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Full Length Research Paper

The effect of carbon sources on the expression level of thermostable L2 lipase in *Pichia pastoris*

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Thermostable lipases are enzymes that are particularly pleasing for industrial purposes such as in the production of detergents, animal skin-based industry and food processing endeavours. Thermostable L2 lipase gene fished out from *Bacillus* sp. L2 was cloned into *Pichia pastoris* strain GS115 under constitutive expression system of pGAPZαA. Study performed on various carbon sources revealed that glucose and glycerol support the growth of *Pichia pastoris* and expression of L2 lipase. In addition, the by-product of sugar refinery processes (molasses) was found to be a potential carbon source for the growth of *P. pastoris* and L2 lipase expression. Since the thermostable L2 lipase will be extracellularly secreted into the medium, the use of *P. pastoris* expression system is seen as an alternative to the conventional expression system of *Escherichia coli* that applies intracellular expression of the L2 lipase gene encoded.

Key words: Thermostable lipase, *Pichia pastoris*, constitutive expression, carbon source.

INTRODUCTION

The use of lipases (EC 3.1.1.3) for industrial applications is the driving force to enhance research and development on this enzyme. They have a key role in providing substances in flavouring, biodiesel production, pharmaceuticals, and other commercial uses (Zhao et al., 2008). Thermostable enzymes offer more advantages over thermolabile type. Thermostability is always linked to higher resistant to chemical denaturants. Furthermore, by conducting enzyme catalyzed reactions at higher temperature, the use of higher substrate concentration and minimal microbial contamination are achievable (Demirjian et al., 2001).

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Abbreviations: **AOX**, Alcohol oxidase; **FLD**, formaldehyde dehydrogenase; **GAP**, gylceraldehyde-3-phosphate dehydrogenase; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **DHAP**, dihydroxyacetone phosphate; **F6P**, fructose-6-phophate; **G3P**, glyceraldehyde -3-phophate; **3PG**, 3-phosphoglycerate.

Pichia pastoris is one of the methylotrophic yeasts that have been used enormously to express heterologous proteins (Higgins and Cregg, 1998). Besides alcohol oxidase (AOX) promoters, there are other promoters available. These promoters include formaldehyde dehydrogenase (FLD) (Sunga and Cregg, 2004) and gylceraldehyde-3-phosphate dehydrogenase (GAP) (Waterham et al., 1997). GAP gene is constitutively expressed for the process of glycolysis and gluconeogenesis. The strength of constitutive expression level of GAP promoter is dependent on the carbon source used for cell growth.

There are reports on thermostable enzymes that have been successfully expressed in *P. pastoris*. These include thermostable xylanase 2 from *Hypocrea jecorina* (*Trichoderma reesei*) (Hokanson et al., 2011), acetylxylan esterase from *Thermobifida fusca* (Yang et al., 2010), xylanase from an alkaliphilic *Bacillus* sp. (Zhang et al., 2010), and thermostable GH5 mannan endo-1,4-β-mannosidase from *Aspergillus niger* BK01 (Bien-Cuong et al., 2009). There are also other lipases which have been successfully cloned and expressed in *P. pastoris*. Zhao et al. (2007) scaled-up the production of *Candida*

rugosa lipase expressed in *P. pastoris* from 5 to 800 L production. In addition, heterologous production of *Rhizopus oryzae* lipase in *P. pastoris* was performed using two different promoter systems of *P. pastoris*: the alcohol oxidase and formaldehyde dehydrogenase promoters (Cos et al., 2005). Other examples include lipase Lip 2 from *Yarrowia lipolytica* (Yu et al., 2010), human stimulated-bile salt lipase (Murasugi et al., 2001), and lipase B from *Candida antarctica (*Rotticci-Mulder et al., 2001).

Bacillus sp. L2 carries 1251 bp thermostable lipase gene (GeneBank Accession No. AY855077), and was isolated from a hot spring in Slim River, Perak, Malaysia (Shariff et al., 2007). In this study, the recombinant *P. pastoris* harbouring pGAPZαA with L2 lipase gene was grown in different carbon sources to determine the best carbon source that gave the highest expression level of thermostable L2 lipase. The objective of this paper was to evaluate the potential use of untreated molasses as carbon source for yeast growth and protein expression of *P. pastoris* utilizing GAP expression system.

