British Journal of Applied Science & Technology
4(10): 1472-1484, 2014

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Fermentation Optimization of *Mycobacterium smegmatis* Using Experimental Design

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Authors' contributions

This work was carried out in collaboration between all authors. Author SOW performed the fermentation procedure and designed the Doehlert matrix together with author ASP and written the first draft of the manuscript. Author ASP has developed the extraction procedure for MspA and designed the Doehlert matrix together with author SOW. Author PC has supervised the mathematical modeling. Author PHP has supervised the reactor design and operation. Author SHB has supervised the engineering endeavor and has supervised the writing of the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 18th August 2013
Accepted 1st October 2013
Published 5th February 2014

ABSTRACT

The exceptionally stable mycobacterial protein porin A (MspA) from *Mycobacterium smegmatis* has potential applications in protein-based solar cells, and as a biotemplate for nano-wires and nano-dots. These applications would be enabled by an efficient and cost effective method to grow the host organism at high cell mass yields, and recover purified MspA.
In this work, the cell mass yield was maximized and costs lowered by applying experimental design (varying nitrogen and iron contents according to a Doehlert matrix) based on a minimal fermentation medium that was reported earlier. Glucose use was minimized by adjusting glucose feed based on analyzing residual glucose after fermentation. The costs for extracted and purified MspA were lowered by 67% for the

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minimum medium and the optimized composition derived here compared to commercial medium (7H9 Middlebrook).

Keywords: Mycobacterium smegmatis; MspA; medium optimization; protein extraction.

1. INTRODUCTION

The mycobacterial porin A (MspA) forms a pore in the outer membrane of *Mycobacterium smegmatis* [1-3]. It provides the major diffusion pathway for hydrophilic nutrients. X-ray crystallography revealed an octameric assembly to form the goblet shaped superstructure of MspA [4]. Each monomer includes two consecutive 16-stranded β-barrels, forming the constriction zone in the octamer. The constriction zone, marked in Fig. 1, is the narrowest point throughout the pore with a diameter of about one nanometer.

Particular interest in MspA as a nanomaterial was raised due to its extreme stability. Its high thermal stability (92–112ºC) was confirmed by infrared and circular dichroism spectroscopy with MspA's stability exceeding even the highly stable porins of gram negative bacteria. MspA maintains its native structure in 2% SDS and at any pH. [5].

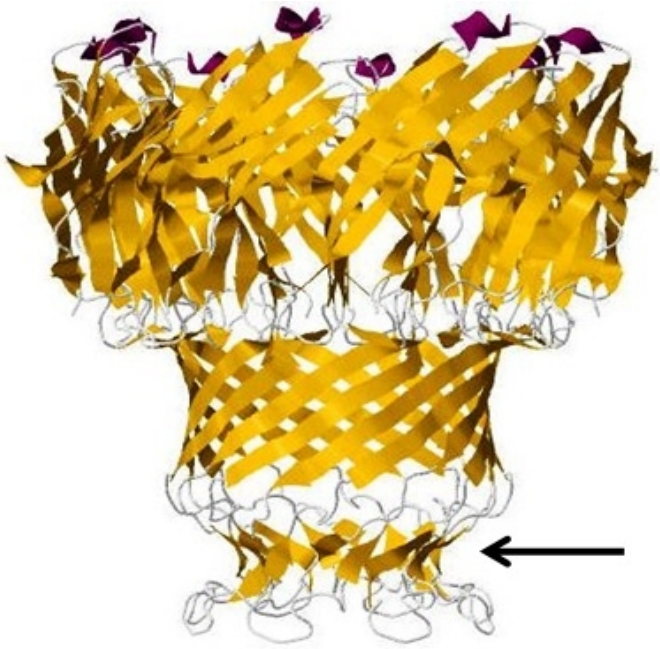

Fig. 1. MspA porin with its goblet like shape. The arrow marks the 16 stranded beta barrels. It is also the narrowest part of the pore (constriction zone) with an approximate inner diameter of 1nm (source: Protein Data Bank, code 1UUN [19]

These key features, as well as the ability to form hydrophilic homopores, allow many different applications for MspA: It has been deposited on HOPG-surfaces (highly ordered pyrolytic graphite), forming nanopores [6], complex protein networks and "letters"[7]- or star-shaped microstructures when deposited together with PMMA (Poly methyl methacrylate) [8]. MspA also reinserts in natural, as well as artificial, membranes and polymer layers [9-11].

