Chemostat Fermentation of *Xanthomonas Campestris* with a Small Working Volume

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Abstract:

A strategy for chemostat fermentation of *Xanthomonas campestris* XWCM-1 for xanthan gum production by using a small working volume is given. *X. campestris* growth kinetics were determined during growth in five media and a defined media, DM1/AA, was selected for use. The maximum growth rate was $0.09hr^{-1}$. Samples taken from the chemostat had colonies of differing morphology (smaller) than normal XWCM-1. BIOLOG analysis showed these colonies to be a variant. A variant is problematic in maintaining steady state conditions as its growth kinetics may differ. The media likely has insufficient supply of a nutrient for stable growth and production that may be causing mutation. Nitrogen additions did not have any observable affect. In a subsequent run, a possible variant that may be better adapted to the media conditions and prove to be a better producer of xanthan than already established strains was detected. Viscosity from xanthan production decreases mixing abilities in the small apparatus. Production optimization experiments are thus limited in this fermentation apparatus, as these will increase the viscosity. The chemostat may be useful for future work in evaluating physiological processes of *Xanthomonas* and ultimately genetic manipulation experiments.

Introduction:

Xanthan gum is a polysaccharide produced by the obligate aerobic bacterium *Xanthomonas campestris*. Xanthan gum provides stable viscosity over a wide range of temperatures and has compatibility with high ionic environments. The commercial application of xanthan gum includes food additives, industrial goods, and in oil recovery.

Maintaining a chemostat for this fermentation would be highly valuable for evaluating physiological processes of *Xanthomonas*. Chemostat is a continuous fermentation regulated by a limiting nutrient (Goldberg and Er-el, 1981). Steady state conditions, in which growth rate is defined by dilution rate (Calcott, 1978), can be maintained as a result of this limitation. In non-viscous fermentation, steady state

conditions are useful for basic understanding and optimization of growth and product formation (Elwood, 1987; Goldberg and Er-el, 1981; Stanbury et al., 1984). Once a steady state is reached in a chemostat, changes to the media or environmental conditions can be correlated directly to corresponding changes in growth and production of product from the organism under study (Elwood, 1987; Goldberg and Er-el, 1981). At the industrial level this is highly valuable as an easy way to maximize quality and production of the sought after product, in this case xanthan gum.

Viscosity development in polysaccharide fermentation creates a challenge in applying chemostat technology to these processes. Accumulation of polysaccharide produced by *Xanthomonas campestris* during fermentation processes causes high broth viscosity. Physical and biochemical limitations normally associated with batch fermentation processes such as bulk mixing, mass transfer, gas exchange and sample handling and analysis are exacerbated by viscosity (Stanbury et al.,1984). Work in chemostat with high viscosity is further complicated by the effects of hold-up (volumetric increase associated with entrained gas in viscous broth), which upsets the dilution rate constant required for the maintenance of steady state conditions (Calcott, 1978).

Media is the principal component for the organism's growth in fermentation. Two forms of media considered are complex and defined media. Complex media is composed of substances with variable and undefined elemental compositions. Defined media has a known and replicable elemental composition. A defined media is best to use in chemostat fermentations. The elemental composition must be known in order to proceed with physiological studies in which the composition of the media is manipulated.

The goal of this project is to develop a process for evaluating xanthan fermentation using chemostat fermentation at a small working volume (1L).

Materials and Methods

Growth rates

Five media from previous studies were selected for analysis, three defined media and two complex media as controls. Tryptone-Yeast Extracted plus glucose (TYEG) and E-1 are the two complex media; DM1/AA, GATES, and Nitrate are the three defined media formulations. E-1 is a minimal medium that contains a complex nitrogen source and is used at CP Kelco as a standard flask media. The TYEG medium is a rich medium designed to provide optimum nutrition for cell growth. DM1/AA is a medium that had been formulated by Peter Mirrasoul (2000) during previous work done to develop a defined medium for productivity. GATES medium was found in work done by Smith (Tate & Lyle; 1976) and Nitrate medium in work done by Tompsett (Tate & Lyle; 1979). The media were prepared in four different groups with the first group placed in nephelometer flasks. Remaining groups were placed in Erlenmeyer flasks and added to the nephelomenter flasks after autoclave sterilization. A strain 4142 seed was prepared with YM media and incubated in a Kuhner Shaker model ISF-1-W with a 1" throw at 300rpm and held at 30^0C for a 24 hr period. Nephelometer flasks were inoculated with 0.1ml of the prepared seed. Photoelectric colorimeter readings were taken at the time of inoculation and every hour subsequently with a Klett-Summerson instrument model 900-3. Maximum growth rates were obtained by plotting data on a linear scale with an exponential trend line along points of greatest growth (Fig.1). The resulting equation for the line ($X=X_0e^{\mu t}$) was then given in which μ is maximum growth rate, X_0 is initial density, X is final density, and t is the time between density readings.

