial fermentation for ethanol production. The kinetics of *G. thermoglucosidasius* M10EXG and the capability of using glucose as a single carbon source were investigated using defined TMM supplemented with glucose (TGTV). Glucose is a hexose sugar which is commonly found in the degradation of cellulosic or lignocellulosic materials.

In the 1% (w/v) glucose medium (TGTV), this strain could grow and produce 1.4 g l^{-1} biomass in 24 hours with a maximum specific growth rate (μ_{max}) of 0.19 h^{-1}. Half the amount of glucose was remained in the medium at the end of fermentation with a biomass yield of 0.34 g g^{-1} based on glucose consumed. Acetate was maintained during growth at about 0.2 g l^{-1}, while L-lactate was detected at the beginning of growth at about 0.2 g l^{-1} and then declined during log phase. Ethanol was produced in the log phase at about 0.2 g l^{-1} (Fig. 1).

Low concentration of sugar could be utilized by this strain compared to recombinant ethanol producer mesophile, *Zymomonas mobilis*, which could use sugar up to 25 g l^{-1} (Joachimstal and Roger 2000; Joachimstal 2001; Jeon 2004). Low biomass production is one of the characteristics of thermophilic bacteria compared to mesophiles. This character is an advantage for enzyme production. At the other hand, as relatively slow growth bacteria compared to mesophiles, low biomass production would be

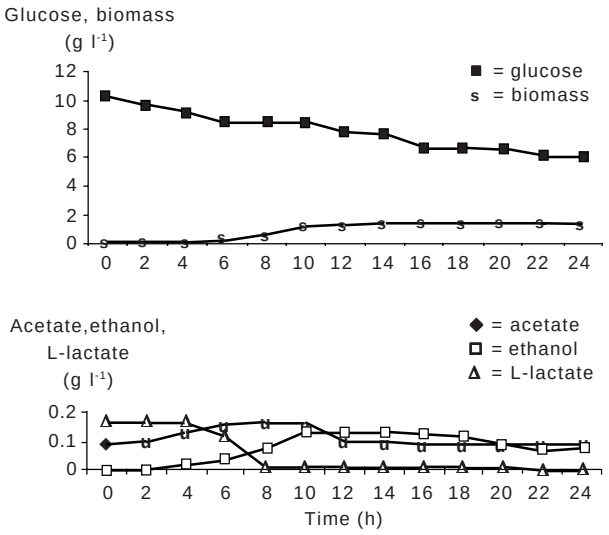

Fig. 1. Growth of *G. thermoglucosidasius* M10EXG on TGTV (1% glucose) medium.

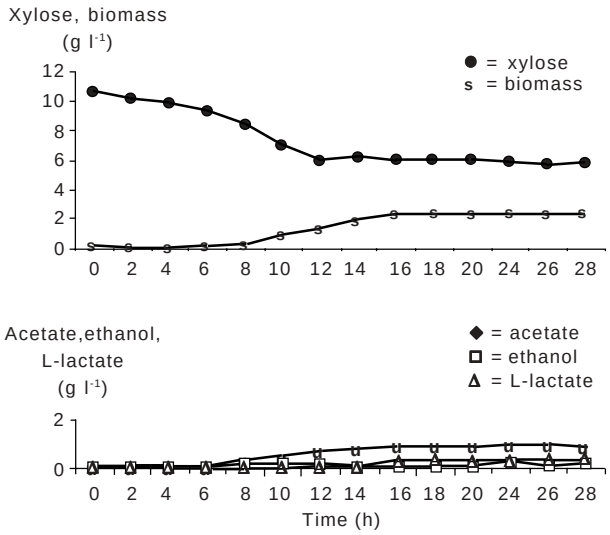

Fig. 2. Growth of *G. thermoglucosidasius* M10EXG on TXTV (1% (w/v) xylose) medium.

disadvantage to obtain certain amount of biomass for transformation purpose for genetic manipulation. Therefore, those data are very useful for further investigation to optimize the biomass production for transformation purpose.