MATERIALS AND METHODS

Strains and plasmids

P. pastoris strain GS115 incorporating pGAPZαA/L2 was used as a host. L2 lipase gene was previously cloned into the constitutive system of pGAPZαA (unpublished data). Plasmid extraction of pGAPZαA/L2 from *E. coli* Top 10 was conducted using Exprep-Plasmid Quick kit (GeneAll, Korea). Linearized pGAPZαA/L2 was transformed into *P. pastoris* GS115 through electroporation method as described in EasySelect™ *Pichia* Expression Kit manual. Culture was spread on yeast extract peptone dextrose medium with sorbitol (YPDS) (1% (w/v) yeast extract (Difco); 2% (w/v) bacteriological peptone (Difco); 2% (w/v) dextrose (Merck); 1 M sorbitol (Merck) containing 100 µg/ml Zeocin (Invitrogen, USA). Colonies obtained were screened through colony PCR. L2 lipase was expressed constitutively and secreted into the culture broth.

Screening of thermostable L2 lipase activity

Nine single colonies of positive transformants were inoculated into 10 ml YPD [1% (w/v) yeast extract (Difco); 2% (w/v) bacteriological peptone (Difco); 2% (w/v) dextrose (Merck)] and grown overnight. 1% of inoculum of each transformant was transferred into nine 500 ml baffled flasks containing 100 ml of YPD. After 48 h of incubation, 5 ml culture from each flask was harvested *via* centrifugation at 1500 *g* for 5 min (Hitachi, Japan). Supernatant obtained was stored on ice for subsequent lipase assay. The best clone which gave the highest reading of lipase activity was chosen for further study.

Study on fermentative and non-fermentative carbon sources for growth and L2 lipase expression

A single colony of GS115/pGAPZαA/L2 Clone No. 17 obtained from 100 µg/ml zeocin YPD plate after three days of incubation at 30°C was inoculated into 10 ml YPD (1% Yeast extract; 2% bacteriological peptone; 2% dextrose) and grown overnight. 1 ml of culture was transferred into total volume of 100 ml YP (1% Yeast extract, and 2% bacteriological peptone) with glucose and other carbon sources at various concentrations in 500 ml baffled flasks. The cultures were left to grow for 120 h at 30°C, 300 rpm (Infors, Switzerland), and 1 ml of culture was harvested from each flask every 24 h. The culture was subjected for lipase assay and optical density determination at 600nm. Every experiment was performed twice to ensure consistency of the results. For optimization of fermentative carbon source, glucose concentration for each flask was set as followed: 1, 2, 3, 4, 5 and 6% (w/v). Carbon source for each flask in optimization study of non-fermentative carbon sources was set as followed: 1% (v/v) oleic acid, 1% (w/v) trehalose, 1% (v/v) glycerol, 1% (w/v) lactose, 1% (v/v) methanol, 1% (w/v) sorbitol and mixture of glucose (0.5%) and glycerol (0.5%).

Study on different concentrations of fifth grade molasses as potential carbon source for growth and L2 lipase expression

Molasses concentrations for each flask were set as 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5% (v/v) with 2% (w/v) glucose as positive control. The fifth grade molasses used in this study was kindly donated by the Central Sugar Refinery Sdn. Bhd., Malaysia. Molasses constitutes reduced polymeric sugars which may be converted to fermentation sugar during enzymatic hydrolysis. Normal cane molasses usually has water content around 17 to 25%, sugar content (sucrose, glucose, and fructose) of 45 to 50% as well as polysaccharides namely dextrin, pentosans, and polyuronic acids of 2 to 5% (Najafpour and Shan, 2003).

Study on mud cake as potential carbon source

Mud cake concentrations for each flask were set as 1, 2, 5, and 100% (w/v) with 1% (v/v) glycerol as positive control. Mud cake used in this study was also obtained from Central Sugar Refinery Sdn. Bhd., Malaysia. Mud cake is the result of the precipitation of calcium carbonate which traps colour and impurities from raw sugars (Personal communication, 2010). Since there is possibility that mud cake may contain a trace of sugar, different concentrations of mud cake were tested for its potential as carbon source for *P. pastoris* growth and lipase expression.

Analytical procedures

Cell density measurement

The growth of *P. pastoris* was monitored by measuring the optical density at absorbance 600 nm using Biophotometer (Eppendorf, Germany).

