International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.4, No.2, pp 713-719, April-June 2012

Effects of Biodiesel Waste Glycerol on the Growth Characteristics of *Pichia pastoris* Genetically Modified to Produce Spidroin

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Abstract: Spider silk poccesses a unique combination of excellent tensile strength and elasticity; in fact, dragline silk is among the toughest fibers known. Silk as a biomaterial scaffold has been widely used in the field of tissue engineering and regenerative medicine. Commercial production of spider silk is problematic because of the territorial nature of spiders. Nonetheless, there are alternative ways to produce spider silk (spidroin) proteins. Recombinant technology has been used to produce spidroin in plant cells, yeast cells, milk from transgenic goats and other systems. However, the expensive cost of carbon source for silk protein production at a scaled up system for yeast fermentation maintains a critical issue. In this article, we evaluated the growth characteristics of a *Pichia pastoris* yeast strain which was genetically modified to produce spidroin. We examined the effects on yeast cell growth in shake-flask cultures of using biodiesel waste glycerol (BWG), a by-product of the biodiesel production process and thus a relatively inexpensive replacement for the carbon source in the yeast culture media. We produced biodiesel from several off-the-shelf cooking oils made from different plant sources to obtain the BWG. A commercial BWG and laboratory grade glycerol were also used for the study. In this study we addressed the following questions: (1) could yeast be cultured using BWG and (2) does the source of the oil (e.g., corn vs. canola) used in BWG production influence cell growth? In summary, the BWG, irrespective of source oil, resulted in better cell growth than did laboratory grade glycerol. Thus, we conclude that using BWG as the carbon source for yeast culture can substantially reduce the costs for genetically engineered proteins production.
Keywords: *Pichia pastoris*, biodiesel, glycerol, spidroin, cell density.

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Introduction

Spider silk is an extraordinary material, with a tensile strength comparable to commercially available high performance carbon and aramid fibers, but having a greater toughness and energy to break than these or any other common materials (1). However, unlike that of the silk from the silkworms because of the territorial nature of spiders (2), commercial production of spider silk is problematic. Nonetheless, there are alternative ways to produce spider silk (spidroin) proteins. Recombinant DNA technology has been used to produce spidroin in plant cells (3), yeast cells (4), microbial cells (5), transgenic silkworms (6) and other systems. Based on the repetitive sequences in native gene encoding the dragline silk of *Nephila clavipes* discovered by Xu and coworkers (7), we have successfully cloned three types of homopolymer spidroin1-like recombinant proteins individually into *Pichia pastoris* (*P. pastoris*). *P. pastoris*, a methylotrophic yeast, is commonly used as a host for the production of recombinant proteins of commercial interest (8). Its capacity to grow to very high cell density in a defined media and in the presence of a strong inducible alcohol oxidase promoter (AOX1) makes *P. pastoris* a popular expression system (9). This yeast system is capable to scale up into industrial fermentation level with high cell density and relatively low cost of media. Part of the expressed foreign proteins can be secreted into media, which is an advantage for down-scream protein recovery (8). *P. pastoris* genetically modified to express collagen fused spidroin1-like copolymer (10) was used for this study.

High cell density fermentation of *P. pastoris* requires a great amount of glycerol in culture media as the carbon source, especially at industrial scale (11). Glycerol is the single carbon source for yeast fermentation and it is the biggest investment in industrial media (12). Since glycerol has literally thousands of uses and it is relatively expensive, we are interested to replace commercially available glycerol with biodiesel waste glycerol (BWG), which is the main by-product of biodiesel production industry (13). Biodiesel is a relatively new and renewable diesel fuel made from the triglycerides in oils, fats, waste cooking oils, etc (14). BWG made from used cooking oils may be expected to be highly variable. Thus, in order to obtain results on cell growth using BWG from a variety of sources, we produced BWG from six commercially available cooking oils. We tested BWG from canola, corn, sunflower, vegetable and a blend oil of canola and vegetable cooking oil. All were "as-received" from local grocery.

Materials and methods

Materials

Geneticcally engineered *P. pastoris* GS115 (15) yeast strain and waste glycerol from commercial biodiesel (C-BWG) were kindly provided by Professor Albert Abbott at the Department of Genetics and Biochemistry, Clemson University. Cooking oils for biodiesel waste glycerol production in laboratory were obtained from local grocery store. All other chemicals were from Sigma Chemicals (St. Louis, MO) unless noted otherwise.