Once inserted in a membrane it can form cation selective ion channels that show voltage gating [12]. When deposited on a MICA surface, MspA can stand perpendicular to its axis of symmetry freely and without the support of a self-assembled monolayer or polymer layer [13]. Based on MspA's ability to act as nanoscopic surfactant, the formation of vesicles has been studied [14,15]. These studies led to the development of MspA-based solar cells, in which sensitizer diads were tethered to the periplasmatic loop region of MspA. Octameric sensitizer-diad modified MspA form layers on electrode materials, such as TiO_2 in solar cells. This geometry is suitable for MspA's participation in photo-driven electron transfer processes [16].

The goal of the work reported here is to produce MspA efficiently and cost effectively. Therefore the MspA producing organism, *M. smegmatis,* is cultivated in an inexpensive and well-defined minimal medium. The known MspA extraction procedure for *M. smegmatis* cultivated in 7H9 Middlebrook medium [17] had to be adapted. To purify MspA, the cell membrane is disrupted with 0.5% n-OPOE (n-octyloligooxyethylene) in aqueous medium at 65°C, exploiting the thermal stability of MspA. Gel electrophoresis is used to determine presence and purity of MspA. Coomassie blue staining shows a single band in the extract (MspA) and very little contamination by protein fragments. A background expression of Msp porins is however still detectable in immunoblots using an Msp-specific antibody [17].

To lower the cost of the cultivation medium, this project relies on the use of basic carbon-, nitrogen-, and phosphorous-sources. The expensive antibiotic hygromycin is replaced by a copper complex ($CuSO_4$-Malachite green) to ensure the cultivation of monocultures [18].

2. MATERIALS AND METHODS

- Centrifuge Thermo Scientific Sorvall Legend RT+, Asheville, NC
- Gel electrophoresis equipment: Power Pac Basic, Mini PROTEAN Tetra cell, Bio Rad, Hercules, CA
- Gel electrophoresis chemicals: TEMED (Tetramethylethylenediamine), APS (Ammonium persulfate), Acrylamide / N,N'-Methylenbisacrylamide solution (30:1), Bio Rad, Hercules, CA
- Gel buffer 3x concentrated: 1l distilled water, 3M TRIS (tris-hydroxymethyl-aminomethane), 0.3 w% SDS, pH 8.45 adjusted with NaOH / HCl
- Cathode Buffer 10x concentrated: 1M TRIS, 1M Tricine (N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine), 1w% SDS, pH 8,25 adjusted with NaOH / HCl
- Anode Buffer 10x concentrated: 1M TRIS, ph 8,9 adjusted with NaOH / HCl
- Controlled environment incubation shaker, New Brunswick Scientific Co. Inc, New Brunswick, NJ
- 14ml polystyrene round bottom tube Falcon, Becton Dickinson Labware, Franklin Lakes NJ
- KIMAX 2l culture flasks, 125ml culture flasks, KIMAX, Janesville WI
- Phosphate buffered saline (PBS buffer) 10x concentrated: 1l distilled water, 1,36M NaCl, 45mM KCl, 100mM Na_2HPO_4, 18mM KH_2PO_4, pH 7,4 adjusted with NaOH / HCl
- PEN buffer 3x concentrated: 750ml distilled water, 300mM Na_2HPO_4, 0,3mM Na_2-EDTA, 450 mMNaCl
- Freestyle Freedom light blood sugar analyzer & test strips, Abbott Diabetes Care Inc., Alameda, CA

