Optimizing a nitrogen-supplemented, condensed corn soluble medium for growth of the Polyhydroxyalkanoate producer *Pseudomonas putida* KT217

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Abstract: *Pseudomonas putida* KT217 produces medium-chain-length polyhydroxyalkanoate (mcl-PHA) that is of commercial interest as a biodegradable plastic. To reduce PHA production costs, a less expensive medium to grow *P. putida* KT217 to a high cell dry weight (CDW) was developed. *P. putida* KT217 was grown in aerated shake flasks on a condensed corn solubles (CCS) based medium that provided several organic acids and carbohydrates that were utilized for growth. The medium was prepared by adding various amounts of fresh CCS (100-600 g/L, or 34.9-209.4 g/L dry basis) to water and then centrifuging and filtering. The CCS permeate media contained dry matter levels of 28.8-164.9 g/L. The CCS permeate medium containing 108.4 g/L solids produced approximately 6 g CDW/L, at a growth rate of 1.03 per hour, and maximum cell population of 4×10^9 CFU/mL. Because CCS is low in nitrogen, ammonium hydroxide was added at a level of 1.73 g/L, and this significantly increased the CDW (> 20 g/L) produced in an aerated bioreactor. Therefore, a nitrogen supplemented CCS medium could provide a cheap source of nutrients for production of *P. putida* KT217 and PHA.

Keywords: cell density, cell dry weight (CDW), biodegradable plastic, biocreator, pH, nitrogen supplement, growth rate
DOI: 10.3965/j.ijabe.20120504.007

Citation: Javers J, Gibbons W, Karunanithy C. Optimizing a nitrogen-supplemented, condensed corn soluble medium for growth of the Polyhydroxyalkanoate producer *Pseudomonas putida* KT217. Int J Agric & Biol Eng, 2012; 5(4): 62－67.

1 Introduction

Pseudomonas putida KT217 is a mutant of *P. putida* KT2442, affected in its glyoxylate pathway by means of an *aceA* knockout[1]. This causes an increased flux of acetyl-CoA to the fatty acid synthesis pathways and leads to increased production of medium chain length polyhydroxyalkanoate (mcl-PHA).

PHAs are a class of biodegradeable polymers that are suitable replacements for synthetic polymers in several applications. Costs of PHA production have been high and limited their entry into the marketplace[2]. Two of the main areas of concern include the high cost of the fermentation medium, and costs associated with PHA extraction[2,3]. As with any commodity chemical produced by fermentation, production costs could be lowered if a cheaper substrate could be used for growth and PHA production. Numerous studies have evaluated PHA production using low cost substrates from industrial waste streams. Starch wastewater[4], beer malt[5], soya waste[5], hydrolyzed whey permeate[6], glycerol water[6], olive oil mill wastewater[7], waste frying oil[8], food scraps[9,10], hemicellulose[11], molasses and corn steep

Received date: 2012-08-07 **Accepted date:** 2012-11-14
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liquor[12,13] (CSL) are just a few of the industrial by-products evaluated for PHA production. Unfortunately, most of these substrates have not resulted in high PHA production rates.

Condensed corn solubles (CCS) is a by-product of the dry-mill ethanol process, in which the whole corn kernel is the first ground, mixed with water and enzymes to saccharify the starch into glucose, and then fermented to ethanol by yeast. The resulting beer is then distilled, and the remaining solution is called whole stillage. The whole stillage is centrifuged to remove the course suspended solids, and the remaining supernatant (i.e., thin stillage) is evaporated to a syrup-like material. This syrup, CCS, contains proteins, corn oil, starches and cellulose of varying chain lengths, glycerol, glucose, organic acids, and a number of macro and micro-nutrients. The CCS produced by each ethanol plant varies slightly, depending on their respective processes. These processing variables can include oil removal, fractionation of the corn kernel prior to fermentation (bran, germ, and endosperm), fermentation recipes, temperature profiles in the process, microbial contamination levels, etc. In general, CCS is sold as liquid cattle feed or is added to the solids from the whole stillage and dried to form dried distillers grains with solubles (DDGS). With the rapid expansion of corn ethanol production, the volume of these byproducts is saturating feed markets and reducing prices. Therefore, CCS represents an inexpensive nutrient base for a fermentation medium.

