High Yield Protein and DNA Expression in Flasks

Robert F Smith[1], Sam Ellis[2], Marinko Sremac [3], Craig Woodard[3], Steve Ellis[2], Paul F Torrence[1]

[1]Department of Chemistry and Biochemistry, Northern Arizona University, Flagstaff AZ 86011,

[2]Biological Sciences Division, Thomson Instrument Company Inc, Oceanside CA 92054

[3] Department of Biological Sciences, Mt. Holyoke College, South Hadley MA 01075

Abstract

Thomson Flasks* have provided an opportunity to increase the growth capacity of shake flasks to a capacity rivaling fermentors. Using Thomson flasks and a new media formulation, *E.coli* cultures obtained growth densities of 20 $OD_{(600)}$ or more. A comparison of generic glass and Thomson flasks of protein expression of soluble *vaccinia* thymidine kinase and DNA plasmid expression in a *XL1Blue* cell line demonstrated enhanced bacterial growth and increased protein/DNA yields.

Introduction

Historically, the expression of soluble recombinant protein in *E.coli* has been confined to milligram amounts of purified materials in traditional glass shake flasks. Difficultly expressed proteins were often limited to production of microgram amounts. This has largely been due to growth limitations from poor aeration [1,2]. We report here that the new design of Thomson flasks, combined with an improved media, overcomes these production barriers using common laboratory equipment.

Materials and Methods

TB Media was modified from a previous protocol [3] and contained yeast extract (24g), tryptone (16g), casamino acids (10g) (Fisher BL14242), 1% glycerol w/v, and 100 mM Tris-HCl buffer (pH 8.0) per L of media. This improved media was referred to as SRAM.

The addition of casamino acids allowed for greater cell density growth, and the substitution of Tris-HCl buffer for MOPS buffer allows for a better buffering capacity with reduced cost. As cell

lines differ in the pH needed for optimal growth, buffer pH should be adapted to the cell line of interest.

Bacterial Growth:

SRAM (2.5 L containing chloramphenicol 33µg/mL and carbenicillin 50µg/mL) was inoculated with an overnight culture of BL21 (DE3) (Invitrogen) containing plasmids pLysS (Invitrogen) and pET-16b:TK [3]. This was split into 500 mL aliquots, and then divided into 3 Thomson and 2 generic glass 2 L flasks. The cultures were grown in flasks at 37°C at 400 rpm with Thomson seals* for optimal aeration. At various time points up to 29 h, pH and $OD_{(600)}$ were recorded. A Beckman pH Meter and HP 8452A DAD UV-Vis spectrophotometer were used respectively.

A similar experiment was conducted with three Thomson flasks containing 250 mL of SRAM media over 36 h of growth at 37°C at 400 rpm.

DNA Expression:

TB media (50 mL containing ampicillin 50µg/mL and casamino acids (10g/L)) was inoculated with 250 µL of glycerol stock transformed *XL1-Blue* Competent Cells (Stratagene 200249) with plasmid pπ25.1 [6]. Identical cultures were grown in 3 Thomson 125 mL flasks with Thomson seals and 3 generic 125 mL glass flasks with aluminum foil seals. The cultures were grown for 18 h at 37°C at 400 rpm in a New Brunswick Scientific Series 25 incubator shaker.

DNA Quantitation:

A Qiagen Plasmid Midi Kit was used for DNA preparation. Purified Midi prep DNA was resuspended in 200 µL 10 mM Tris-HCl (pH 8.8). DNA was diluted 1:100 in H2O and the OD_{260} and OD_{280} were measured using a Beckman DU® 640 UV/Visible spectrophotometer.

Protein Expression:

SRAM (500 mL with chloramphenicol 33 µg/mL, carbenicillin 50 µg/mL) was inoculated with an overnight culture of *BL21 (DE3)* (Invitrogen) containing plasmids pLysS (Invitrogen) and pET-16b:TK [3] at 37°C. The culture was induced with 1 mM IPTG* at $0.5OD_{(600)}$ and then grown at 20°C at 400 rpm in a VWR Shaker (1575R). Flasks were covered with Thomson seals to provide optimal aeration.