Lipase assay

The activity of L2 lipase was assayed colorimetrically by using Kwon and Rhee's method (1986) with slight modifications. Cupric acetate-pyridine reagent was prepared as follow: 5 % (w/v) of aqueous solution of cupric acetate was prepared with its pH adjusted to 6.1 with pyridine. Substrate emulsion on the other hand was prepared by homogenizing (Heidolph, Germany) an equal ratio (1:1) of olive oil (Bertolli, Italy) and 50 mM pH 7.0 phosphate buffer. The reaction mixture (that is, 0.05 ml enzyme, 0.95 ml of 50 mM phosphate buffer, 2.5 ml substrate emulsion and 20 µl of 20 mM $CaCl_2$) was incubated at 70°C for 30 min in a water bath (Protech, Malaysia) with 200 rpm agitation rate. The enzymatic reaction was stopped by adding 1 ml of 6 N HCl, followed by 5 ml of isooctane. The mixture was vortexed vigorously for 30 s. The upper layer of isooctane (4 ml) was transferred into a clean test tube before the addition of 1 ml cupric-acetate-pyridine reagent. The mixture was

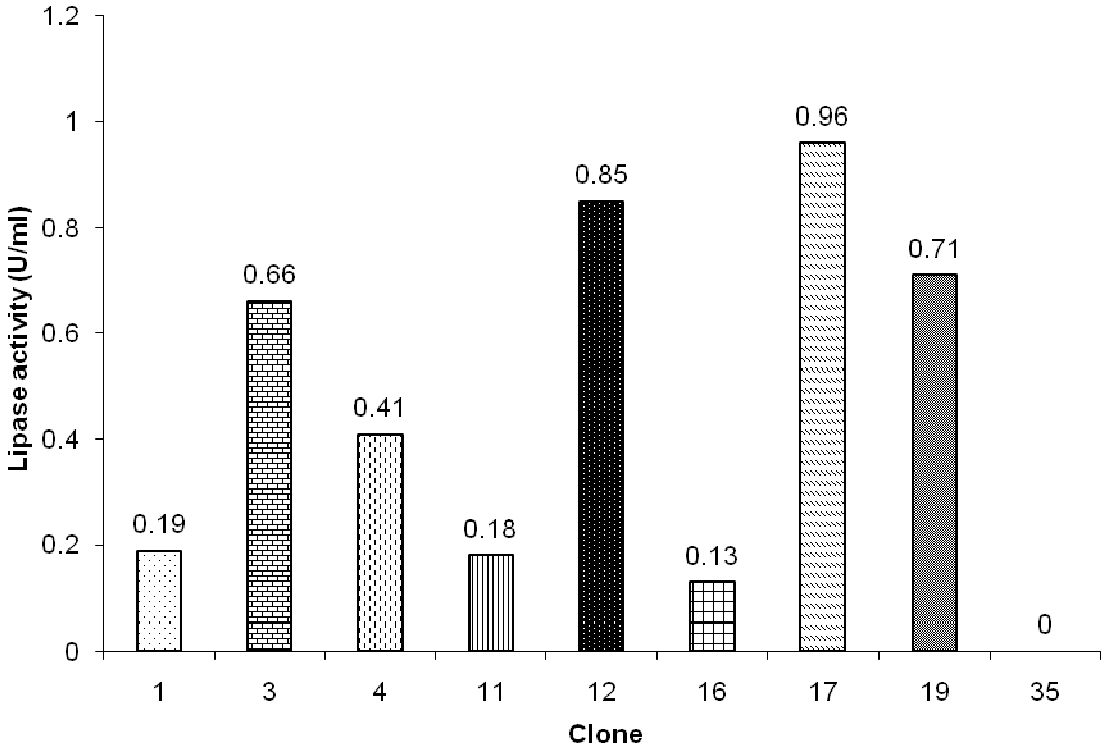

Figure 1. Lipase production of positive transformants obtained after 48 h of growth at shake-flask scale. Clone No. 17 which produced the highest lipase production level was selected for further study.

then vortexed vigorously (30 s) and left for an hour. The absorbance of upper layer was measured at 715 nm. Lipase assay was carried out in triplicate for every sample and the average of three reading was taken as a result. One unit (U) of lipase activity is defined as 1 µmol of fatty acid released per minute under standard assay condition.

RESULTS AND DISCUSSION

Screening of positive transformants of GS115/pGAPZαA/L2

Through colony PCR, nine colonies were found to have pGAPZαA/L2 incorporated in the *P. pastoris* genome. These positive transformants were then subjected to shake-flask study to screen for lipase expression. Clone No. 17 which gave the highest lipase expression level of 0.96 U/ml was selected for further study (Figure 1).