Prior work in our lab[14-18] has shown that a number of bacteria and fungi can grow on a fermentation medium derived from CCS. The objective of this study is to determine the optimal concentration of a CCS permeate-based growth medium for *P. putida* KT217 and to examine addition of nitrogen for increasing carbon utilization and growth of *P. putida*.

2 Materials and methods

2.1 Bacterial strain and maintenance

The bacterium used was *P. putida* KT217, obtained from Dr. Bernard Witholt (Institute of Biotechnology, Swiss Federal Institute of Technology, Zurich, Switzerland). Long term storage was via lyophilization, while short term storage was on tryptic soy agar slants. The culture was routinely transferred in tryptic soy broth (supplemented with tetracycline) shake flasks incubated for 24-48 hours at 30℃ and 250 r/min. Subcultures were also transferred to shake flasks containing various concentrations of the CCS based medium described below (also containing tetracycline).

2.2 Experimental design

Initial experiments to determine the optimal CCS concentration were carried out in one liter aerated shake flasks (800 mL working volume) with aeration at 1 V/V·min (as determined by rotameter), agitation at 250 r/min and 30℃. A one percent inoculum (v/v) was utilized in all experiments, with 24-h *P. putida* KT217 culture grown on the same medium used in the respective experiments (to minimize lag phase). Antifoam from Cognis (Clerol FBA 3107) was utilized as needed.

CCS used to prepare the growth media was obtained from a local dry mill ethanol plants. CCS was analyzed at the Olson Biochemistry laboratories of South Dakota State University. The CCS permeate-derived growth medium was prepared by mixing a given amount of CCS (100, 200, 400, or 600 g fresh weight, or 34.9, 69.8, 139.6, or 209.4 g dry basis) with deionized water to a volume of one liter. The medium pH value was adjusted with sodium hydroxide to 7.3 for the aerated shake flask trials. The resulting mixture was centrifuged at 11 000 r/min (18 150 g) for seven minutes at 15-25℃ to remove suspended solids. The liquid portion was then filtered through Whatman 113 filter paper to remove most of the remaining suspended solids and oils. The final CCS permeate media contained 28.8 g/L, 54.3 g/L, 108.4 g/L, or 164.9 g/L of dry solids.

After the optimal CCS permeate concentration was determined, trials were conducted in five liters Bioflo III bioreactors containing three liters of the 108.4 g/L CCS permeate medium (dry basis), supplemented with 1.73 g/L of ammonium hydroxide. The medium pH value was dropped to 6.7 after autoclaving. A concentrated solution of sodium hydroxide was utilized to adjust the pH value to 7.0 at the beginning of incubation. The pH value remained stable during incubation so that

further pH value adjustments were not needed. Aeration was held constant at 1 v/v/m with agitation at 500 r/min. Cerol FBA 3107 (Cognis) was utilized as needed for foam control.

2.3 Cell dry weight and colony forming unit measurement

Cell dry weight (CDW) was determined by harvesting 30-50 mL samples and centrifuging at 4 000 r/min for 30 min. Cell pellets were washed with 30 mL deionized water, re-centrifuged, and dried to a constant weight in a 60℃ oven. Colony forming units (CFU) were determined by serial dilution and plating in triplicate on Tryptic Soy Agar (TSA).

2.4 HPLC analysis

A Waters HPLC system with refractive index detector was used to quantify sugars, organic acids, and glycerol in the samples. Prior to analysis, samples were filtered through 0.2 μm filters, then 50 μL injections were made. The mobile phase was helium-degassed 4 mM sulfuric acid at 0.6 mL/min, through a Biorad HPX-87H organic acid analysis column operated at 65℃. Standard solutions of maltose, glucose, lactic acid, acetic acid, propionic acid, succinic acid and glycerol (at 3 g/L and 30 g/L) were used to calibrate the integrator.

2.5 Nitrogen and phosphorus analysis

Samples were assayed for nitrogen and phosphorus by using Hach test kits and a Hach DR/2010 colorimeter. Samples were filtered through 0.2 μm filters, then diluted 1:100 dilution with double distilled water. Nitrogen was measured as ammonia by using the Hach HCT 102 Unicel kit. Phosphorus was measured as free phosphates by using the Hach HCT 122 Unicel kit.