Cells were pelleted 24 hours later by centrifugation at 3,000 x g for 20 minutes at 4°C and frozen at -80°C.

Protein Harvesting:

Cells were thawed and resuspended in IMAC* buffer (50 mM Sodium Phosphate pH 7.0, 1 M NaCl) with 1 X CØmplete Protease Inhibitor (Roche). Cells were lysed with 1X BugBuster (Novagen) and incubated with 2500 units Benzonase (Novagen). After a 30 minute incubation with rocking at room temperature, the supernatant was collected after centrifugation at 16,000 x g at 4°C for 20 minutes. The supernatant was incubated for 18 h at 4°C with 2 mLs Ni-NTA (Qiagen). The nickel resin was loaded onto a polypropylene column and washed with IMAC buffer with gradually increasing amounts of imidazole (0-100 mM). *Vaccinia* thymidine kinase was eluted with 5 volumes of IMAC containing 250 mM imidazole. Protein concentrations were measured via the BioRad Protein Assay using BSA as a concentration standard.[5]

Results

- The use of Thomson Flasks allowed cell growth to continue to at least twice (30 $OD_{(600)}$) the density of generic glass flasks (15 $OD_{(600)}$) at 30 h. (Figure 1)
- In cultures grown in glass flasks, the pH dropped steadily from 8.0 to below 6 over 12 h. Cultures grown in Thomson flasks showed a net decrease of only 1 pH unit (from pH 8.0-7.0) over 30 h. (Figure 2)
- When the volume of culture was decreased to 250 mL, higher cell densities were possible. After 30 h (at pH 7.6) three separate cultures had an $OD_{(600)}$ of at least 40, and after 49 h, an $OD_{(600)}$ of 79 was achievable. (Figure 3)
- When differences in bacterial cell mass were standardized by comparing µg purified soluble *vaccinia* thymidine kinase/gram bacterial cell mass, a tenfold increase of protein in Thomson flasks compared to generic glass flasks was observed (Table 1)
- When differences in bacterial cell mass was standardized by comparing µg DNA/gram bacterial cell mass, a twofold increase of plasmid DNA was observed. (Table 2)
- Soluble *vaccinia* thymidine kinase has been repeatedly purified in purities of 98-99% at concentrations of 8-15 mgs/mL from cultures harvested at 20-27 OD. (Figure 4)

