Utilization of waste glycerol as a carbon source for *Pichia pastoris* cultivation

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Abstract

Waste glycerol is a by-product of the biodiesel production process. Therefore, waste glycerol price is very inexpensive. The feasibility of cultivating *Pichia pastoris* using waste glycerol as a carbon source was investigated. In this study, the composition of waste glycerol from 2 sources, E-ester company, Chiang Rai and Maecham pork cracker community enterprise, Chiang Mai was analyzed. The average glycerol content was 27.70% and 29.82%, respectively. The simple methods to pretreat the waste glycerol were employed. Then the effect of treated and untreated waste glycerol on *P. pastoris* growth compared with pure glycerol was investigated. The suitable concentration of waste glycerol for *P. pastoris* cultivation was also assessed. Media containing 1, 2 and 5% (v/v) waste glycerol were used to cultivate *P. pastoris*. The highest dry cell weight was achieved using 5% untreated waste glycerol. The maximum dry cell weight using waste glycerol from E-ester company, Chiang Rai and Maecham pork cracker community enterprise, Chiang Mai were 11.47 and 14.38 g/L, respectively. The high density of biomass obtained from fermentation in a 5-L bioreactor by using waste glycerol as a carbon source without any negative effect. This study revealed that waste glycerol from E-ester company, Chiang Rai and Maecham pork cracker community enterprise, Chiang Mai can be directly used as a carbon source for *P. pastoris* cultivation.

Keywords: Waste glycerol, Biodiesel, *Pichia pastoris*

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1. Introduction

Biodiesel is produced by a trans-esterification reaction using vegetable oils or animal fats with methanol, a process that generates large amounts of glycerol as a by-product, at levels of approximately 10% (w/w) of the total biodiesel generated. The rapid increase of biodiesel production has led to the creation of a glut in the glycerol market (Johnson and Taconi, 2007). Thailand was ranked seventh of the world's biggest biodiesel producing countries according to their production volume in 2014 with a total production volume of around 3.1 billion liters in 2014, Thailand biodiesel production was estimated at to be 1.2 billion liters (Statista Inc, 2015). As the number of biodiesel plants grows, increasing amounts of waste glycerol are generated and thus affecting glycerol's market price. The current market value is US$ 0.27–0.41 per pound of pure glycerol (Yang *et al.,* 2012) and as low as US$ 0.04–0.09 per pound of waste glycerol (Sims, 2011). The waste glycerol in Thailand also has a relatively low price at about 4–10 Baht/kg and many biodiesel producer are currently stocking waste glycerol to wait for better market or using for bunker oil (Loedaech *et al.,* 2009). One reason of the low price stemmed from the presence of impurities in the waste glycerol. While some large scale producers are able to refine waste glycerol to high purity glycerol for the industrial applications such as the food, beverage, pharmaceutical and cosmetic industries, but small scale producers are unable to justify refining waste glycerol due to expensive cost and energy-intensive requirement (Jitrwung and Yargeau, 2015). It is urgent to develop utilization methods for waste glycerol that can make biodiesel production more profitable. Many applications have been considered to exploit the potential of converting waste glycerol from biodiesel to value-added products such as hydrogen, ethanol, butanol, citric acid, docosahexaenoic acid (DHA), lipase and 1, 3-propanediol (Yang *et al*., 2012). If feasible, using waste glycerol for microbial cultivation will create a substantial demand for this type of raw materials.