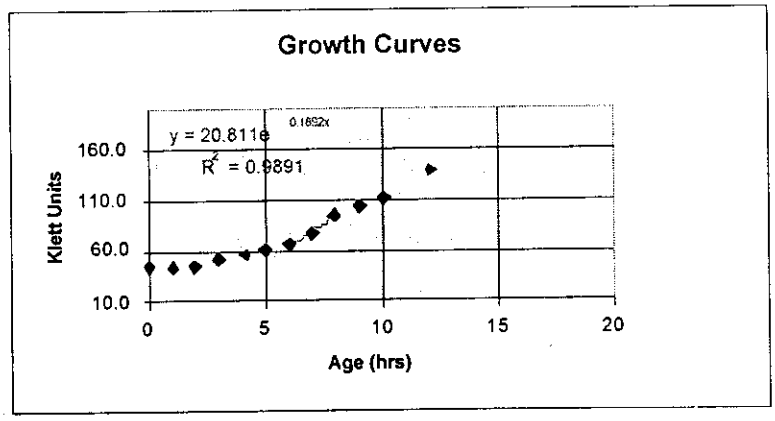

Figure 1. Growth curve of DM1/AA from the five media growth curve analysis with a trend line at the greatest growth fluctuation and an equation for maximum growth.

DM1+AA evaluation

Growth curves were obtained using the same methodology as described previously with adjusted DM1/AA media while using non-adjusted DM1/AA and TYEG as controls. DM1/AA media was adjusted in three ways by removing amino acids, removing amino acids and HoLe Salts, and by removing amino acids and supplementing with added nitrogen. Data was evaluated as before for maximum growth rate.

Batch run

A batch fermentation of strain 4142 was done in a 2L Applikon apparatus. DM1/AA medium was prepared as before except the first group was placed into the Applikon before sterilization with 0.5 ml of Hodag K-60-K antifoam added. All other media components, after separate sterilization, were added together and aseptically transferred into the Applikon. Environmental conditions such as mixing RPM, airflow, pH, and temperature were controlled by GENESIS programming using set conditions in a 14L station. A sample of the medium inside the Applikon was taken before inoculation and checked for contamination by preparation of a streak plate and a microscopic examination. This was repeated immediately after inoculation (0 hr) and then twice daily (only one steak plate prepared each day). Analyses for pH, optical density, viscosity, and residual sugar concentration were performed on samples taken after inoculation. Seed for inoculation was prepared as for the growth rates experiment.

Chemostat

Chemostat fermentation was done in the 2L Applikon apparatus that was modified with an inlet port and J-tube effluent port added to the head plate. Tubing connecting the feeding port and effluent weir were run through duplex pump heads mounted to a variable speed drive motor. The tubes for each line were loaded in opposite orientations to insure flow in the proper direction for each. The tubing on the effluent

weir was of a larger internal diameter than that used for the feed line. This was done to insure the effluent rate would always be greater than that of the feed and the level of the medium in the vessel would be controlled by the height of the weir tube A sampling port was added to the effluent line with a Y connector so that samples would not affect growth vessel working volume. Two impellers and two small baffles provided mixing in the Applikon.

Weighing feed and waste containers, acid and base containers, and samples taken from effluent line provided a means to monitor the working volume. Any difference in media added (feed, acid, and base) and removed (waste and samples) would be the difference in the growth vessel volume. Weights of these were taken at each sampling period.

Seed for inoculation was the same seed as for the DM1/AA evaluation experiment. The vessel was first run in batch mode with the same parameters as in the "Batch run" experiment. Dm1/AA media, as described in "Growth Rates" experiment, with 20g/L of glucose was used as the feed media. Once glucose levels were minimal the pump for media feed and waste was started at $0.09hr^{-1}$-dilution rate (1.5ml/min). After a time this dilution rate was reduced to $0.045hr^{-1}$ (0.75ml/min) once wash out was apparent.

Sampling and analysis was performed as in the "Batch run" experiment. All other parameters were measured and controlled by the GENESIS computer program. For this run, the chemostat fermentation proceeded for approximately 270 hours.