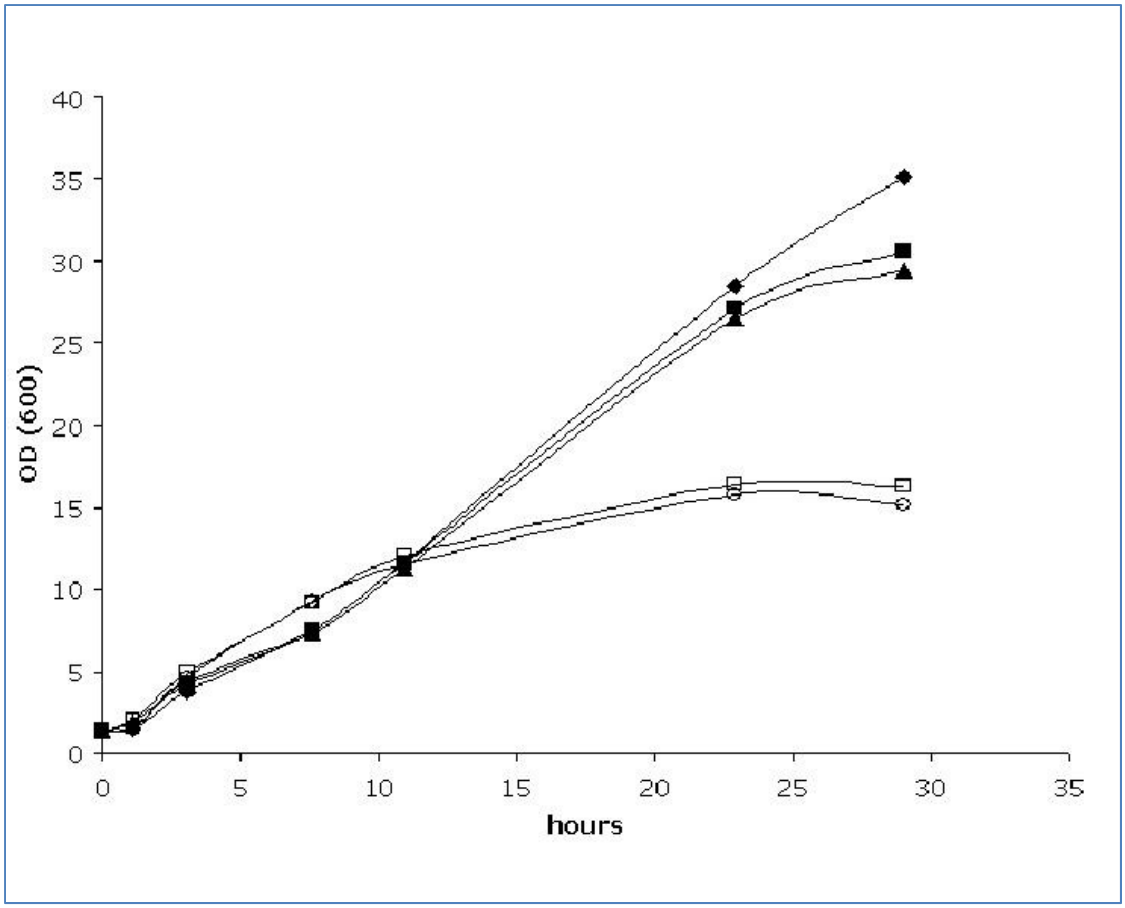


Figure 1. Cultures (500 mL) were grown as described in Materials and Methods. Cell densities were measured at the time points indicated. Closed symbols denote Thomson flasks, Open symbols denote glass flasks.