Study on fermentative and non-fermentative carbon source

Optimization of glucose concentrations

Different concentrations of glucose were tested to see any significant effect in terms of growth and expression level of L2 lipase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a tetrametric NAD-binding enzyme involved in glycolysis and gluconeogeneosis, therefore, this enzyme is always expressed constitutively at high level (Waterham et al., 1997). Figure 2A to F shows the growth curve and expression of L2 lipase of *P. pastoris* cells grown on glucose at different concentrations. As concentration of glucose increased, the specific growth rate of cells also increased (data not shown). Conversely, the level of lipase expression was found to be inhibited as the level of glucose concentration increased. The highest value of lipase activity (36.19 U/ml) was given by glucose concentration of 2% (w/v) at 120 h of cultivation, followed by 1% (w/v) and 3% (w/v) (Figure 2). Concentration of glucose between 4 to 6% clearly exhibited low level of lipase expression, even though at these concentrations the cell densities were high.

Since GAP constitutive system was employed in this study, carbon source that induced the GAP promoter play a big role to determine the level of lipase expression. Outcome from this experiment conform to the report by Waterham et al. (1997) as they mentioned that the expression level of heterologous protein was closely correlated with the activity of GAPDH. We found out that the highest L2 lipase expression was obtained when the cells were grown on glucose, followed by glycerol. L2 lipase expression was acceptably high when glucose concentration was ≤ 3%. Higher glucose concentration