The methylotrophic yeast, *Pichia pastoris* is a highly successful expression system for the production of a variety of heterologous proteins. In recent years, more than 500 proteins have been cloned and expressed using this system (Cereghino and Cregg, 2000). *P. pastoris* can be grown to high cell density by basal salt medium with glycerol as a carbon source. Thus, if possible, the use of waste glycerol for microbial cultivation (Tan *et al.,* 2013) will reduce the cost of production and dispose the surplus of waste glycerol. Although impurities in waste glycerol can inhibit cell and fungal growth and result in lower production rate and product yield when compare with pure glycerol under the same condition culture. To achieve that, composition of waste glycerol and pretreatment methods fermentation condition should be studied and optimized (Yang *et al.,* 2012). Several pretreatment techniques

such as centrifugation (Tang *et al.*, 2009), solvent-assist (Anand *et al.*, 2012), and dilution (Chi *et al*., 2007) were utilized with better yields compared to untreated waste glycerol. On the other hand, waste glycerol can be used directly as a carbon source. For instance, Celik *et al*. (2008) employed waste glycerol in *P. pastoris* fermentation process to produce recombinant human erythropoietin. The product and cell yield obtained were approximately 1.4 folds higher than using pure glycerol. This study demonstrated a potential use for of waste glycerol, without any treatment.

The aims of this study were to test the feasibility of cultivating *P. pastoris* using waste glycerol as a carbon source and to investigate the effect of treated and untreated waste glycerol on *P. pastoris* growth compared with pure glycerol.

2. Materials and Methods

2.1 Yeast strain

P. pastoris strain X-33 (Invitrogen Inc, USA) was kindly provided by Dr. Christopher P. Marquis, (School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Australia).

2.2 Waste glycerol analysis

The waste glycerol, a by-product from biodiesel process, was kindly provided by E-ester company, Chiang Rai (CR) and Maecham pork cracker community enterprise, Chiang Mai (CM). Composition of waste glycerol was analyzed by Central Laboratory (Thailand) Co., Ltd. Total glycerol content was determined by GC/FID. The methanol was determined by GC/MS. The free fatty acid was determined in accordance with AOAC (2010) 969.17. The potassium hydroxide (KOH) was determined by titrimetric method and ash was determined in accordance with AOAC (2010) 923.153.

2.3 Waste glycerol pretreatment

Typically, 200 mL of the waste glycerol was transferred to a beaker and subjected to pretreatment. Simple methods to pretreat the waste glycerol were centrifugation (Tang *et al*., 2009), precipitation at room temperature of 25.4°C (Ministry of transport, 2015) and 4°C (Anand *et al.,* 2012), 30% (v/v) H_2SO_4 treatment (Chi *et al*., 2007), and boiling (Supachai *et al.*, 2009). After treatment, the glycerol part was recovered and replenished with reverse osmosis water.

2.4 Cultivation medium

YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) was used as a starter medium for inoculum preparation. Buffer Glycerol-complex Medium (BMGY) contained 10 g/L yeast extract, 20 g/L peptone, 1.34 g/L YNB (Yeast nitrogen base), 10 g/L glycerol, 400

μg/L biotin and 100 mM potassium phosphate pH 6.0. Buffer Waste Glycerol-complex Medium (BMWGY) was made from treated or untreated waste glycerol (1, 2 and 5% v/v) and the composition of media was similar to BMGY medium except the glycerol was replaced by treated or untreated waste glycerol.

Fermentation basal salts medium with 1% glycerol or 5% waste glycerol (BSM-G and BSM-WG) containing $CaSO_4$, 0.93 g/L; K_2SO_4, 18.2 g/L; $MgSO_4.7H_2O$, 14.9 g/L; H_3PO_4 (85%), 26.7 mL; KOH, 4.13 g/L; glycerol 1% (w/v) or waste glycerol 5% (w/v) was sterilized for 15 min at 121°C, then 4.25 mL trace element solution (PTM_1) were separately filtered through 0.25 μm filter and were added to the above solution aseptically. The trace element solution (PTM_1) was composed of $CuSO_4.5H_2O$, 6 g/L; NaI 0.08 g/L; $MnSO_4$, 3.0 g/L; Na_2MoO_4, 0.2 g/L; H_3BO_3, 0.2 g/L; $CoCl_2$, 0.5 g/L; $ZnCl_2$, 20 g/L; $FeSO_4.7H_2O$, 65 g/L; biotin, 0.2 g/L; 98% w/w H_2SO_4, 5 mL).