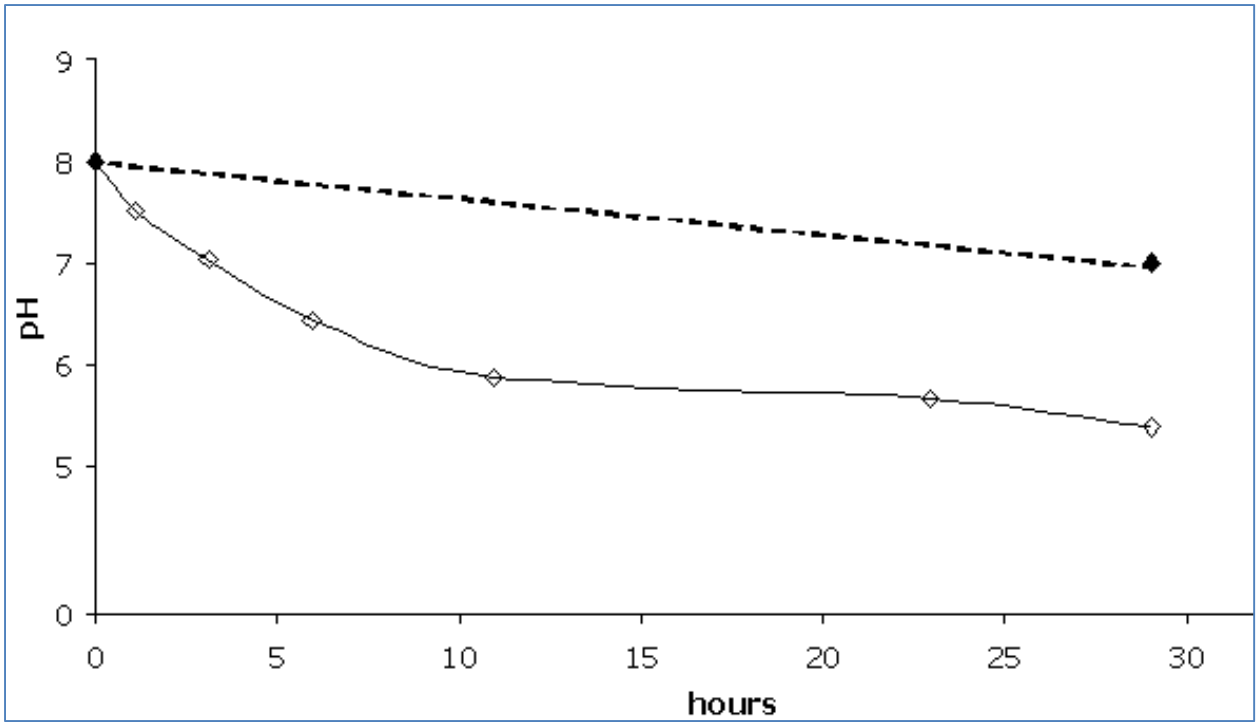


Figure 2. Culture (500 mL) was grown as described in Materials and Methods. pH measurements were taken at the time points indicated. Closed symbol denotes Thomson flasks, open symbol denote glass flasks.