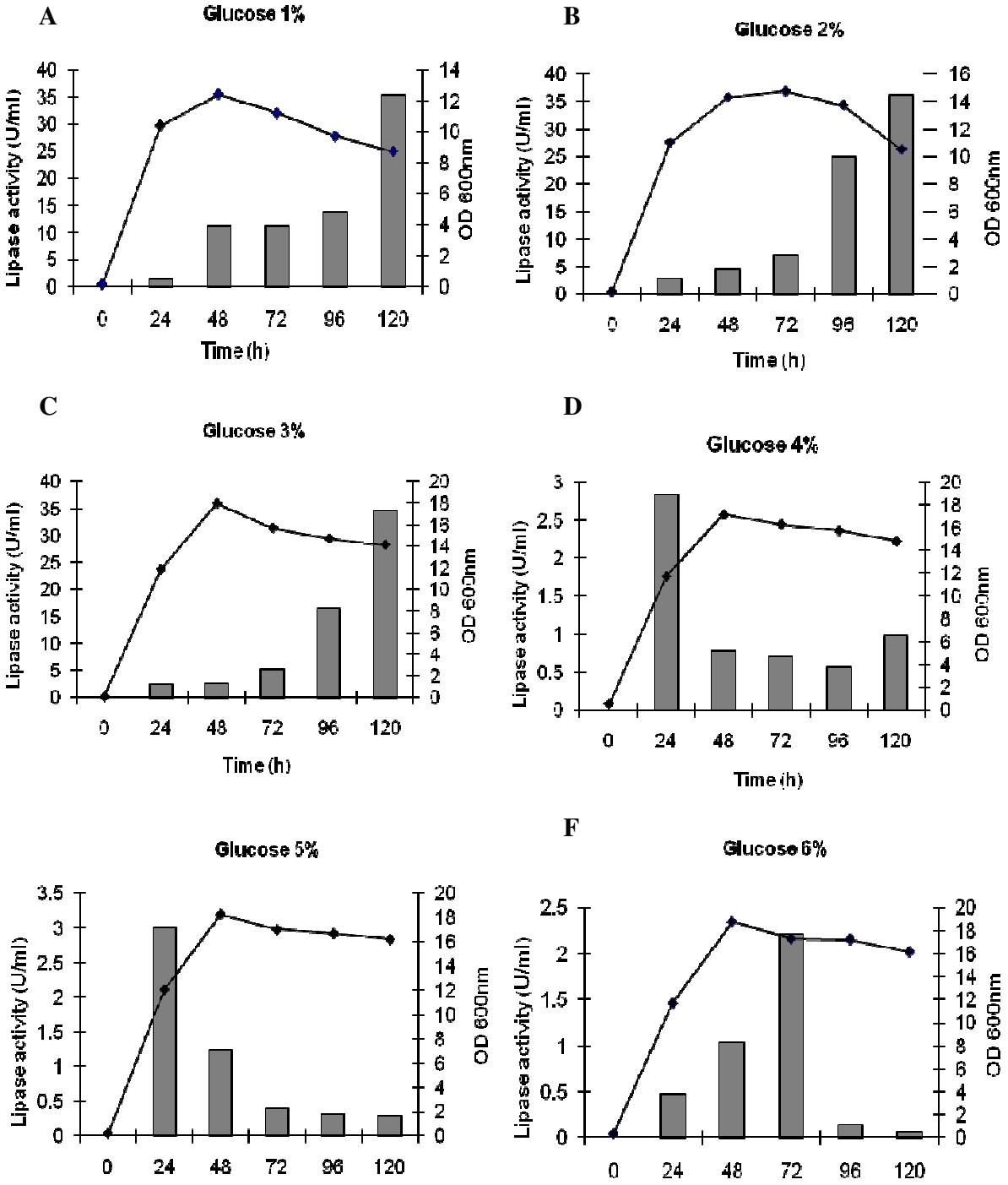

Figure 2. Growth curve (-●-, OD 600 nm) and lipase activity (U/ml, ▒) of GS115/pGAPZαA/L2 grown on different concentrations of glucose: (A) 1%; (B) 2%; (C) 3%; (D) 4%; (E) 5% and (F) 6% (w/v).

resulted in the decrease of L2 lipase production; most probably due to negative feedback inhibition. High concentration of glucose contributed to high production of fermentative by-products namely acetic acid and ethanol, which might slow down the cell growth (Tang et al., 2009).

Optimization of non-fermentative carbon sources

Figure 3A to G displays the growth curve and lipase activity of GS115/pGAPZαA/L2 grown on various types of carbon sources. Glycerol gave the highest lipase activity (33.8 U/ml) followed by the mixture of glucose and