2.5 Cultivation of *P. pastoris*

A single colony of *P. pastoris* from YPD agar was inoculated into a 50 mL conical tube containing 5 mL of YPD medium and cultivated in a shaker (30°C, 250 rpm) for 16 h. Five hundred microliters of overnight culture was then inoculated into 50 mL of BMGY or BMWGY media in 500 mL baffled flask. The culture was further incubated for 30 h. Two milliliters of sample were taken every 6 h for biomass analysis.

2.6 Fermentation

A single colony was inoculated in 5 mL of YPD medium in a 50 mL conical tube, the culture was grown at 30°C, 250 rpm for 16-18 h. The starter culture (1%) was inoculated into 25 mL of BMGY media in a 250 mL baffled flask for 16–18 h. The culture was then pooled and cells density was measured. The cells were collected by centrifugation at 4000 rpm for 10 min and then re-suspended to an OD_{600} of 3.0 in 4 L of BSM-G or BSM-WG medium in a 5-L bioreactor (Biostat C, Germany). The fermentation conditions were as follows: temperature: 30°C; maximum agitation speed: 800 rpm; aeration rate: 1–2 VVM and pH 5.0.

2.7 Biomass analysis

One milliliter of cell suspension in a pre-weight 1.5 mL microcentrifuge tube was centrifuged, followed by twice washing with hexane and distilled water, respectively. The cell pellet was dried in a hot air oven at 105°C for 24 h. Maximum specific growth rate (μ_{max}) was determined from the slope of ln biomass vs. time. Biomass yield ($Y_{x/s}$) was calculated from the growth of organism (biomass) vs. substrate consumed.

3. Results and Discussion

3.1 Waste glycerol analysis

The waste glycerol from 2 sources, E-ester company, Chiang Rai and Maecham pork cracker community enterprise, Chiang Mai, which was obtained from biodiesel production were dark brown liquid with high initial pH values of 10.02 and 10.47, respectively**.** The content of glycerol were found to be 27.70% and 29.82% w/w, respectively with the relatively high methanol content (Table 1) from biodiesel production process (transesterification). It ended up in the glycerol phase due to its polar nature (Dasari, 2007). The waste glycerol from biodiesel production usually contained methanol, soap, catalysts, salts, non-glycerol organic matter, and water impurities (Hansen *et al*., 2009). The composition of waste glycerol by the type of catalyst used to produce biodiesel, the transesterification efficiency, recovery efficiency of the biodiesel, other impurities in the feedstock, and whether the methanol and catalysts were recovered (Cui and Ellison, 2012). For example, Yong *et al*. (2001) reported that composition of waste glycerol from biodiesel contained 20.20% glycerol, 64.30% ash, 3.0% water, 12.40% matter organic-glycerol and pH 12.8 (20% in water). The waste glycerol extracted from waste cooking biodiesel had a composition (w/w) of 36–37% glycerol, 3.3–4.1% ash, 12.3–14.5% water, 53.5–64.5 matter organic non glycerol (MONG) and pH 9.6-10.8 (Saifuddin *et al*., 2014). In contrast, Hansen *et al*. (2009) reported glycerol content ranging between 38–96% of 11 waste glycerol samples collected from different 7 Australian biodiesel producers. Some of those samples contained more than 14% methanol and 29% ash since biodiesel production involved the use of low-grade methanol and homogeneous alkaline catalysts, such as sodium methoxide or potassium hydroxide.

Table 1 Composition of waste glycerol from 2 different sources.