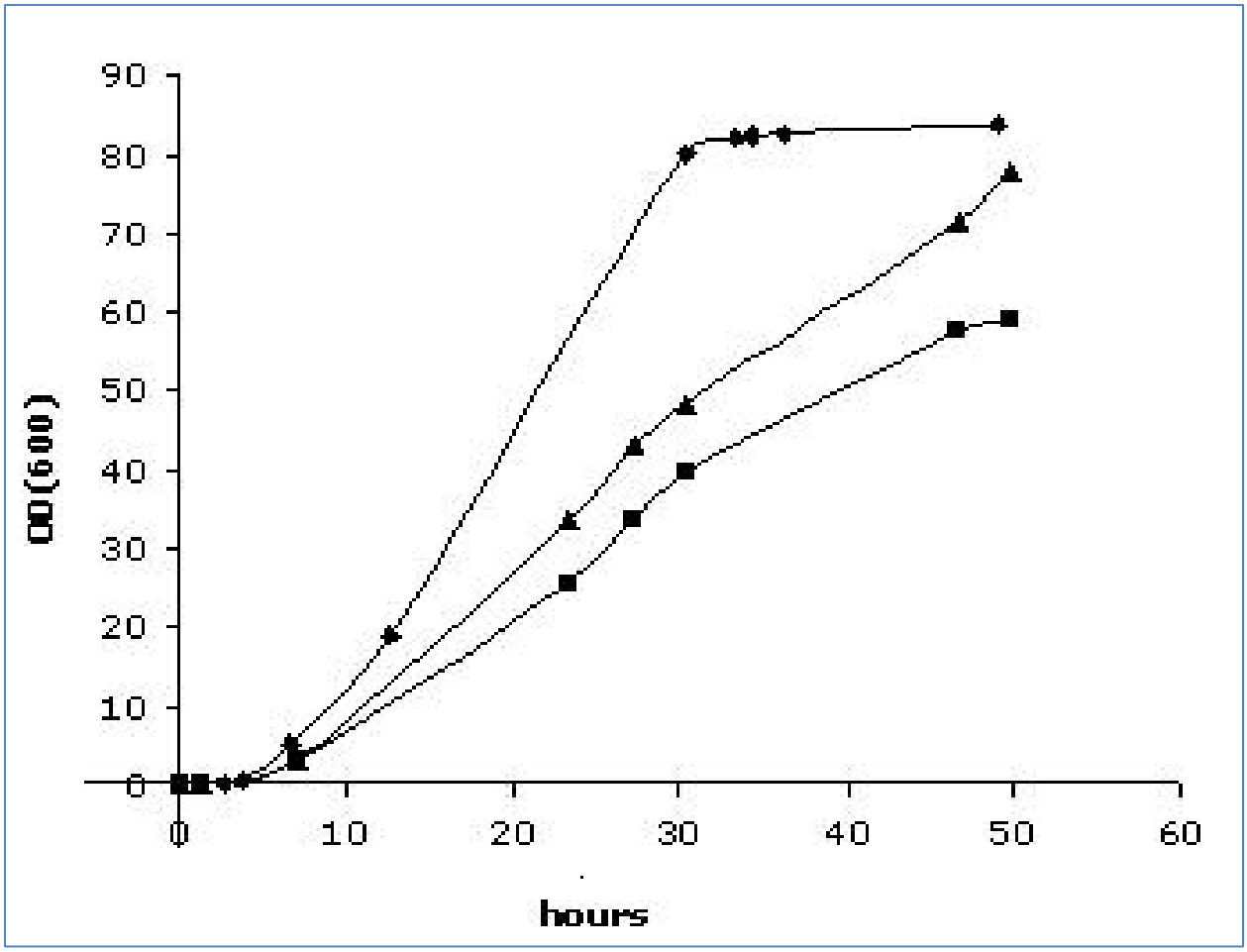
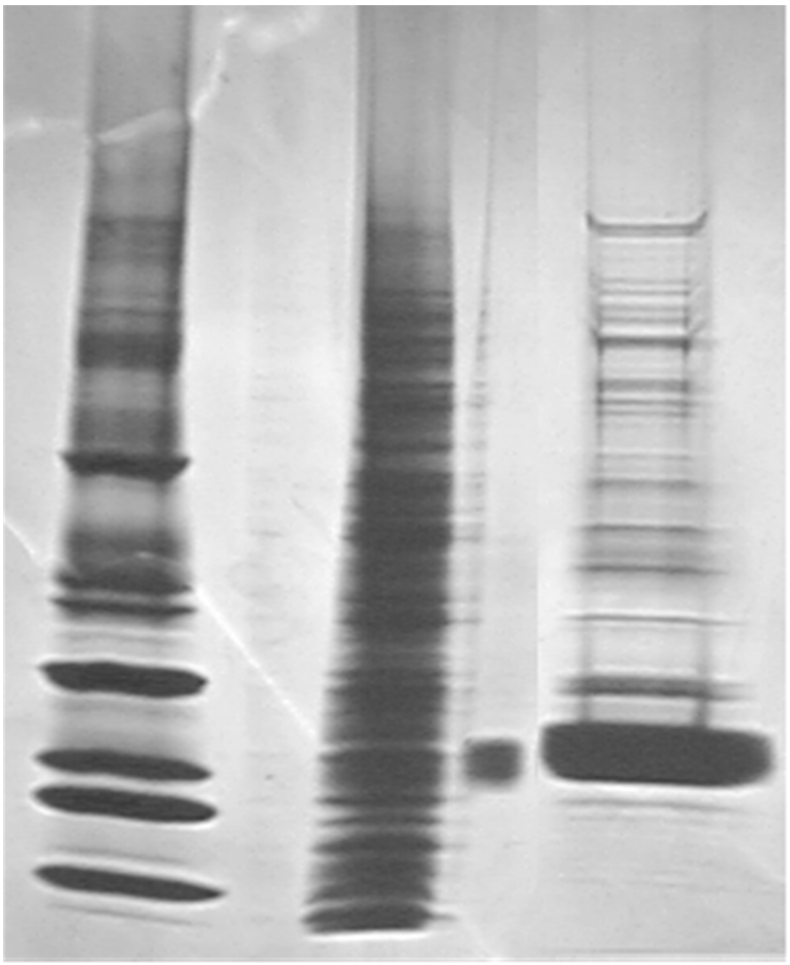