- The mc^2155 strain of *Mycobacterium smegmatis* was used in all experiments (courtesy of the Niederweis group, University of Alabama at Birmingham, purchased from ATCC)
- Millipore Centrifugal Filter Units MWCO 3000 Dalton, Millipore, Billerica, MA
- Yamato Sterilizer SM 200, Santa Clara, CA 95050, USA

2.1 Medium Preparation

A 1l Nalgene bottle was filled with 400ml of distilled H_2O. Potassium phosphate, sodium chloride, sodium nitrate and ferric chloride were added (amounts see Table 1) and the pH was adjusted to 7.2 with hydrochloric acid. A second 1l Nalgene bottle was filled with 560ml H_2O and Zinc Chloride and Glucose were added and pH was adjusted to pH 7.2 (HCl). Both bottles were autoclaved and the contents were then combined. 15ml mineral solution, 2ml Tween 80, and 2ml of a 0.01% Copper (II) sulfate-Malachite green solution were added. For the preparation of 1l 7H9 middlebrook medium see Table 2.

Table 1. Different media and their components

Chemicals	Simple medium	Improved simple medium after experimental design & glucose reduction
distilled water	960 ml	960 ml
Tween 80	2 ml	2 ml
glucose	20.0 g	12.0 g
sodium nitrate	0.5 g	0.5 g
Liquid Minerals	15.0 ml	15.0 ml
monobasic sodium phosphate	1.0 g	1.0 g
sodium chloride	1.2 g	1.2 g
zinc chloride	0.05 g	0.05 g
Copper Complex (to replace the antibiotic hygromycin)	2.0 ml	2.0 ml
Ferric chloride	50	20, 50, 100 mg
Ammonium chloride	1 g	0.6 g

Table 2. 7H9 Middlebrook Medium Composition (Fluka Analytical)

0.5 g/l Ammonium sulfate
2.5 g/l Disodium phosphate
1 g/l Monopotassium phosphate
0.1 g/l Sodium chloride
0.05 g/l Magnesium sulfate
0.0005 g/l Calcium chloride
0.001 g/l Zinc sulfate
0.001 g/l Copper sulfate
0.04 g/l Ferric ammonium citrate
0.5 g/l L-Glutamic acid
0.001 g/l Pyridoxine
0.0005 g/l Biotin

4ml Glycerol, 2.25ml Tween 80, 300µl Hygromycin was added

All chemicals mentioned are purchased from Sigma Aldrich, >99% purity if not otherwise noted

2.2 Growth Cycle

5ml maintenance cultures are constantly kept to serve as inoculum for growth cycles. The maintenance cultures are refreshed every two days by using 4.5 ml of 7H9 Middlebrook medium and 0.5ml from the previous culture to inoculate.

The Growth cycle is structured in three steps: small-, medium size and large cultures. To start a new small culture, 4.5ml medium and 0.5ml inoculum were combined in a 14 ml polystyrene round bottom Falcon tube allowing 2-3 days of growth time, using the shaker at 75 rpm, 37°C. A sterile KIMAX 125ml shake flask was filled with 36ml medium on the clean bench and inoculated with 4ml cell suspension from a maintenance culture without further shaking. The flask was covered with a metal lid and placed in an orbital shaker for two days.

The growth of the large cultures was carried out in 2000ml KIMAX shake flasks. The flasks were filled with 960ml medium and inoculated with a complete medium size culture, bringing the total volume in the flask to 1000ml. The flasks were sealed with aluminum foil to prevent contamination and placed in the shaker for 3 days (see medium size cultures for conditions).