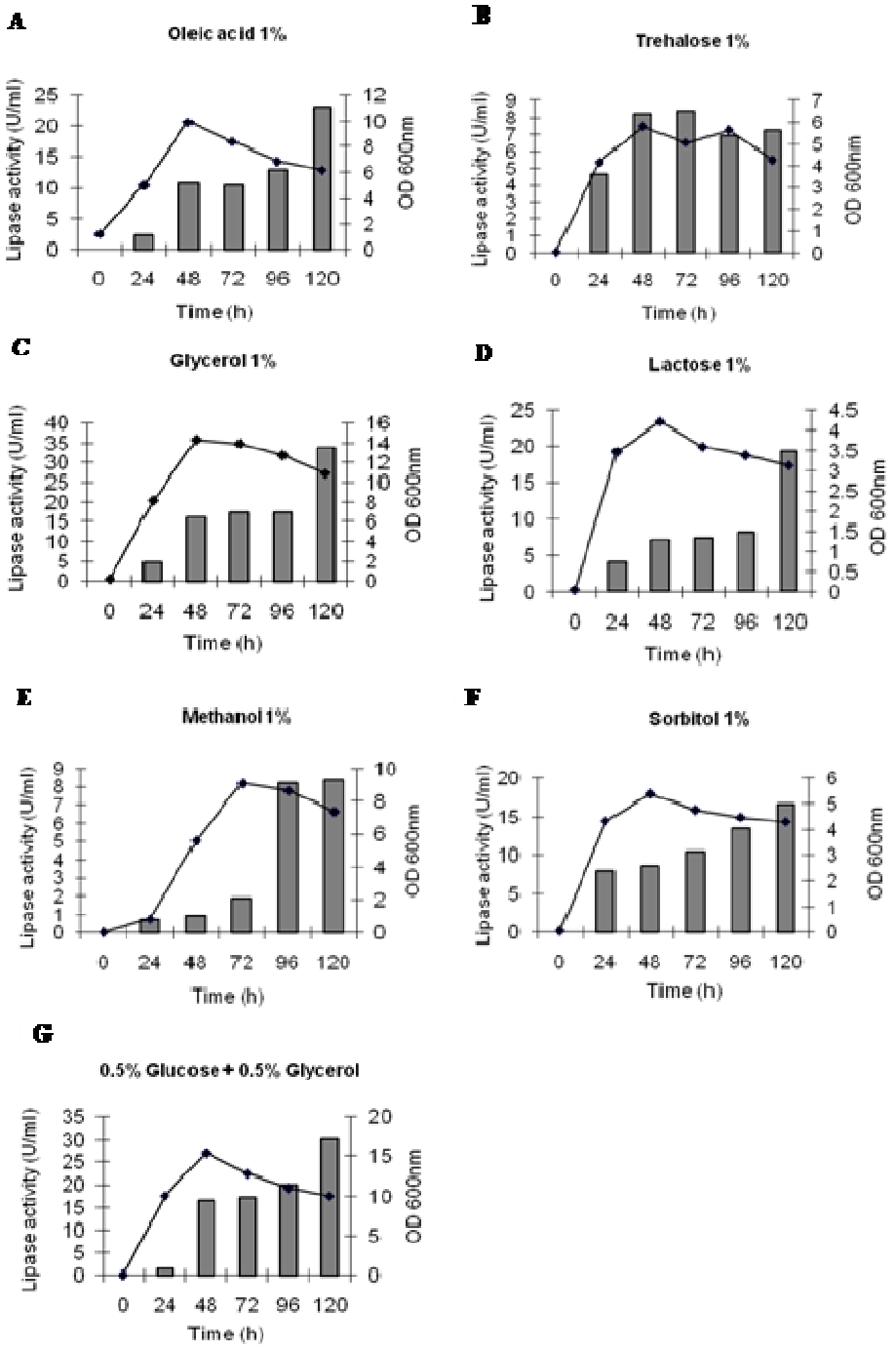

Figure 3. Growth curve (-_-, OD 600 nm) and lipase activity (U/ml, _) of GS115/pGAPZ_A/L2 grown on different concentrations of carbon sources: (A) 1% (v/v) oleic acid; (B) 1% (w/v) trehalose; (C) 1% (v/v) glycerol; (D) 1% (w/v) lactose; (E) 1% (v/v) methanol; (F) 1% (w/v) sorbitol, and (G) 0.5% glucose + 0.5% glycerol.

glycerol, oleic acid, lactose, sorbitol, methanol and trehalose. Growth on glycerol was observed to accumulate the highest cell density (Figure 3C). Study performed by Sola et al. (2004) provides several insights on the central carbon metabolisms and amino acid biosynthesis in *P. pastoris*. Interestingly, they claimed that the regulation of central carbon metabolism in *P. pastoris* is likely more similar to *Saccharomyces cerevisiae* than to *Pichia stipilis*. In addition, the conventional baker's yeast, *S. cerevisiae* is Crabtree-effect yeast, whilst *P. pastoris*