Laboratory biodiesel waste glycerol (L-BWG) preparation

The biodiesel reaction may be represented as transesterification of the triglyceride:

Triglyceride + Methanol =====> Biodiesel + Glycerol
(Alkali catalyst)

The reaction substitutes methanol for the glycerol in the triglyceride to make the methyl esters called biodiesel using lye as catalyst in commercial process. The lye also converts a small amount of the oil to soap and neutralizes any excess fatty acids in used cooking oil. After the reaction is completed, the glycerol and soap settle to the bottom of the vessel and the biodiesel floats on top (13).

Cooking oil was first warmed to 55°C in water bath. Then alkali catalyst of 2% NaOH in methanol was added into the warm oil. The mixture was stirred for 60 minutes for reaction and then settled overnight for separation into expected two layers, with the L-BWG in the bottom layer. BWG pH was adjusted to 6 prior to addition to the culture media.

Yeast culture media preparation

Buffered minimal glycerol media with histidine (BMGH) was used for yeast cells shake flask culture. Please refer to **Table 1** for the detailed ingredients in the media. When we studied cell growth effects using different carbon sources, we also prepared buffered minimal BWG media with histidine (BMBWGH) and buffered minimal glucose media with histidine (BM(G)H). In BM(G)H media, glucose replaced glycerol in BMGH as the carbon source. Other ingredients in BM(G)H remained the same as BMGH. In BMBWGH, BWG replaced the glycerol in BMGH as the carbon source. Other ingredients in BMBWGH remained the same as BMGH (BMC-BWGH was prepared from C-BWG and BML-BWGH was prepared from L-BWG).

Table 1: Media and stock solution recipes

Media/Solution (1 L)	**Recipe**
1M KPi [10X to BMGH]	118.1g KH_2PO_4 and 23g K_2HPO_4 in a total of 1000mL H_2O, pH 6.0 with KOH. Autoclave
YNB (10X)	$(NH_4)_2SO_4$ 100g, YNB 34g. Add water to 1 L. Autoclave
Glycerol (10X)	100mL glycerol. Add water to 1 L. Autoclave
Histidine (100 g/L) [2500X to BMGH]	100 g histidine, add water to 1 L. Sterilized by filtering
Biotin (500X) 0.2g/L [500X to BMGH]	20mg D-biotin in 100mL water. Sterilized by filtering
PTM_1 Trace Metals Solution (1000X)	Cupric sulfate-5 H_2O ($CuSO_4$-$5H_2O$):6.0g [3.84 g for $CuSO_4$] Sodium iodide (NaI): 0.08 g Manganese sulfate-H_2O ($MnSO_4$-H_2O): 3.0 g Sodium molybdate-$2H_2O$: 0.2 g Boric Acid: 0.02 g Cobalt chloride: 0.5 g Zinc chloride: 20.0 g Ferrous sulfate-$7H_2O$: 65.0 g Biotin: 0.2 g Sulfuric Acid: 5.0 mL Water: to a final volume of 1 liter. Sterilized by filtering
Buffered Minimal Glycerol Medium with Histidine (BMGH)	1M KPi Buffer, pH 6.0 (10X): 100 mL YNB (10X): 100 mL Glycerol (100 g/L, 10X): 100 mL Histidine (2500X): 0.4 mL Biotin (500X): 2 mL Water: 697.6 mL (Autoclaved with Glycerol solution) Agar (only for frozen stock): 15 g

Yeast cell growth evaluation from different carbon sources

P. pastoris growth using different carbon sources was evaluated. BMGH (commercial glycerol), BMC-BWGH (C-BWG), and BM(G)H (glucose) media as stated above were used for yeast culture. Cells were first cultured in a 50mL sized conical tube with 10mL culture media at 30°C for 5 h at 270 RPM. Cell suspensions were then transferred into a shake flask containing 30mL fresh media. Cells in flasks were then cultured at 30°C for additional 18 h at 300 RPM. Cell samples were taken each hour during the exponential growth phase and less frequently during the stationary phase for cell density and pH evaluations. A Spectronic 20 Genesys spectrophotometer (Thermo Scientific, Wilmington, DE) was used to measure cell density at 600nm wavelength.