2.3 MspA Purification Process

The methods of MspA purification and characterization are based on the procedure that has been published in reference 19. A large culture (about 1 l) was centrifuged for one hour, 3700g using 800 g capacity bottles. The supernatant was decanted while the cells were suspended in 50ml PBS buffer and the resulting suspension was divided in 50ml conical tubes for centrifugation at 10,000g for one hour using a fixed rotor. 10ml PEN buffer was added to the cell pellet to disperse the cells in the 50ml conical tubes after the supernatant was removed. The dispersed cells are collected in a 200 ml Nalgene bottle and kept at 60-65°C in a water bath under agitation (magnetic stir bar, 200rpm, 2cm length). The temperature range is very important for optimum results. 10 µl nOPOE was added and the cell suspension was kept in the water bath for one hour. The suspension was then transferred into a 50 ml conical tube and again centrifuged at 10,000 g for one hour. A 50 ml conical tube with water was used as a counterweight. The supernatant was collected in a 100ml nalgene bottle and 10 ml ice cold acetone (approx. -20°C) was added before storing the bottle in a freezer (-20 C) overnight. After centrifuging the cold suspension for 30 minutes at 10,000g the supernatant was discarded and the protein pellet was dissolved in 10ml PBS buffer before ultrafiltration. Centrifuging at 10000g for 30 minutes concentrates the product further.

2.4 Product Analysis: Gel Electrophoresis

To prepare an acrylamide separation gel for the gel electrophoresis 3.3ml acrylamide-BIS solution (30:1) was added into a 15 ml conical tube. Also 4.5ml gel buffer, 3.3ml bidest. H_2O and 2.25ml glycerol were added and mixed well. Next, 0.015ml TEMED and 0.135ml 10%APS were added and the solution filled into the glass chamber and the top covered with a layer of water. For the collection gel, 0.8ml acrylamide-BIS (30:1), 2.6ml gel buffer and 4.2ml dest. Water were added to a 15ml conical tube. Then 0.015ml TEMED and 0.160ml 10% APS were added and mixed. The covering water from the separation gel was removed and the collection gel solution was added. A comb is placed for pocket formation.

The gel was placed after 30 minutes in the electrophoresis cartridge and the comb was removed. After loading the samples and molecular marker into pockets, the electrophoresis process was started using 125V constant voltage.

2.5 Sterilization

Autoclaving at 110°C, 30 min holding time, was used for sterilization. Distilled ethanol in water at 70 %vol was used to sterilize equipment that could not be autoclaved.

2.6 Experimental design using a Doehlert system

The goal of a Doehlert experimental design is optimization of a process within a given window of process parameters by using a reasonably small number of experiments. The matrix of the Doehlert design [20] forms a hexagon with a center point (Fig. 2). The total number of experiments for one series is therefore seven (the corners of the hexagon plus the center) as indicated by the x in Fig. 2. The ferric chloride and ammonium chloride concentrations were varied at 5 and 3 levels, respectively (Table 3). The values were normalized for easier data handling. The two best results of the first series of experiments then form the center points of the hexagons for the second and third series.