Sample	Composition (%, w/w)					pH (20% in water)
	Glycerol	Methanol	FFA[1]	KOH[2]	Ash	
CR[3]	27.70	13.57	1.18	3.97	6.18	10.02
CM[4]	29.82	15.34	0.26	3.51	5.31	10.47

Note: [1] mean Free Fatty Acid, [2] mean Potassium Hydroxide,

[3] mean Waste glycerol from E-ester company, Chiang Rai,

[4] mean Waste glycerol from Maecham pork cracker community enterprise, Chiang Mai

3.2 Effect of treated and untreated waste glycerol

The simple methods to treat the waste glycerol were centrifugation, precipitation at room temperature and 4°C, 30% H_2SO_4 treatment and boiling. These methods were effective

in removing impurities from waste glycerol. The total glycerol content of treated and untreated waste glycerol were analyzed by titration method as describes in TISI 336 (2523). The analytical results are shown in Table 2. The glycerol content after treatment was found in the range of 27–55% v/v. The glycerol content after treatment and replenished to initial volume before treatment was found to be at low level in the range of 17–29% v/v. The total glycerol content of treated waste glycerol was lost up to 42%. The presence of low glycerol content after treatment and diluted to initial volume was due to recovery efficiency of purified glycerol and glycerol lost during pretreatment process.

Table 2 Total glycerol content in waste glycerol from Maecham pork cracker community enterprise, Chiang Mai and E-ester company, Chiang Rai after treatment.

Method	Maecham, Chiang Mai (%, v/v)		E-ester, Chiang Rai (%, v/v)	
	Undiluted	Diluted (H_2O)	Undiluted	Diluted (H_2O)
Boiling	34.00	28.40	32.32	17.50
Centrifugation	33.77	27.33	30.42	21.58
Precipitation at RT	36.13	29.40	27.90	19.28
Precipitation at 4°C	39.71	23.24	30.10	25.06
30% H_2SO_4	54.70	20.79	39.49	18.13

3.3 Effect of treated and untreated waste glycerol on *P. pastoris* growth in shake flask

The *P. pastoris* growth rates using pure glycerol, treated waste glycerol, and untreated waste glycerol as carbon sources were evaluated. The cultivation was performed in shake flask with media containing three concentration levels of waste glycerol (1, 2 and 5% (v/v)). The actual untreated waste glycerol concentration levels were calculated to be 0.3, 0.5 and 1.5% (v/v), respectively. Differences in growth rates obtained from treated and untreated BMWGY was observed. The *P. pastoris* culture using treated and untreated BMWGY from 1, 2 and 5% (v/v) waste glycerol had lower growth rate than BMGY medium culture (Fig. 1). The dry cell weight using treated BMWGY media was lower than using untreated BMWGY media due to the relatively lower glycerol concentration level of treated waste glycerol. The maximum dry cell weight using 5% untreated BMWGY from Maecham pork cracker community enterprise, Chiang Mai and E-ester company, Chiang Rai were 14.38±0.87 and 11.47±0.29 g/L, respectively.