Yeast cell growth toxicity study using C-BWG as single carbon source

C-BWG is a complex mixture with glycerol, lye, soap, and methanol from the biodiesel production (16). It may be likely that some components in C-BWG are harmful to yeast growth when the C-BWG concentration in the media is too high. Therefore, potential toxicity to yeast cells using C-BWG as single carbon source was carefully evaluated. Yeast cells were cultured as the same manner as described above. We used 1%, 2%, and 4% C-BWG to replace glycerol

in BMGH culture media. Cell samples were collected during the culture. Cell density and pH of samples were measured.

Yeast cell growth using L-BWG as single carbon source

Different effects on yeast cell growth using L-BWG and C-BWG were studied. Cells were cultured as the same manner as described above. BMC-BWGH and BML-BWGH were prepared using 2% C-BWG and 2% various L-BWG respectively. Cell samples were collected during the culture and cell density was measured. Cell exponential growth rates were determined based on the cell growth curves.

Results and discussion

Yeast growth using different carbon sources

There were minimal differences between initial cell growth rate when using BMGH (commercial glycerol), BMC-BWGH (C-BWG), and BM(G)H (commercial glucose) as single carbon source for cell growth (Figure 1). The pH values of the media in the shake flasks during the culture of C-BWG and glycerol are almost identical. Since glucose is a fast consuming carbon source, the media pH of glucose cell culture decreased much faster than did the others (Figure 2). There was no perceptible difference in the overall cell growth rates due to the three different carbon sources. We thus, initially conclude that BWG is an acceptable carbon source.

C-BWG Toxicity Study

C-BWG at 1%, 2% and 4% were tested for yeast cells culture in the shake flasks. The difference between the cell growth rates and cell densities using the three C-BWG concentrations was minimal (Figure 3). The *P. pastoris* culture using 2% C-BWG had a slightly higher growth rate than the 1% or 4% C-BWG cultures. The pH value for the 4% C-BWG implied a lower cell metabolism rate at this concentration (Figure 4). Thus, we selected 2% BWG as the standard concentration for future BWG experiments.

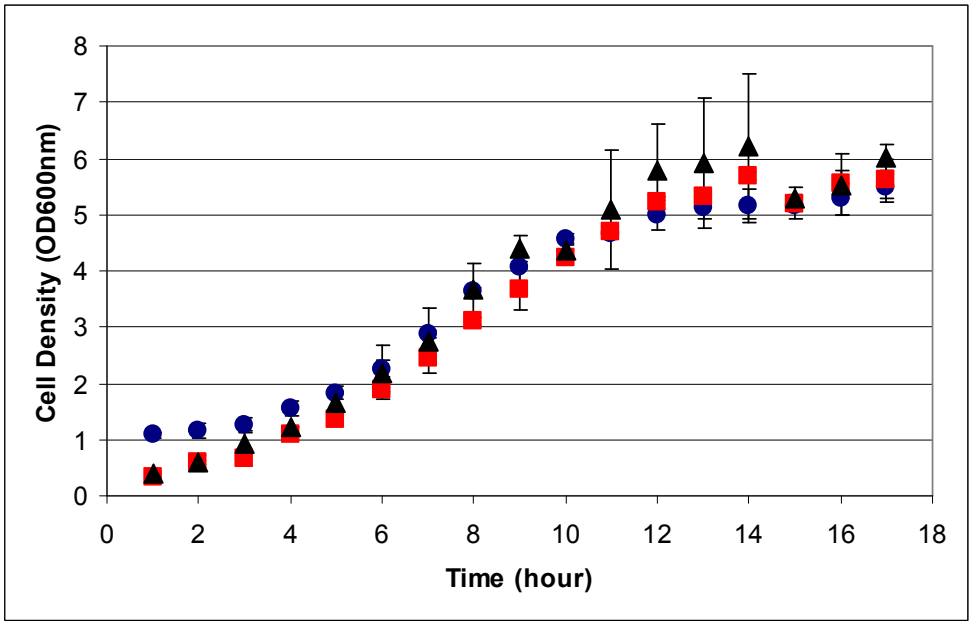

Figure 1: Yeast growth using commercial glycerol (squares), C-BWG (circles), and glucose (triangles) as single carbon source. The difference in initial cell growth rates using different carbon sources was not significant, which showed BWG as an acceptable carbon source for yeast cell culture.