The second and third chemostat fermentations were started as in the "Batch run" both differing from the first run by the DM1/AA media having 20g/L glucose and the seed started in YM media. Once glucose was minimal, the feed of DM1/AA at 32.75g/L glucose was started at $0.045hr^{-1}$-dilution rate. Sampling and all other parameters were the same as in the first chemostat run.

In the third chemostat fermentation media optimization experiments began. Once steady state was achieved elemental supplements were added individually to the vessel at twice the concentration as found in the feedstock for each element tested. After a one

third turnover time (approximately 7.5 hrs) a sample was taken from the effluent stream and analyzed and compared to previous samples for response. Nitrogen was the first nutrient to be supplemented at approximately 114.5 hours and then sulfur at approximately 163 hours.

Results

Growth rates

All defined media had slower growth rates than complex media (Table 1). Overall, TYEG complex media had greatest growth and, amongst the defined media, DM1/AA had greatest growth.

Table 1. Maximum growth rates for five media by optical densities with a comparison for two media of values found in a later evaluation.

Media	"Growth" experiment:	"DM1/AA evaluation":
TYEG	0.2544 hr^{-1}	0.1983 hr^{-1}
E-1	0.077 hr^{-1}	
DM1/AA	0.189 hr^{-1}	0.098 hr^{-1}
GATES	0.057 hr^{-1}	
Nitrate	0.054 hr^{-1}	

DM1/AA evaluation

DM1/AA exhibited a greater growth rate then the adjusted versions of this defined media (Fig.2). Maximum growth rate values for TYEG and DM1/AA were lower compared to previous work (Table 1).

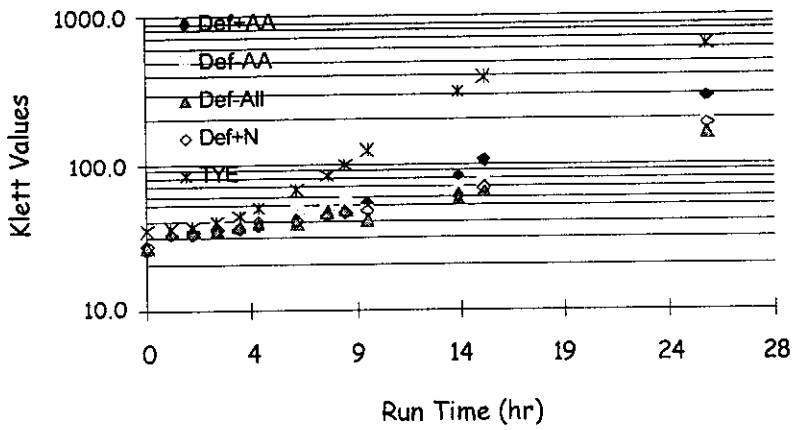

Figure 2. Growth curves for DM1/AA (Def/AA) adjusted media and non-adjusted media (Def-AA = without Amino Acids; Def-all = without amino acids and Hole salts; Defend = without amino acids and added nitrogen; Def/AA = non-adjusted; TYE = control).

Batch run

No contamination occurred throughout the fermentation. Problems with airflow occurred. The mass flow controller was not calibrated properly and the inlet air filter needed to be tightly joined with the connector. Once viscosity was established, visibly the outer portions of media in the Applikon were not mixing adequately.

Chemostat

Chemostat run 01062819

After feeding of medium began the glucose concentration increased exponentially while optical density and viscosity decreased (figs. 3 &4). The feed rate for the medium was then slowed to half the previous value. Initially it was believed that the maximum growth rate for Xanthomonas in DM1/AA media was $0.18hr^{-1}$. With a dilution rate set at half this amount ($0.09hr^{-1}$) wash out occurred. Maximum growth rate was then re-calculated from growth results from the DM1/AA evaluation, which had lower rates, and the value from this was $0.09hr^{-1}$. By slowing the feed rate to half the dilution rate was set to half the actual maximum growth rate. Following dilution rate adjustment, steady state

conditions were slowly approached. At about 200 hours the residual sugar concentrations began to rise with optical density and viscosity decreasing.

The 217-hour plate had colonies of differing morphology (smaller) than normal strain 4142; a contaminant was detected as well. The new colony, normal colony, and contaminant were isolated and analyzed with a BIOLOG. The contaminant found was a gram-negative rod of the genus Leclercia. The new colony typed out as a variant of *Xanthomonas campestris*. The colony with the normal morphology was confirmed to be strain 4142.