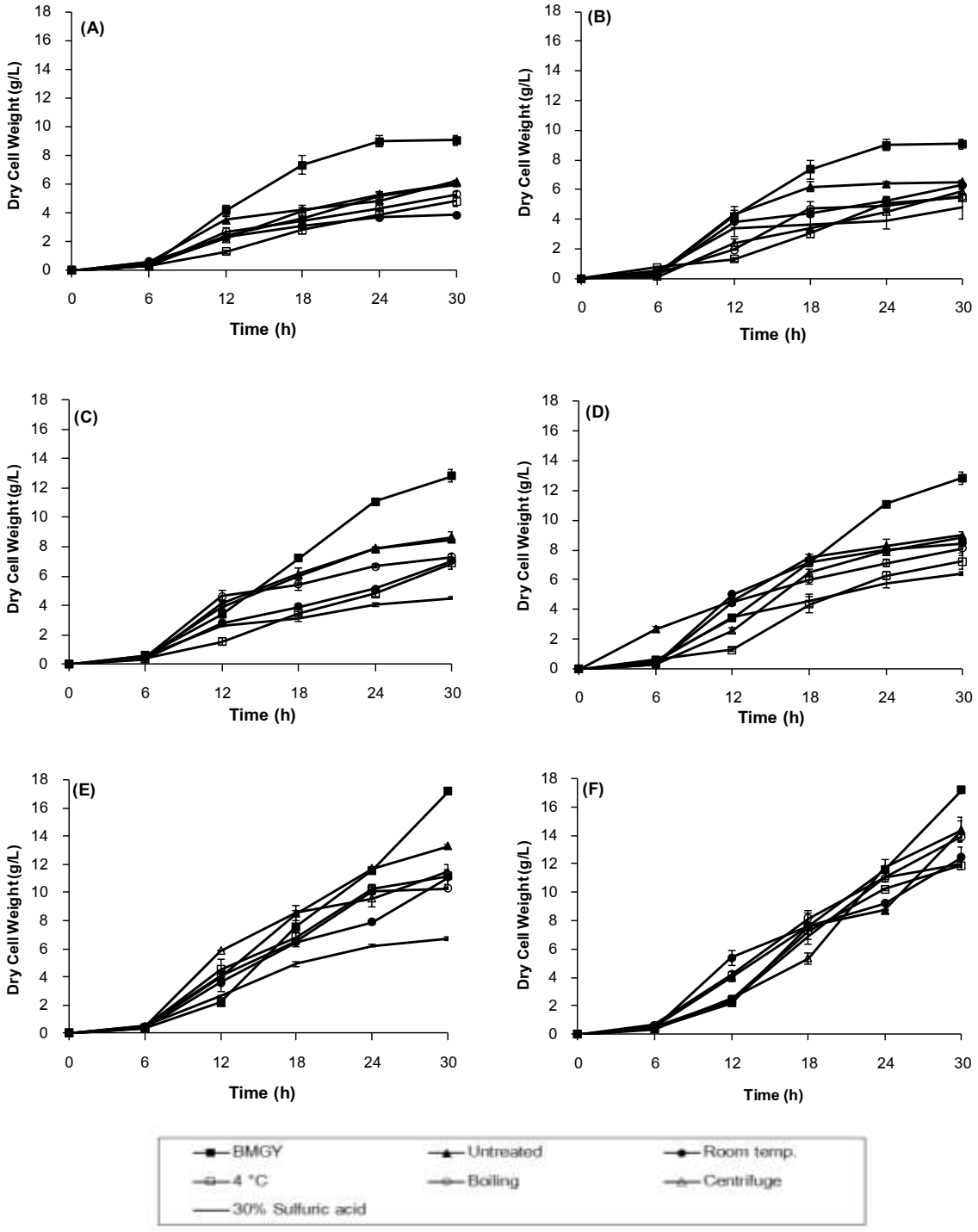

Fig.1 Cell growth using BMGY medium, treated and untreated BMWGY. (A, C, E) Waste glycerol from E-ester company, Chiang Rai at 1, 2 and 5%, respectively and (B, D, F) waste glycerol from Maecham pork cracker community enterprise, Chiang Mai at 1, 2 and 5%, respectively.

Glycerol has been regularly used as the main initial carbon source in *P. pastoris* fermentation to increase the cell concentration. The results showed that *P. pastoris* could be cultured in untreated BMWGY media without any negative effect. The dry cell weight from BMGY media was lower than untreated BMWGY media using waste glycerol from Maecham