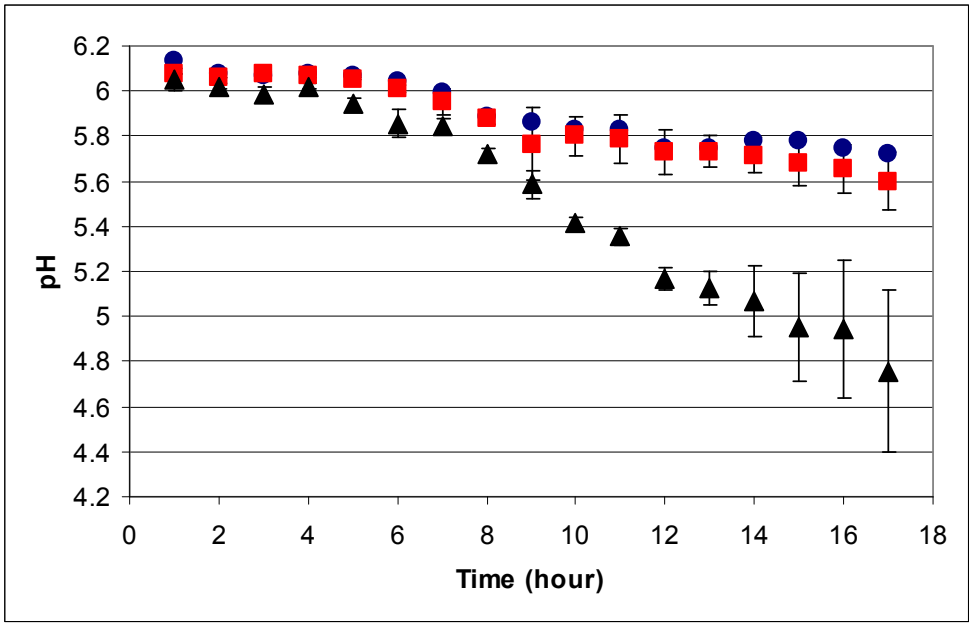

Figure 2: Media pH of yeast growth using commercial glycerol (squares), C-BWG (circles), and glucose (triangles) as single carbon source. Media pH during the culture of glycerol and BWG were almost identical. Media pH of glucose culture decreased much faster due to fast consuming carbon source feature of glucose.

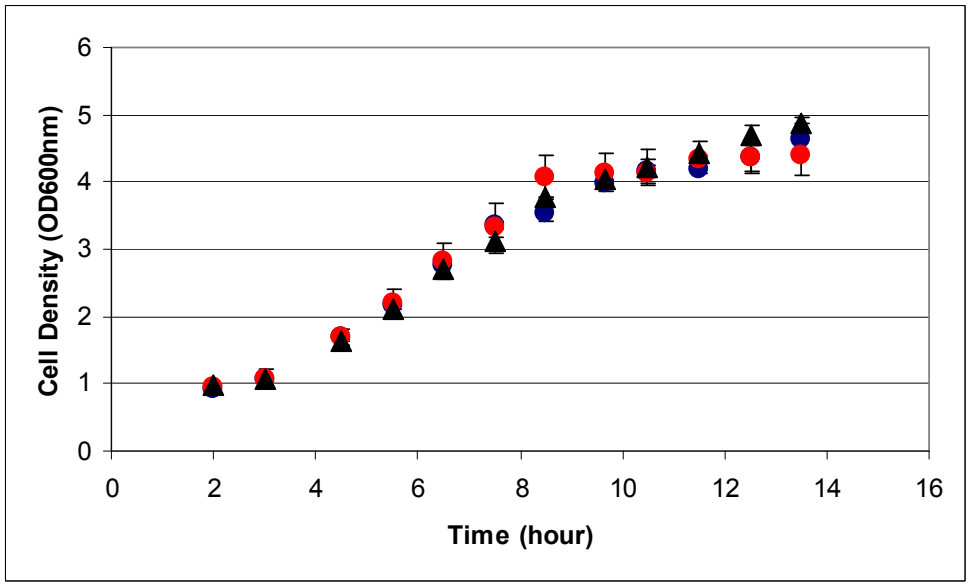

Figure 3: Yeast growth using 1% BWG (squares), 2% BWG (circles), and 4% BWG (triangles) as carbon source. Cell growth rate difference using the three C-BWG concentrations was minimal. Cell culture using 2% BWG had a slightly higher growth rate.