Growth of *G. thermoglucosidasius* M10EXG on TXTV medium

Xylose fermentation is a more significant issue for agricultural residues and hardwood than for softwood as a result of hemicellulose hydrolysis. Xylose is not metabolized by wild-type *S. cerevisiae*, apart from a minor reduction to xylitol. Xylose 1% (w/v) as a single carbon source can be utilized by *G. thermoglucosidasius* M10EXG strain (Fig. 2), which seemed to grow better in this medium compared to the growth on glucose medium (TGTV). About half of the xylose was utilized by the end of the growth, and the higher final biomass reached was 2.4 g l^{-1}. The maximum specific growth rate was 0.2 h^{-1} and biomass yield was about 0.5 g g^{-1} based on xylose utilized. Acetate was produced while the biomass concentration increased to about 1 g l^{-1}, and low concentrations of L-lactate and ethanol (below 0.5 g l^{-1}) were detected also during this time.

Xylose is a pentose sugar which is normally found in the hemicellulose in lignocellulose hydrolysate mixture. The capability of the strain of using different carbon sources is preferable for ethanol production from cheap materials, lignocellulose from agricultural waste. The commercially used for ethanol production, wild type of yeast does not posses genes to ferment xylose sugar.

Growth of *G. thermoglucosidasius* M10EXG on TGXTV medium

Figure 3 shows the growth of M10EXG on TMM containing 0.5% (w/v) glucose and 0.5% (w/v) xylose (TGXTV medium). This strain utilized both of sugars simultaneously, but used more glucose than xylose.

Two growth phases (diauxic growth) were observed with a higher maximum specific growth rate for first phase. Diauxic growth phases often occur as the strain metabolizes a mixture of sugars. During the first phase, cells preferentially metabolize the sugar whose catabolism is most efficient (often glucose). Only after the first sugar has been exhausted, the cells switch to the second sugar. At the time of the "diauxic shift", there is often a lag period during which the cells produce the enzymes needed to metabolize the second sugar. Diauxic growth occurs because organisms use multiple sets of genes to metabolize the different nutrients they encounter. If an organism allocates energy to metabolize nutrients that are processed inefficiently or that are not highly abundant, it may be put at a reproductive disadvantage.

At the end of growth, about 1.5 g l^{-1} glucose and 3 g l^{-1} xylose remained in the medium. Biomass was produced to a concentration of 1.4 g l^{-1} and a maximum specific growth rate was 0.22 h^{-1}. A biomass yield of

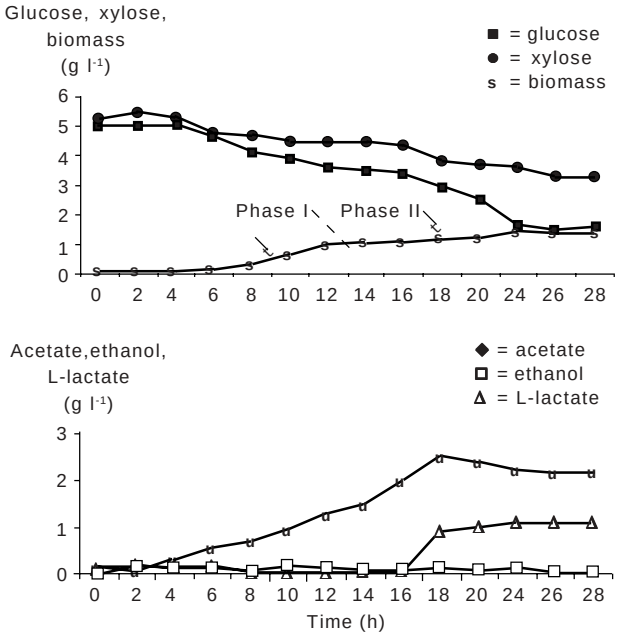

Fig. 3. Growth of *G. thermoglucosidasius* M10EXG on TGXTV (0.5% (w/v) glucose and 0.5% (w/v) xylose) medium.