pork cracker community enterprise, Chiang Mai but higher than untreated BMWGY media using waste glycerol from E-ester company, Chiang Rai when compared by 1% total glycerol content. There was no huge difference in growth yield and maximum specific growth rate using 1% pure glycerol and 5% untreated waste glycerol (Table 2). The maximum specific growth rate (μ_{max}) using BMGY media was 0.1225, which was slightly higher than BMWGY-CM and BMWGY-CR. The highest $Y_{x/s}$ value was obtained with BMWGY-CM (0.765±0.14 g/g_{subs}) followed by the pure glycerol (BMGY) (0.720±0.32 g/g_{subs}) and BMWGY-CR (0.670±0.15 g/g_{subs}). $Y_{x/s}$ for most yeast and fungi grown aerobically is typically between 0.4–0.8 g/g_{subs} (Potvin *et al*., 2012). The cell yields obtained from untreated BMWGY are in accordance with biomass yield coefficient of 0.62 g/g of waste glycerol reported by Noseda (2014). Therefore, it was conclude that untreated glycerol from biodiesel production can be used as a carbon source for *P. pastoris* cultivation. Several research groups also reported the utilization of waste glycerol to culture microorganisms without pretreatment (Cavalheiro *et al*., 2012). For example, Celik *et al*. (2008) have directly used waste glycerol as a carbon source to produce human erythropoietin by *P. pastoris*. The cell yield using waste glycerol was 1.4 folds higher than using pure glycerol. This improved growth could also be due to the transfer of micronutrients from the vegetable or animal fats, which are used as substrate to produce biodiesel (Cui and Ellison, 2012).

Table 2 Cell growth, cell yields and maximum specific growth rate of *P. pastoris* using pure glycerol, waste glycerol from E-ester company, Chiang Rai and waste glycerol from Maecham pork cracker community enterprise, Chiang Mai as a substrate.

Culture media	Fermentation method	Dry cell Weight (g/L)	Dry cell Weight (g/L/1% glycerol)	μ_{max} (h^{-1})	$Y_{x/s}$ (g/g_{subs})
BMGY	Shake flask	9.07±0.32	9.070	0.1225	0.720±0.32
BMWGY-CM	Shake flask	14.38±0.87	9.645	0.1052	0.765±0.14
BMWGY-CR	Shake flask	11.47±0.29	8.448	0.1042	0.670±0.15
BSM-G	Bioreactor	8.52±0.004	8.520	NA*	0.676±0.001
BSM-WGCM	Bioreactor	13.04±0.059	8.746	NA*	0.694±0.014
BSM-WGCR	Bioreactor	11.81±0.053	8.698	NA*	0.615±0.016

Note: *NA: not available

3.4 Fermentation of *P. pastoris* using pure glycerol and waste glycerol as substrate

The fermentation for high cell density production was carried out in 5-L bioreactor with 4 L of BSM-G, BSM-WGCM or BSM-WGCR medium. When pure glycerol was used as a substrate, the maximum dry cell weight was 8.52±0.004 g/L which was lower than using waste glycerol. The final dry cell weight using BSM-WGCM and BSM-WGCR were 13.04±0.059 g/L and 11.81±0.053 g/L, respectively as shown in Table 2. The dry cell weigh obtained from fermentation 5-L bioreactor was lower than shake flask due to limitation of other nutrients found in yeast extract and peptone used in BMGY medium. The $Y_{x/s}$ from fermentation 5-L bioreactor of *P. pastoris* by using pure glycerol, waste glycerol from E-ester company and Maecham pork cracker community enterprise, Chiang Mai were 0.676±0.001, 0.694±0.014 and 0.615±0.016 g/g_{subs}, respectively. This demonstrates that *P. pastoris* can use waste glycerol directly, as carbon source, for fermentation.

4. Conclusion

The work presented here showed the potential of using less expensive waste glycerol from biodiesel production process. The results showed that *P. pastoris* were able to use untreated waste glycerol as a carbon source efficiently for their growth. It grew in 5% (v/v) untreated waste glycerol better than pure glycerol and 1 and 2% treated and untreated waste glycerol media. The high density biomass obtained from cultivation of *P. pastoris* in a 5-L bioreactor using waste glycerol as a carbon source did not reveal any negative effect. *P. pastoris* were able to directly use waste glycerol efficiently for their growth similar to pure glycerol. The results indicated that untreated waste glycerol from biodiesel production process is one of the efficient carbon source for *P. pastoris* cultivation.

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