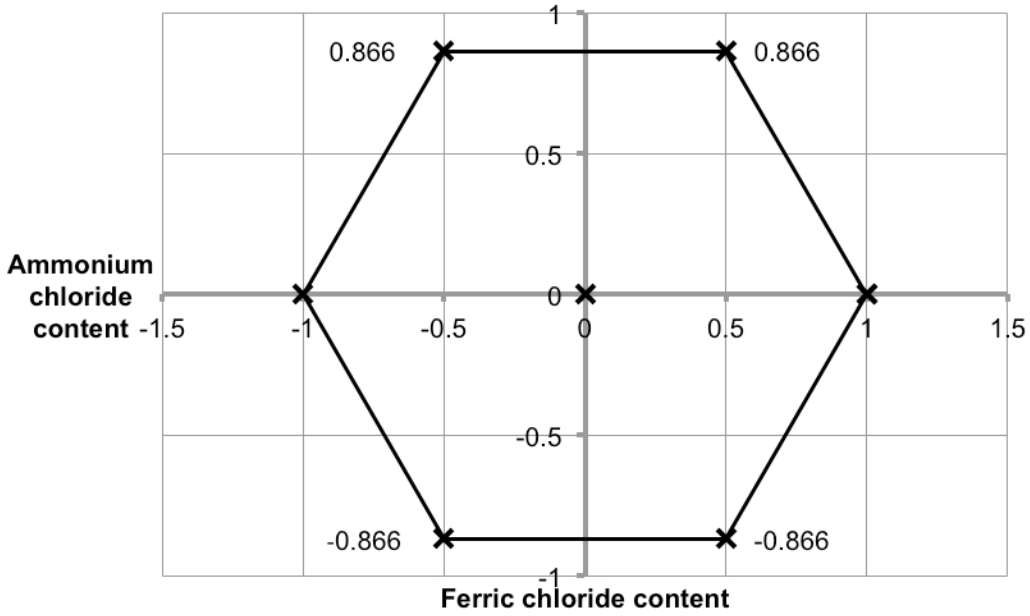

Fig. 2. The basic model of a Doehlert matrix for two variables: Iron content & Ammonium chloride content

2.7 Residual Glucose Analysis

The residual glucose after fermentation was determined with a commercial blood sugar analysis (Freestyle Freedom, Abbott) and standard operating procedure according to the manufacturer was followed. Due to the fact that these samples don't match the physical conditions of blood (e.g. salt concentration, viscosity, pH) the error is significantly higher than

the error proposed by Abbott (rel. error: 5%). This method will generally determine the sugar content with an accuracy of 29.26 mg/100 ml in the range used here. A glucose standard was used to determine the standard deviation and calculate the error.

3. RESULTS AND DISCUSSION

3.1 Experimental Design Results

A concentration of 0.6 g ammonium chloride per liter medium was found to suffice to reach the cell mass of 3±0.2 g observed previously at 1.0 g ammonium chloride per liter medium[18], resulting in a savings of 1.5 cents per liter medium shown in Table 4. There was no impact of the iron concentration on the growth of *M. smegmatis* in the range studied here. Fig. 3 and Table 3 show that the impact of ammonium chloride variation on mycobacterial growth is significant while iron chloride variation was not.

Table 3. Wet cell mass yields in g for various iron chloride and ammonium chloride concentrations. The non-bold numbers are the cell mass yields

		mg Iron chloride						
		12.5	**50**	**87.5**	**125**	**162.5**	**200**	**237.5**
g Ammonium chloride	**1.95**	1.9	1.9	1.9	1.8	1.7	1.7	1.7
	1.25	2.2	2.2	2.15	2.1	2.1	2.1	2.1
	0.6	2.7	2.75	2.8	2.7	2.6	2.6	2.6
	0.288	2.1	2.1	2.15	2.2	2.2	2.2	2.2