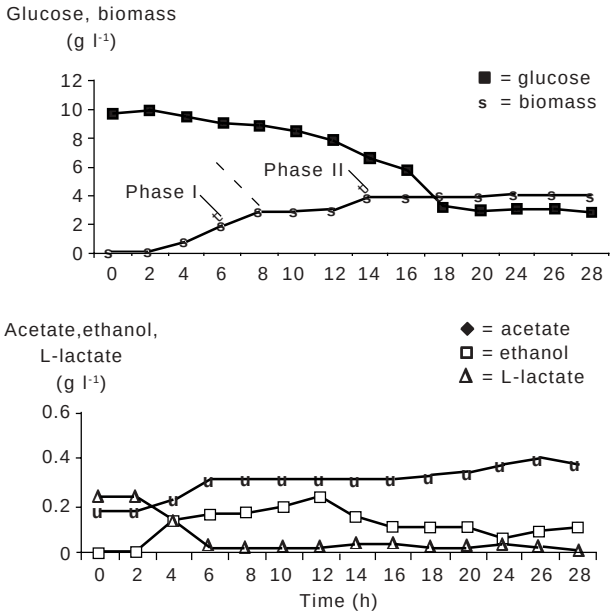

Fig. 4. Growth of *G. thermoglucosidasius* M10EXG with addition of 0.2% (w/v) yeast extract on TGTV medium (1% (w/v) glucose).

about 0.27 g g^{-1} was achieved (based on total sugars utilized), which was lower than when grown on glucose only (0.34 g g^{-1}) or xylose only (0.50 g g^{-1}). Acetate was produced in association with growth to a biomass concentration of 2.5 g l^{-1}, and L-lactate was produced at the end of growth at 1 g l^{-1}. Only very low levels of ethanol were detected.

Table 3 shows that *G. thermoglucosidasius* M10EX strain is capable of co-fermenting glucose and xylose. The character of co-fermentation (using two or more sugars simultaneously) is an advantage trait for fermentation using materials from lignocellulosic hydrolysate. As known that biomass hydrolysate consisted of the mixture of pentose and hexose sugars.

Growth of *G. thermoglucosidasius* M10EXG with Addition of 0.2% (w/v) Yeast Extract

Addition of yeast extract to glucose medium

Addition of 0.2% (w/v) yeast extract to the 1% (w/v) glucose medium resulted better growth of the strain (Fig. 4). More glucose was utilized even though it was not used up completely. Two phase growths were again observed. In the first phase, the maximum specific growth rate was higher compared to that in the second phase, viz. estimates of 0.5 and 0.16 h^{-1}, respectively. In the first phase, readily assimilable components in the yeast extract were used. As shown in Figure 4, the glucose concentration decreased relatively slowly in the first phase. It is probable that during the second phase this strain utilized glucose rapidly only when the main carbon source nutrients from the yeast extract had been depleted, as it was shown that glucose decreased faster in the second phase. The final biomass concentration obtained was 4.1 g l^{-1} compared to 1.4 g l^{-1} when grown without yeast extract. The biomass yield obtained was 0.59 g g^{-1} based on sugar utilized, although the yield has been increased by the presence of yeast extract. Additional acetate was produced during the process to a final concentration of 0.3 g l^{-1}, and L-lactate was detected in the beginning of process about 0.2 g l^{-1}. Ethanol was detected to about 0.4 g l^{-1} after 10 hours although its concentration finally decreased.

Addition of yeast extract to xylose medium

The results of the addition of 0.2% (w/v) yeast extract for growth on 1% (w/v) xylose medium are shown in Figure 5. More xylose was utilized with the final biomass of about 2.3 g l^{-1}. In the first 6 hours, xylose

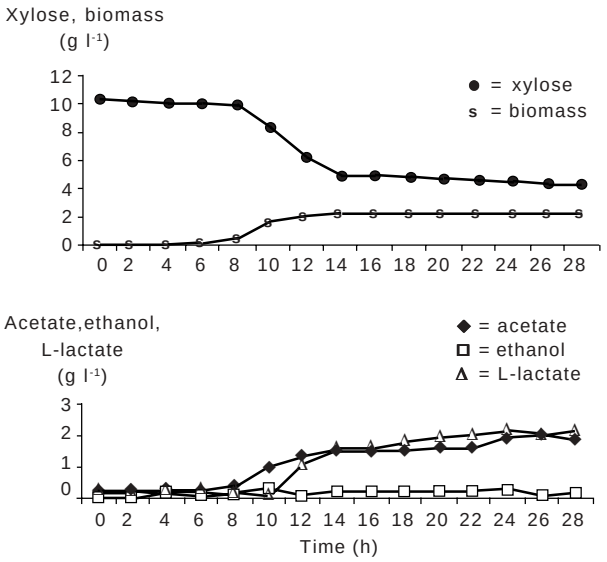

Fig. 5. Growth of *G. thermoglucosidasius* M10EXG on TXTV medium (1% (w/v) glucose) with addition of 0.2% (w/v) yeast extract.