2.6 Statistical analysis

All trials were replicated in triplicate, with statistical analysis by using the Tukey-Kramer test via Jump® software package (SAS institute).

3 Results and discussion

3.1 CCS analysis

The CCS from a local ethanol plant was analyzed for chemical composition and the results were shown in Table 1. Many of these components could serve as necessary nutrients for growth of *P. putida*. During medium preparation, the CCS was diluted (34.9-209.4 g/L on dry basis) and filtered, thus removing suspended solids. The permeate composition from the 34.9 g/L CCS medium was also shown in Table 1. The protein in the permeate could serve as a nitrogen source for cellular protein needs of *P. putida* KT217. The fat component consisted of both triglycerides and free fatty acids; however, because *P. putida* is deficient in lipase activity[19], only the free fatty acids would be available for phospholipids synthesis. The nitrogen free extract component in CCS permeate represents glycerol, monosaccharides, polysaccharides, and organic acids, and most of these should have remained in the water soluble fraction, thus providing the carbon and energy source for metabolism. The ash component of CCS permeate represents the inorganic macro- and micro-nutrients which are important in structures such as phospholipids as well as enzyme cofactors.

Table 1 CCS and CCS permeate composition from a dry mill ethanol plant

Component[a]	CCS[b]	CCS permeate[c]
Dry matter, %	34.9	2.9
Crude protein, %	13.7	15.6
Crude Fat (Ether extract), %	16.2	10.5
Ash, %	9.5	16.0
Fat: Roese Gottlieb	16.2	10.6
Crude fiber, %	0.8	2.0
Nitrogen free extract, %	59.9	55.8
Copper, ppm	8.3	13.2
Sodium, ppm	5 620	7 587
Calcium, ppm	487	564
Magnesium, ppm	6 850	8 080
Zinc, ppm	49	37.6
Phosphorus, ppm	15 400	19 900
Potassium, ppm	22 900	34 600

Note: [a]Composition reported on a dry matter basis; [b]CCS as received from the ethanol plant; [c]CCS permeate from the 34.9 g/L (fresh w.t.) CCS medium.

3.2 Optimization of CCS concentration in growth medium

The concentration of nutrients in a medium can affect the growth characteristics of the microorganism. The CCS from dry mill ethanol plants contains a mixture of nutrients that shows variability from plant to plant, and even over time in the same facility. The first objective of this project was to characterize the growth of *P. putida* KT217 on CCS permeate media containing final dry

solids levels of 28.8-164.9 g/L (100-600 g/L fresh w.t.). Figure 1 shows the average growth curves for *P. putida* KT217 grown in aerated shake flasks.

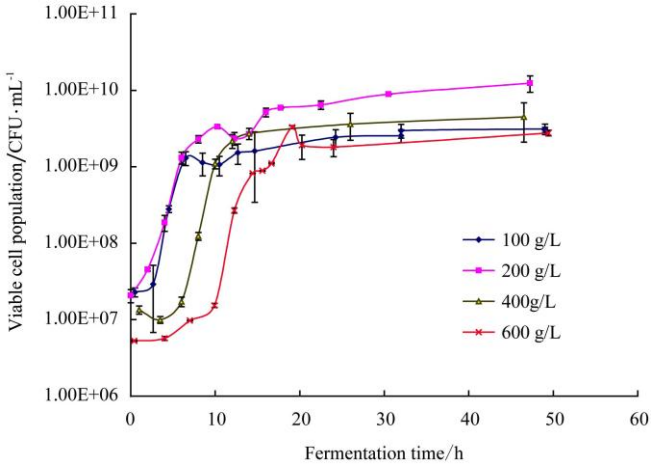

Figure 1 Growth curve of *P. putida* KT217 on CCS derived culture media. Error bars represent the standard deviation from three replications. 100 g/L fresh w.t. = 28.8 g/L dry; 200 g/L fresh w.t. = 54.3 g/L dry; 400 g/L fresh w.t. = 108.4 g/L dry; 600 g/L fresh w.t. = 164.9 g/L dry

Even though each of the media formulations used inoculum grown in the same CCS permeate formulation, there was a significant difference in lag times. The 28.8 g/L and 54.3 g/L CCS permeate media showed the shortest lag time, while the 108.4 g/L formulation was intermediate and the 164.9 g/L medium exhibited a lag phase of approximately 10 hours. The increase in lag time, as well as the lower initial cell concentration observed as the CCS concentration increased, was likely due to increased osmotic stress on the organism.