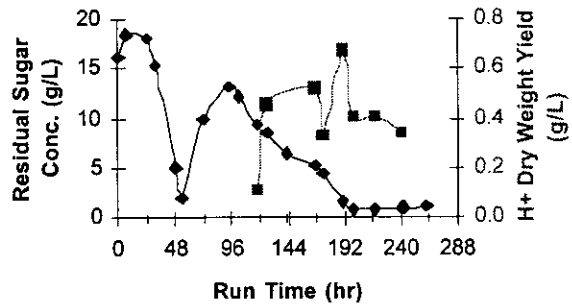

Figure 3. Residual sugar concentrations from YSI analysis for chemostat run 01062819.

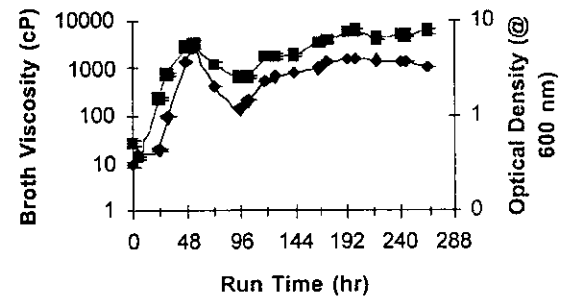

Figure 4. Viscosity and optical density data collected from twice daily sampling and analysis for chemostat run 01062819.

Chemostat run 01071019

At approximately 50 hours the effluent stream rate had slowed and was exceeded by the influent stream rate. Thus, the vessel filled with

media. After different attempts to fix the situation, contamination was a threat. The run was terminated and reset. The effluent line was replaced with larger tubing (size 16).

Chemostat run 01071619

At approximately 114 hours the chemostat appeared to have reached steady state (figs. 5 & 6). The nutrient spike of nitrogen that followed had no immediate affect. After a spiking of sulfur the glucose concentration dropped quickly with no change in optical density or viscosity. After adding additional sulfur to the feedstock the glucose levels increased to levels before any nutrient spikes occurred. Optical density and viscosity decreased to the same level before spiking. Nitrogen was added again at about 259 hours as a check, no changes were observed.

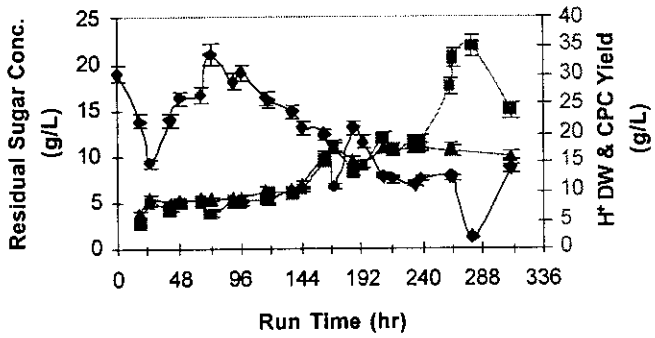

Figure 5. Residual sugar concentration and polymer concentration (H+DW & CPC Yields) measured from samples taken routinely from the 010716-chemostat run.

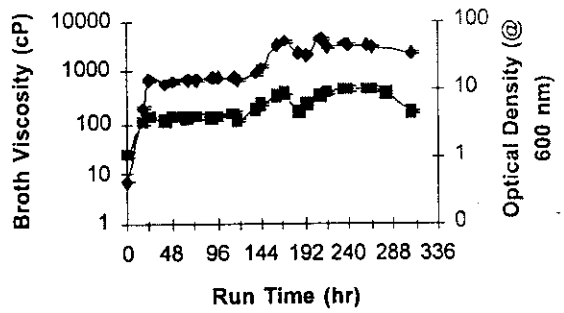

Figure 6. Viscosity and optical density measured from samples taken routinely from the 010716-chemostat run.

At about 300 hours the feed was not adding media to the vessel. The previous feed bottle had emptied before a new bottle was exchanged. This may have caused crystals to accumulate in the feed line. A larger feed line (size 14) was subsequently added. The run was terminated as viscosity had risen to a level in which poor mixing occurred. The pH was then measured improperly and too much base was added, raising the pH to detrimental levels. When viscosity increases to values >1000cP proper mixing becomes a problem, as seen in this incident.

A comparison between the growth kinetics of the culture in the chemostat after the change in measurements occurred and the original culture used to inoculate the chemostat was done. No difference in kinetics was detected.