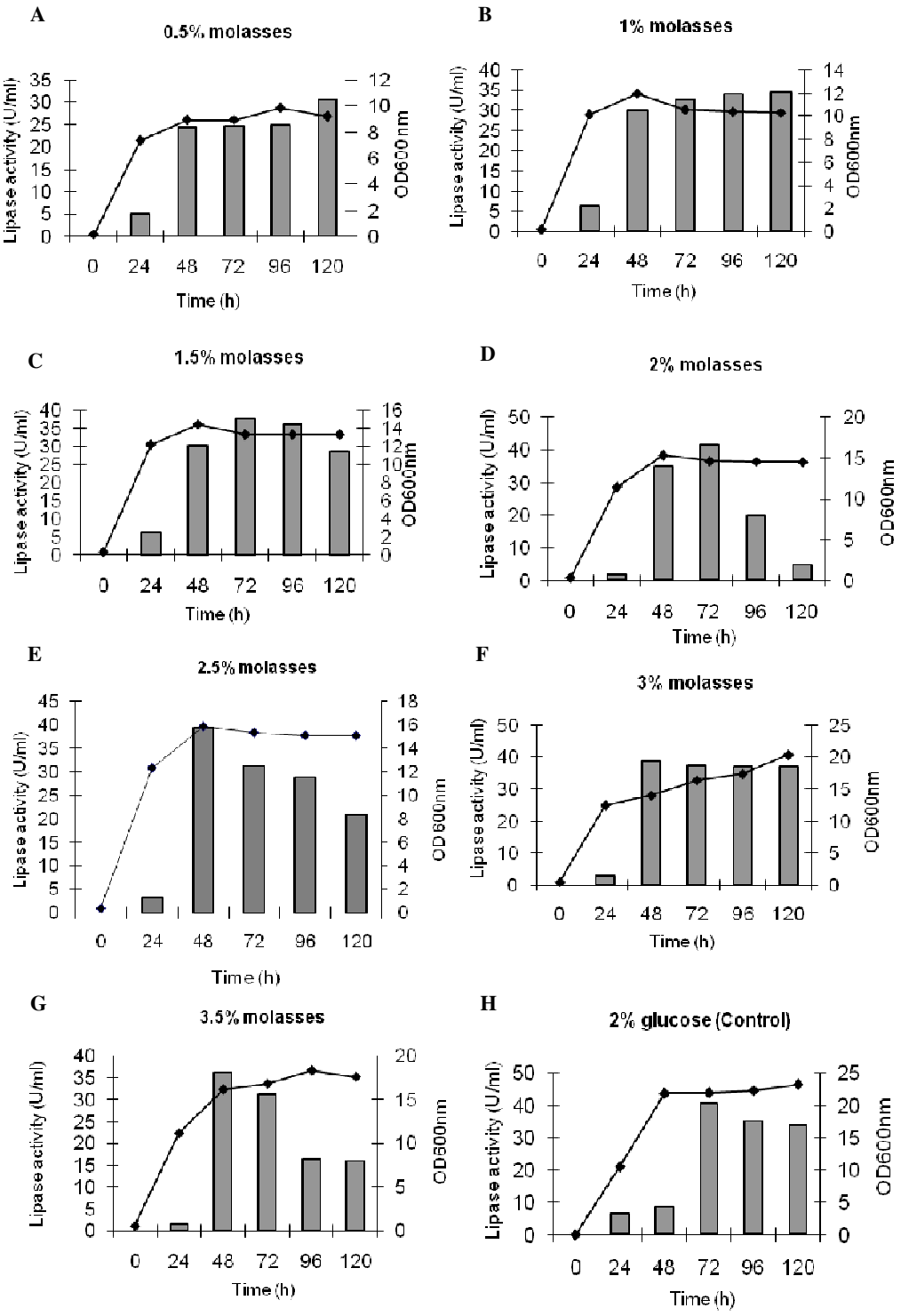

Figure 4. Growth curve (-_-, OD 600 nm) and lipase activity (U/ml, _) of GS115/pGAPZ_A/L2 grown on different concentrations of molasses (v/v): (A) 0.5%; (B) 1.0%; (C) 1.5%; (D) 2.0%; (E) 2.5%; (F) 3.0%; (G) 3.5%; (H) Control – 2% (w/v) glucose.

prefers to grow in respiratory mode, which results in the reduction of fermentative by-products such as acetic acid and ethanol. The pathways of catabolic metabolisms of glucose and glycerol finally reached its merging point when dihydroxyacetone phosphate (DHAP) derived from glycerol and F6P from glucose were both catalyzed by