Table 2 shows the final CDW, growth rates, and 24 hours viable cell populations (CFU/mL) for these trials. The highest growth rates were observed in the 28.8 g/L and 108.4 g/L CCS permeate media (ranging from 1.03 to 1.06 per hour), but the 54.3 g/L formulation was statistically similar at the 0.05 level with a growth rate of 0.85 per hour. The growth rate in the 164.9 g/L medium was significantly lower at 0.67 per hour. The 24 h viable cell counts showed no significant difference among any of the four media, with means ranging from 2.7×10^9 CFU/mL to 8.6×10^9 CFU/mL. However, the final CDW did segregate into two statistical groups. The 28.8 g/L and 164.9 g/L permeate media had means ranging from 2.5 g/L to 2.7 g/L, while the 54.3 g/L and 108.4 g/L formulations showed means ranging from 5.8 g/L to 6.2 g/L. This roughly correlated with viable cell counts, but was also likely impacted by greater accumulation of PHA in cells grown in the 54.3 g/L and 108.4 g/L CCS media as observed by phase bright granules via phase contrast microscopy.

Table 2 Effects of CCS levels on growth of *P. putida* KT217

Parameter	CCS Levels			
	100 g/L	200 g/L	400 g/L	600 g/L
Final CDW (g/L)	2.694^{A}	5.811^{B}	6.159^{B}	2.531^{A}
Growth rate (per hour)	1.06^{A}	$0.85^{A,B}$	1.03^{A}	0.67^{B}
24 h Viable cells	$3.2e^{9\,A}$	$8.6e^{9\,A}$	$4.5e^{9\,A}$	$2.7e^{9\,A}$

Note: For each parameter, different superscript letters are used to denote averages that are statistically different at the 0.05 level.

Overall, the 54.3 g/L and 108.4 g/L CCS permeate media performed better than the formulations with lower or higher CCS levels. This could be explained by the lower nutrient levels in the 28.8 g/L CCS permeate medium, while the 164.9 g/L CCS medium may have caused osmotic stress from high dissolved solids concentration. The 108.4 g/L CCS medium was chosen as optimal concentration because it yielded the highest growth rate and final CDW. This formulation was subject to foaming, but this could be controlled by adding antifoam. The pH in all trials rose from the initial levels of 6.6-6.7 and to final pH of 8.0.

The nitrogen concentration (ammonia) measured at the beginning of the shake flask trials was less than 100 mg/L in all cases. There was also nitrogen available (in the form of protein) to *P. putida* KT217. Analysis of *P. putida* cell mass showed a crude protein of 44.3% on a dry matter basis. This protein content was calculated by multiplying the nitrogen content by 6.25. Therefore approximately 7% of the cell mass is nitrogen by weight. Assuming that the majority of the crude protein measured in the CCS permeate is not available to the organism, and the low amount of nitrogen was measured as ammonia at the beginning of incubation, we concluded that nitrogen was a limiting nutrient, since the carbon sources were not depleted. In all trials the lactic acid, acetic acid, and succinic acid were preferentially utilized during incubation. The 164.9 g/L trial did not use any of the

glucose present, whereas in the other trials glucose was completed consumed. Glycerol was utilized to a very low extent in the 28.8 g/L and 54.3 g/L trials but remained constant in the remainder of the trials, because nitrogen was limiting, and subsequent trials were evaluated supplementation with ammonium hydroxide.

3.3 Effects of nitrogen supplementation of the 400 g/L CCS medium

Ammonium hydroxide was added to the 108.4 g/L CCS permeate medium to determine its effects on cell growth. These trials were carried out in a bioreactor which allowed for more uniform aeration, mixing, and pH control compared to shake flasks. Nutrient concentrations were also monitored more thoroughly in these fermentations, using HPLC for carbon/energy source and testing kits for nitrogen and phosphorus.