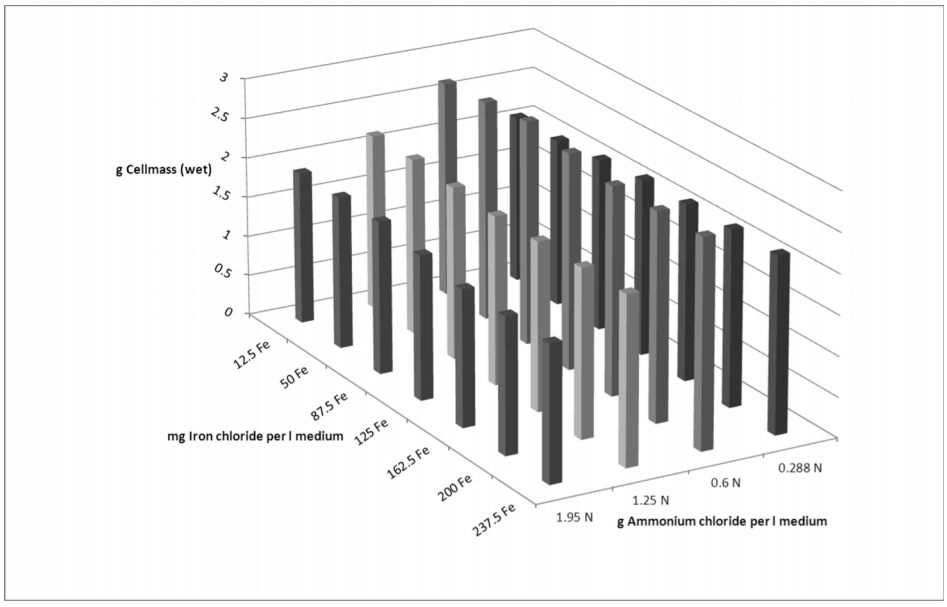

Fig. 3. Wet cell mass yields for various iron chloride and ammonium chloride concentrations. Changes in iron concentration have no significant impact on the cell mass yields ($p>0.05$). The cell mass yields for all data points with 0.6 g ammonium chloride differ significantly ($p<0.005$) from the other data and therefore support an optimal value for the addition of nitrogen source to the medium

The specific carbon-, nitrogen-, and phosphate-yields were calculated (average cell mass composition $C_{250}H_{611}O_{77}N_{55}P_6S$) [21] to ensure that none of these elements were growth-limiting. Dry cell mass consists of 51% carbon, 13.2% nitrogen and 3.1% phosphor. A mass balance for carbon, nitrogen and phosphorous (amount in cell mass vs. amount supplied to fermentation) revealed that the highest yield for nitrogen, calculated as shown by eq. 1, was at 26% of nitrogen supplied found in cell mass. Fig. 4 allows the comparison to other nitrogen/iron source configurations.

$$Y_{\frac{x}{s}Nitrogen} = \frac{Nitrogen\ bound\ in\ cellmass}{Nitrogen\ initially\ supplied\ in\ medium} \cdot 100\% \qquad \text{Equation 1}$$

The phosphorus yield was determined to show that phosphorus is not a limiting factor for mycobacterial growth. With a conversion of about 10-14 wt% it is unlikely that phosphorous limits the growth of *M. smegmatis*. However the conversion of carbon into cell mass is relatively low (5-8 wt%). It is likely that with 20g glucose per liter, too much carbon hydrate is provided. To determine how much glucose is excrescent, the residual glucose in the medium after the fermentation is analyzed and will be discussed later in this paper.