Discussion

Growth rates

Growth rates were not as expected even for the controls. A greater rate of growth was expected with less of a lag period. A defined media was needed for the chemostat work so that when other analysis began it would be known what constituents were available in the media for growth. DM1/AA, the defined media chosen, did not exhibit robust growth but was the better of the defined media studied. The maximum growth rate for this media was low and created a longer turnover time then desired.

DM1/AA evaluation

DM1/AA defined media contains numerous components which makes preparation more time consuming. Ideally some of the components could be eliminated without affecting growth. Removal of components, however, restrained growth more. In this

study a different media was used to grow seed then in the growth rates analysis. The difference in seed media may account for the lower growth rates exhibited for DM1/AA.

Batch run

Inadequate mixing along the walls of the Applikon unit may cause problems in maintaining the chemostat. Homogenous mixing is essential, providing all organisms throughout the vessel the same amount of nutrients. Mixing is also important in maintaining physical parameters by preventing localized reactions that may alter pH monitoring, etc. Oxygen dispersal is directly dependent on mixing, especially in viscous solutions where bubbles coagulate if not consistently disrupted.

Chemostat

Chemostat run 01062819

A difference in maximum growth rates between the two analyses of DM1/AA is likely due to the difference in media used in growing the seed inoculums. The chemostat was inoculated with the same seed as the nephelometer flasks for the evaluation work. The initial dilution rate of $0.09hr^{-1}$ resulted in wash out. According to calculations from the evaluation work, $0.09hr^{-1}$ is close to maximum growth rates. After the dilution rate was reduced to $0.045hr^{-1}$, steady state conditions appeared to be approached. This was evidence that the growth rate previously used was higher than what was occurring in the apparatus. Steady state conditions were approached slowly, a result of the added glucose provided from the higher dilution rate. The addition rate of fresh media was decreased not interrupted after wash out. This resulted in the organism having to metabolize not only the added glucose from the wash out but also what was still being added. Hence the organism had an excess of glucose and had to "catch up" to the glucose limitation that was being set by the lowered dilution rate. This caused a longer time for steady state conditions to be reached than if the feed had been temporarily interrupted, allowing the fermentation to proceed in batch until the excess glucose was metabolized.

As Calcott (1978) stated, the competitive environment of the chemostat, set by limitations, will often promote mutations. Therefore, the divergence in colonial morphology found after 200 hours might be expected.

Chemostat run 01071019

The sudden change in glucose concentrations, optical density, and viscosity that occurred well after the nitrogen spike was unusual. With a total turnover time of approximately 22 hours for a $0.45hr^{-1}$-dilution rate, the nitrogen added in the spike would have been replaced by normal media concentrations by 24 hours when the change occurred. This shift in conditions appears to be independent of the nitrogen spike. Steady state conditions may not have been achieved before the spike, which may account for these variations in analysis. The change that occurred after the sulfur addition may be related to this as well. This may be why the addition of sulfur to the stock resulted in readings that differed from those taken after the sulfur spike. No difference in growth kinetics between the chemostat culture and strain 4142 were detected in a subsequent growth study. This indicates a variant did not account for these changes.

Modifications to the 2L Applikon design should be made to allow for better mixing capabilities. Cooling loops should be expanded in diameter to allow for larger diameter impellers. The area of the baffling should be reduced to help improve bulk mixing as while minimizing mixing shadows, which develop on the down stream side of baffles as viscosity increases.

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References:

Calcott, P.H. (1978). The construction and operation of continuous cultures. In Continuous Cultures of Cells, pp. 13-27.

Elwood et al. (1987). Production of Bacterial Polysaccharides. United States Patent. Patent Number 4,696,900.

Goldberg, I. And Er-el, Z. (1981). The Chemostat-An Efficient technique for Medium Optimization. Process Biochemistry: pp. 2-8.

Mirrasoul, P. (2000). CP Kelco Fermentation Lab. Unpublished work.

Smith, C. E. (1976). Xanthan research and development report no. 8. (1976). Production of xanthan gum in continuous culture by *Xanthomonas campestris* - preliminary studies. Unpublished work

Stanbury et al. (1984). Microbial Growth Kinetics. In Principles of Fermentation Technology, pp.14-18. Pergamon Press, New York.

Tompsett, C. E. (1979). Xanthan research and development report no. 33. (1977). Effect of using a nitrate medium for growth and polysaccharide production by *Xanthomonas campestris* in continuous culture. Unpublished work.