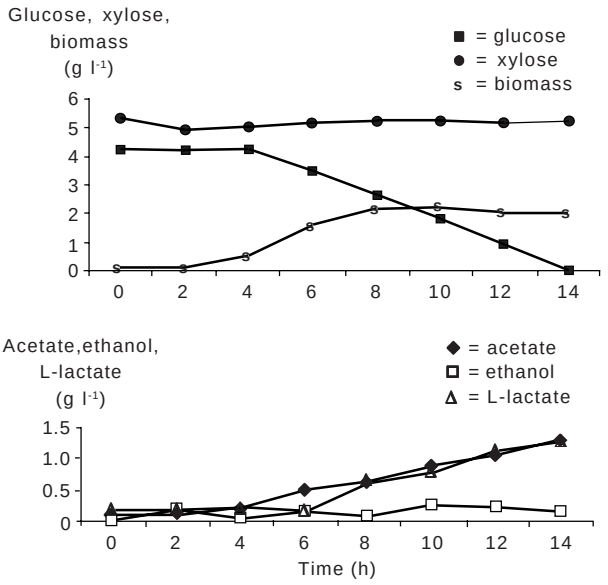

Fig. 6. Growth of *G. thermoglucosidasius* M10EXG on TGXTV medium (0.5% (w/v) glucose and 0.5% (w/v) xylose mixture medium) with the addition of 0.2% (w/v) yeast extract.

was slowly utilized (growth mainly on yeast extract components), and then the xylose concentration decreased rapidly up to 13 hours. A biomass yield was calculated of about 0.38 g g^{-1} based on xylose utilized. Maximum growth rate was higher, about 0.28 h^{-1}, compared to growth on xylose medium only (0.14 h^{-1}), and fermentation ceased sooner at 14 hours compared to 17 hours previously. By-product formation (acetate and L-lactate) up to maximum concentrations each of 2 g l^{-1}, occurred in the second phase. Ethanol concentrations were again low at 0.2 g l^{-1} (Fig. 3 and 5).

Addition of yeast extract to glucose and xylose medium

Addition of 0.2% (w/v) yeast extract on 0.5% (w/v) glucose and 0.5% (w/v) xylose medium enhanced the growth of M10EXG strain, and resulting in a higher biomass increase from 1.4 g to 2.20 g l^{-1} and a slight increase in the maximum specific growth rate from 0.25 h to 0.27 h^{-1} (Figure 6). The pattern of sugar utilization was different when yeast extract was added to the medium and two growth phases were not identified. In the presence of yeast extract, glucose was utilized completely while xylose was used very little. In the first 4 hours, the cells utilized yeast extract for growth as glucose and xylose concentrations showed no reduction. After growth ceased, a biomass yield of approximately 0.36 g g^{-1} was calculated based on the total sugars utilized, although yeast extract addition would influence this result. Both acetate and L-lactate were produced to about 1.5 g l^{-1}, and only a very low level of ethanol was detected (0.1 g l^{-1}).

The newly isolated strain *G. thermoglucosidasius* M10EXG grew on minimal medium. This is one piece of evidence that *G. thermoglucosidasius* M10EXG would be a better host for further genetic manipulation for ethanol production at elevated temperatures compared to *T. thermophilus* HB27 as this organism could not grow in a defined medium and is sensitive to ethanol.

Kinetic Evaluation of *G. thermoglucosidasius* M10EXG

The values of the kinetic parameters for *G. thermoglucosidasius* M10EXG growth on various media are summarized and compared in Table 1. *G. thermoglucosidasius* M10EXG grew on fully defined media, however the addition of yeast extract (0.2% (w/v)) also enhanced its growth for the conditions tested.