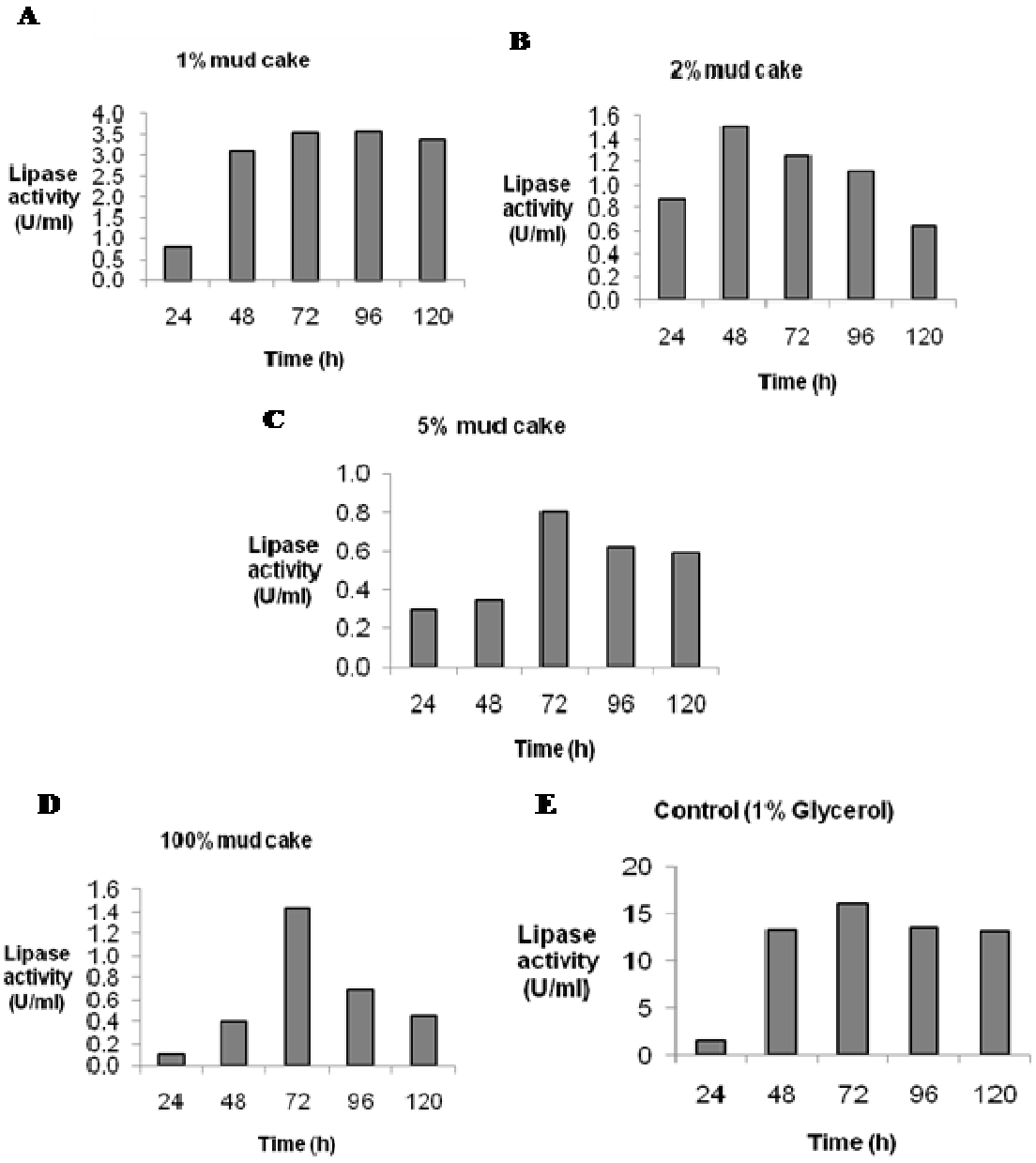

Figure 5. Thermostable L2 lipase activity (U/ml, _) of GS115/pGAPZ_A/L2 grown on different concentrations of mud cake (w/v): (A) 100%; (B) 1%; (C) 2%; (D) 5%; (E) Control – 1% glycerol (v/v).

glyceraldehyde -3-phophate (G3P) to become 3-phosphoglycerate (3PG). Since glycerol only needs to be converted to DHAP to trigger GAP promoter to work compared to glucose, more energy is attributed to the growth of the cells hence higher cell biomass is obtained. The experimental results showed that both glucose and glycerol worked effectively as carbon source for the expression and growth of *P. pastoris* cells even though there was a slight difference in expression level. Other carbon sources, that is, trehalose, lactose and sorbitol worked fairly well as inducers of GAP promoter.

Study on different concentrations of untreated fifth grade molasses as potential carbon source for growth and L2 lipase expression

The maximum value of lipase activity took place at different incubation time. Figure 4A to H shows the growth curve of GS115/pGAPZαA/L2 grown on different concentrations of molasses. The correlation between concentration of molasses and lipase expression level was relatively vague since there was no distinct pattern showed by the graphs.

Study on mud cake as the potential carbon source for growth and L2 lipase expression

Based on the results obtained, the use of mud cake as carbon source was ineffective. Growth of cells on solely 100% mud cake resulted in very low lipase activity (1.43 U/ml) after 72 h of incubation. Figure 5A to D shows the lipase activity of GS115/pGAPZαA/L2 grown on different concentrations of mud cake. Low lipase activity was reflected by the low cell density of *P. pastoris* grown in medium containing mud cake (data not shown). Furthermore, as the concentration of mud cake increased, the lipase activity decreased. In addition, as mud cake naturally exist in solid, it is inconvenient for any fermentation medium which usually requires full solubility in aqueous solution to achieve homogenous condition. The addition of yeast extract and peptone as organic nitrogen source generally helps in promoting the cell growth and lipase production. Nonetheless, since commercial yeast extract and peptone contain a small trace of glucose, this may contribute towards the induction of GAP promoter.

Conclusion

The untreated fifth grade molasses was found promising as sole carbon source for cell growth and production of L2 lipase. The use of molasses is economically feasible for massive production of L2 lipase to supply the Malaysian local need of lipase for industrial purposes. Preliminary study in bioreactor has shown that molasses gave acceptable level of expression of L2 lipase (unpublished data). Commercial carbon source tested namely glycerol or glucose which is proven to give acceptably high expression level of L2 lipase could be mixed with medium containing molasses to boost up the expression level.

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