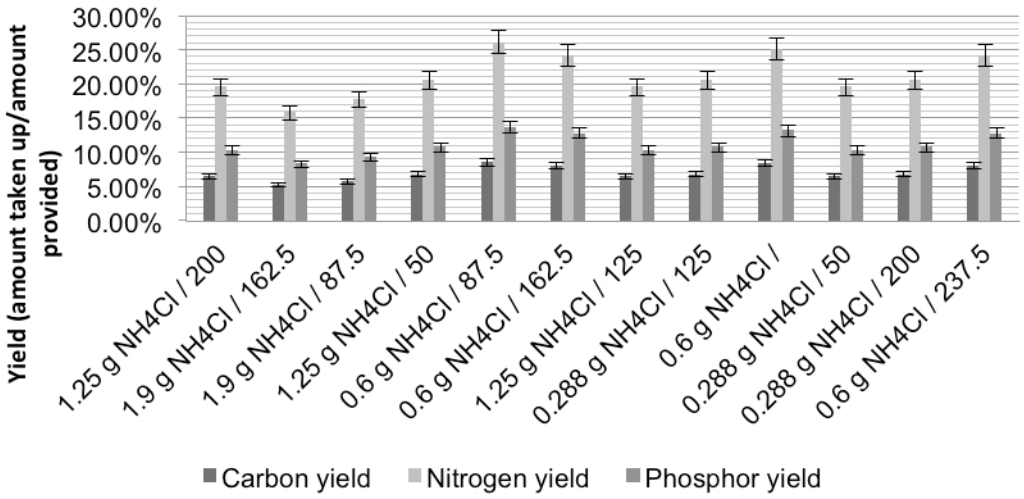

Fig. 4. Comparison of the carbon-, nitrogen- and phosphorus-yields for the experimental design fermentations. Every medium composition was tested 3 times and the standard deviation (< 6.6 %) was determined. The nitrogen yields for the addition of 0.6 g of ammonium chloride are significantly higher than for all other groups ($p<0.0002$)

3.2 Glucose Analysis

All samples showed a large amount of glucose left in the medium (Fig. 5). The data suggests that about 8 g to 10 g glucose remains unused in the medium. This information was used to reduce the amount of glucose provided in the medium by 8 g. This potentially lowers the costs by 24 cents per liter medium.

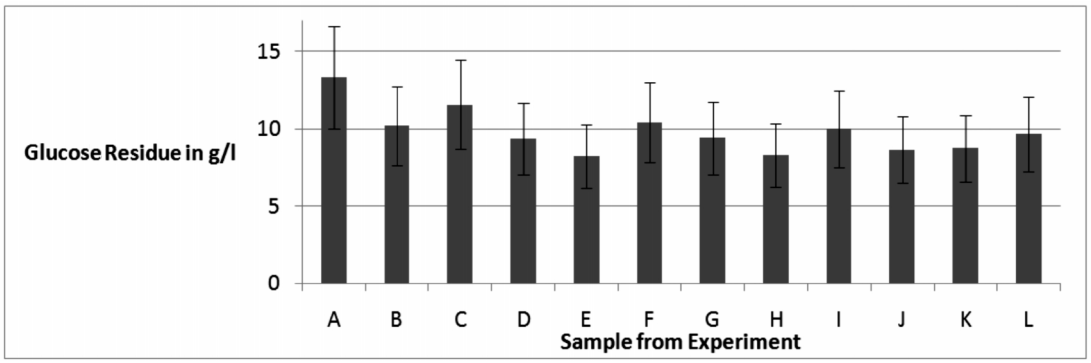

Fig. 5. Residual amounts of glucose in the medium after the fermentation

3.3 MspA Extract Analysis

The product was analyzed using the cells from the 0.6 g ammonium chloride and 125 mg iron chloride sample and gel electrophoresis with a coomassie blue stain. The single band on top of the left column in Fig. 6 is MspA, as previously established in the literature and verified by MALDI [22]. The light streaking below the band indicates the presence of protein fragments but no other band is formed. The right column contains the molecular marker identifying MspA.

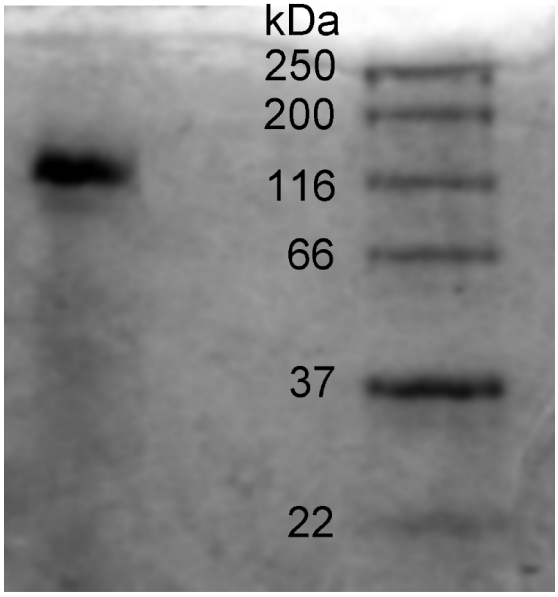

Fig. 6. Gel from MspA analysis with molecular marker

The yield of MspA from a 1 liter culture is 2.9 mg in average for the determined optimal medium conditions. (840µg/ml determined by Bradford assay in 7 ml). If MspA is extracted from a 7H9 Middlebrook culture the average yield is 2.2 mg from a 1 liter culture.