From this result, the effect of different sugar ranges on the thermophilic bacteria growth could be studied. Moreover utilization of sugars in a media containing a mixture of sugars could be investigated. Ethanol production using lignocellulosic materials is the best

Table 1. Growth kinetics of *Geobacillus thermoglucosidasius* M10EXG at 60°C on various media.

Media	Doubling time (h)		Maximum specific growth rate (h^{-1})		Length of lag phase (h)	Sugar uptake (g)		Final biomass (g)	Biomass yield ($g\ g^{-1}$)	Length of fermentation (h)
	Phase I	Phase II	Phase I	Phase II		Glucose	Xylose			
TMM + glucose (1% (w/v))	3.7	-	0.19	-	6	4.71	-	1.42	0.34	15
TMM + xylose (1% (w/v))	5.0	-	0.14	-	7	-	4.77	2.38	0.50	17
Glucose : xylose (0.5%:0.5%)	2.8	21.50	0.25	0.003	6	3.47	2.14	1.44	0.26	24
Glucose : yeast extract (1:0.2)	1.4[1]	4.50	0.50[1]	0.15	2	6.87	-	4.08	0.59[1]	24
Xylose : yeast extract (1:0.2)	2.5	-	0.28	-	7	-	5.96	2.25	0.38	14
Glucose : xylose : yeast extract (0.5 : 0.5 : 0.2)	2.6	-	0.27	-	2	4.23	1.88	2.20	0.36	14

All of the kinetics parameters were calculated values (usually to a two figure accuracy).

[1]Values measured based on sugars utililized, although addition of yeast extract would result in over-estimation of biomass yield values.

option due to the environmentally-friendly and food security issue and a cheap material. The result of lignocellulose hydrolysis contains a mixture of pentose and hexose.

G. thermoglucosidasius produced the highest level of biomass at 4.08 g l^{-1} when grown on 1% (w/v) glucose medium in the presence of 0.2% (w/v) yeast extract with glucose yield of 0.59 g g^{-1} based on the sugar utilized, although addition of yeast extract is likely to have caused this high yield as a maximum theoretical yield would normally be close to 0.50 g g^{-1}. Yeast extract enhanced the growth of *G. thermoglucosidasius* M10EXG in a defined medium. In the absence of yeast extract, *G. thermoglucosidasius* M10EXG grew better on xylose media, while with the addition of yeast extract, this strain grew better on glucose media. Studies with 0.5% (w/v) glucose/xylose medium with 0.2% (w/v) yeast extract showed no significant xylose utilization while glucose was fully utilized in 14 hours. The results provide evidence for some glucose repression of xylose uptake, even at the relatively low glucose concentration in the medium.

Diauxic growth occurred in complex media for *G. thermoglucosidasilus* M10EXG, particularly on glucose medium. Diauxic growth was found for *G. thermoglucosidasius* M10EXG with glucose and xylose (1% w/v each) medium and for glucose (1% w/v) with the addition of 0.2% (w/v) yeast extract (Fig. 3 and 4). *G. thermoglucosidasius* M10EXG utilized xylose in the 1% (w/v) xylose medium, or 1% (w/v) xylose with the addition of 0.2% (w/v) yeast extract. However in the glucose and xylose mixture medium, utilization of xylose was repressed by relatively low concentrations of glucose (Fig. 6).

From this results, M10EXG strain has interesting characteristics such as belong to thermophile, capable of growing on minimal medium, co-fermenting glucose and xylose, produce low level of ethanol, and tolerant to 10% ethanol indicating its potential for fermenting lignocellulosic agricultural wastes for ethanol production. Further studies such as genetic manipulation of this strain for ethanol production at elevated temperature would be challenging.

CONCLUSION

Kinetic evaluations of *G. thermoglucosidasius* M10EXG showed that the strain grew on minimal medium supplemented with single carbon source such as glucose and xylose, or both carbon sources. Glucose at relatively low concentrations (0.5% w/v) repressed xylose, particularly when yeast extract (0.2% w/v) was added to the medium. Diauxic growth, two growth phases were observed when the strain was grown on the glucose medium added with yeast extract. This strain produced low level of ethanol, L-lactate, and acetate. Therefore, the strain has desirable traits for ethanol production of agricultural waste containing lignocellulose in thermophilic system.

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