As shown in Figure 2, a CDW of 22 g/L was obtained, which was statistically different than the trials without nitrogen supplementation. The maximum cell population was 6.0×10^{10} CFU/mL (data not shown), which was also statistically different than that of the 108.4 g/L trials without nitrogen supplementation. *P. putida* KT217 was also found to sequentially utilize certain carbon sources available in the medium. The glucose and organic acids were firstly consumed, followed by the glycerol. Succinic acid levels dropped in the first 15 hours of incubation, but then leveled off. In complex media such as CCS it is possible that other compounds may co-elute with succinic acid, and we believe this explains the succinic acid trend shown in Figure 2. In these trials, the nitrogen was completely utilized along with most of the carbohydrates contained in the medium. There were however some longer chain carbohydrates that were not utilized by this organism. At approximately 25 hours, phosphate levels rose, which was most likely due to lysis of dead cells.

These trials showed that the optimal concentration of CCS in a fermentation medium for *P. putida* KT217 was 400 g/L (fresh weight) or 139.6 g/L (dry basis). After centrifuging and filtration this medium contained 108.4 g/L dry solids. In aerated shake flask trials this formulation resulted in a high growth rate (1.03 per hour) and CDW of 6.1 g/L. Subsequent trials in a bioreactor with additional nitrogen (1.73 g/L of ammonium hydroxide) resulted in higher CDW of 22 g/L. However, these cell concentrations were lower than expected for an aerobic growth phase, since approximately 70 g/L of useable carbon source (total carbohydrates and glycerol) was provided and only 22 g/L of biomass was obtained. This amounts to a conversion efficiency of just over 30%, whereas we would expect conversion efficiency closer to 50%. This was most likely due to nitrogen limitation at 24 hours which coincided with plateuing of CDW.

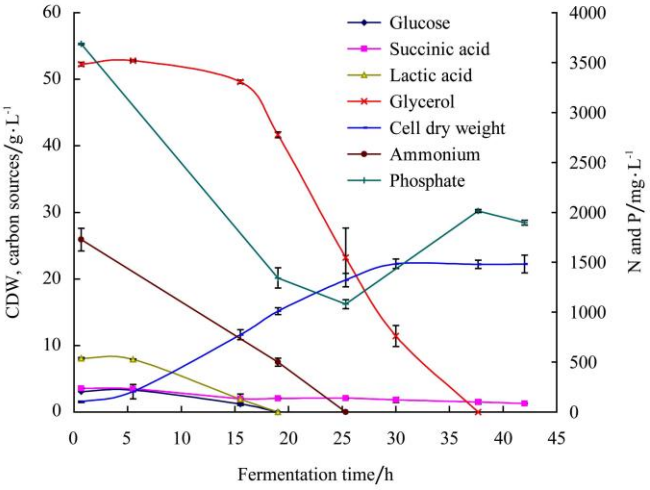

Figure 2 Growth of *P. putida* KT217 on a 400 g/L CCS based medium (108.4 g/L dry w.t.) with 1.73 g/L of ammonia added. Points are the means of measurements obtained from three separate trials. Nitrogen and phosphorus are shown on the right-hand axis

Others have made similar attempts to use co-products of the corn wet-milling industry. For example, CSL was tested as a medium component along with sugarcane molasses for growth of *Bacillus megaterium*[12]. In their report, the best growth was observed when an inorganic form of nitrogen was utilized, but not when CSL was used as a nitrogen source. Similar results were obtained in this experiment when ammonium hydroxide was added as the nitrogen source. In another experiment using CSL and cane molasses[13] the optimal levels for inclusion of molasses and CSL for growth of *Azobacter beijerinckii* were found to be 65.4 g/L and 13.2 g/L, respectively, resulting in biomass production of around 15 g/L. These are much lower inclusion rates than tested in this experiment, which was due to their lower final biomass.

4 Conclusions

This study determined that the optimal concentration of CCS in a fermentation medium for *P. putida* KT217 was 400 g/L (fresh weight) or 139.6 g/L (dry basis). After centrifuging and filtration this medium contained 108.4 g/L dry solids. Nitrogen supplementation through addition of ammonium hydroxide (1.73 g/L) improved the cell dry weight from 6.1 g/L to 22 g/L. Future work will determine the amount of nitrogen necessary to increase carbon conversion to biomass and reduce the amount of carbon used strictly for maintenance energy. In addition, fed-batch fermentations will be carried out with substrates such as glycerol water and soapstock to maximize PHA formation.

Acknowledgments

The authors are grateful to the funding provided by the South Dakota Corn Utilization Council.

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