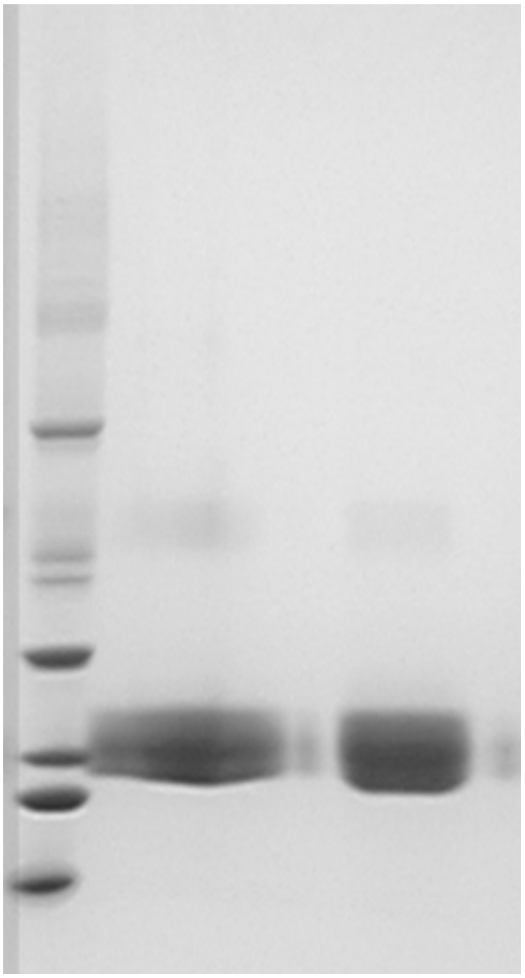
Figure 3. Culture (250 mL) was grown as described in Materials and Methods. Cell densities were measured at the time points indicated. Closed symbols denote Thomson flasks.

Figure 4: Coomassie Stained 4-20% Tris-Glycine SDS-PAGE Gels

Gel 1



Gel 2



* indicates position of *vaccinia* thymidine kinase

Gel 1:

Lane1: Sigma VII Marker

Lane 2: 25 µg total soluble protein from *E.coli* supernatant

Lane 3: 25 µg Ni-NTA purified protein from glass flasks at 0.8 mg/mL from culture at 15 OD

Gel 2:

Lane1: Sigma VII Marker

Lane 2: 30 µg Ni-NTA purified protein from Thomson flasks at 15 mg/mL from culture at 27 OD

Lane 3: 38 µg Ni-NTA purified protein from Thomson flasks at 18 mg/mL from culture at 23 OD

Table 1: Protein Purification	Avg µg purified *vaccinia* thymidine kinase	
Flask Type	500 mL of bacterial culture	µg protein/gram bacterial cell pellet
Glass	345	26.5
Thomson	5000	294.1

Table 2: DNA Purification	Avg µg total DNA	
Flask Type	50 mL of bacterial culture	µg DNA/gram bacterial cell mass
Glass	12.9	11.5
Thomson	93.7	33.3

Conclusions

These results developed from Thomson flasks, Thomson seals, and SRAM media have produced higher densities of cells than previously possible in shake flasks. Thomson flasks allowed for better aeration, thereby resulting in maintence of a lower pH over time, enabling better cell growth. With better cell growth, better protein and DNA expression was achieved. Using this system, the expression of *vaccinia* thymidine kinase, a protein that was only yielding 0.25 mg/L under standard media and glass flasks, was elevated to 10 mg/L of culture. The increased DNA plasmid yield in the *XL1Blue* cell line demonstrated that this system can be used for both protein and DNA.

References

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Abbreviations/Footnotes

Thomson Flasks: Thomson Ultra Yield™ Flasks

Thomson Seal: AirOTop™ Seal

IPTG: isopropyl-beta-D-thiogalactopyranoside

IMAC: Immobilized Metal Affinity Chromatography

pET-16b:T7 encodes for *vaccinnia* virus thymidine kinase with a 6XHis tag on the N-terminus of the protein