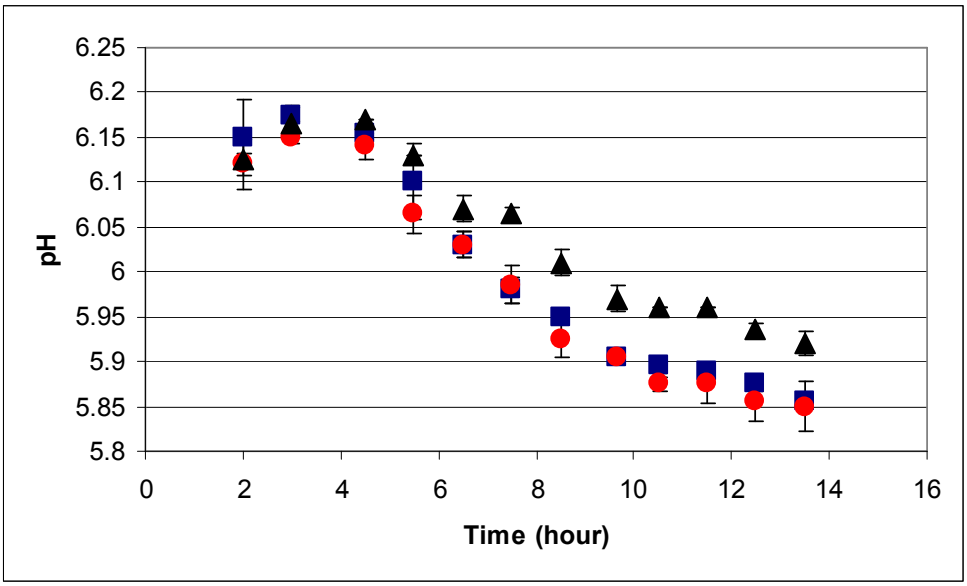

Figure 4: Media pH of yeast growth using 1% BWG (squares), 2% BWG (circles), and 4% BWG (triangles) as carbon source. Media pH curve of cell growth using 4% BWG implied lower cell metabolism rate comparing with others.

Table 2: Exponential growth rates of yeast cultured using different glycerol sources

Carbon source	Glycerol	C-BWG	Canola-BWG	Corn-BWB	Sunflower-BWG	Vegetable-BWG	Blend vege-BWG
Growth rate	0.27 ± 0.01	0.36 ± 0.05	0.34 ± 0.05	0.26 ± 0.02	0.30 ± 0.01	0.31 ± 0.04	0.29 ± 0.01

Study of L-BWG on Yeast Cells Growth

We observed there was no significant difference in growth rates using C-BWG and L-BWG (canola, sunflower, and vegetable) as the carbon sources for yeast cell culture (Table 2). The growth rates were slightly lower when using the corn BWG and blend vegetable BWG as the carbon sources to culture the yeast. The blend vegetable cooking oil was a blend oil of canola and vegetable oil, and contained no corn oil. We found C-BWG and canola L-BWG have the highest growth rate for yeast culture and the differences between other L-BWG as carbon sources were not significant. In comparison, the yeast growth rate cultured using glycerol was 0.265 ± 0.011, which was lower than that of C-BWG. Therefore, we conclude that *P. pastoris* yeast strain had higher growth rates on BWG as the carbon source, regardless of the source of the oil.

We concluded C-BWG is an acceptable replacement for commercially available lab-grade glycerol for *P. pastoris* yeast growth. Yeast cells also showed a strong tolerance to high BWG concentrations in the culture media. Yeast growth using L-BWG prepared from various cooking oil sources also showed higher growth rates than that using commercially available glycerol; specifically, but for unknown reasons, the C-BWG, L-BWG from canola, sunflower, and vegetable cooking oil provided the best growth media for the genetically modified yeasts. L-BWG from corn and blend vegetable cooking oil showed slightly lower growth rates for cell culture. This improved growth could be due to the transfer of micronutrients from the oil, and a deleterious effect from commercial food production. This study mainly focused on the cell mass growth using the BWG as the carbon source because cell mass is critical for industrial fermentation. Future studies are necessary to evaluate the protein production with different BWG sources.

Acknowledgement

We thank Dr. Sarah Harcum and Dr. Gary Lickfield for valuable suggestions, Shamik Sharma for his technical support.

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