3.4 Cost Analysis

A comparison between the costs per liter medium was made. The prices for each chemical were taken from Fisher Scientific. Table 4 outlines the calculation for one liter of each type of medium.

With about $2 per liter the 7H9 Middlebrook option is the most expensive one. In a first attempt, the costs were lowered by 55% to approximately $0.91 per liter of medium. With the recent results from the experimental design fermentations and the reduction of the amount glucose provided the costs were reduced by another 28% to $0.66 per liter. Overall we were able to reduce the costs by 67% from $2 to $0.66 per liter of culture medium.

Table 4. Cost estimations for different types of medium

Substance	Amount	Price $	$/g or ml	$/l 7H9 Medium	$/l Simple Medium	$/l Optimized Simple Medium
NaCl	150 kg	658.4	0.0041	-	0.00492	0.00492
Na5P3O9	1 kg	73	0.073	-	0.073	0.073
NaNO3	500 g	8.2	0.0164	-	0.0082	0.0082
FeCl	3 kg	107.71	0.0359	-	0.001795	0.00314125
ZnCl	1 kg	77.35	0.07735	-	0.0038675	0.0038675
Glucose	5 kg	150.17	0.03	-	0.6	0.36
NH4CL	25 kg	924.73	0.036	-	0.036	0.0216
7H9	500 g	150	0.3	1.26	-	-
Glycerol	4 l	821	0.205	0.492	-	-
Tween	500 ml	42	0.084	0.189	0.168	0.168
Copper Complex	-	-	-	-	0.00053	0.00053
Hygromycin	-	-	-	0.06	-	-
Liquid Minerals	-	-	-	-	0.015	0.015
			$/l total	**$2.00**	**$0.91**	**$0.66**

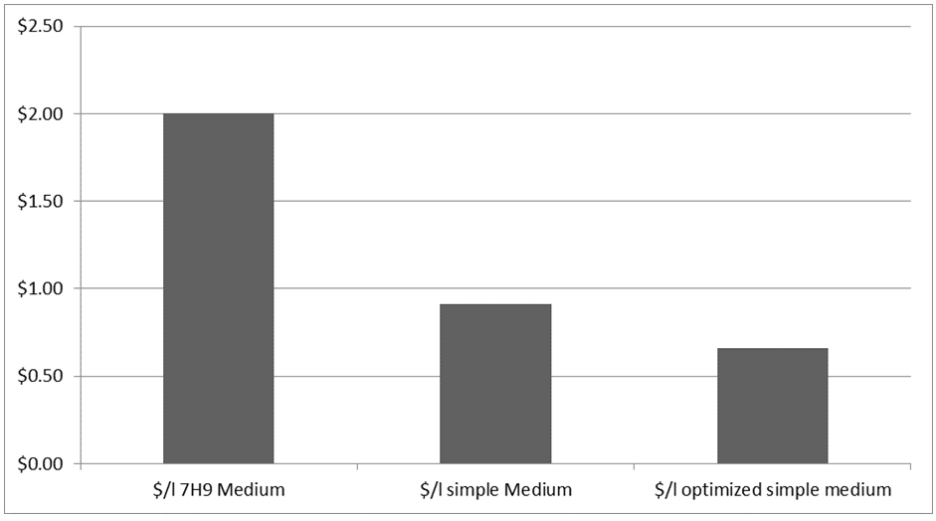

Fig. 7. Comparison of the costs: by optimizing the medium components and substituting antibiotics with a copper-malachite green complex, the costs were lowered by a total of 67%

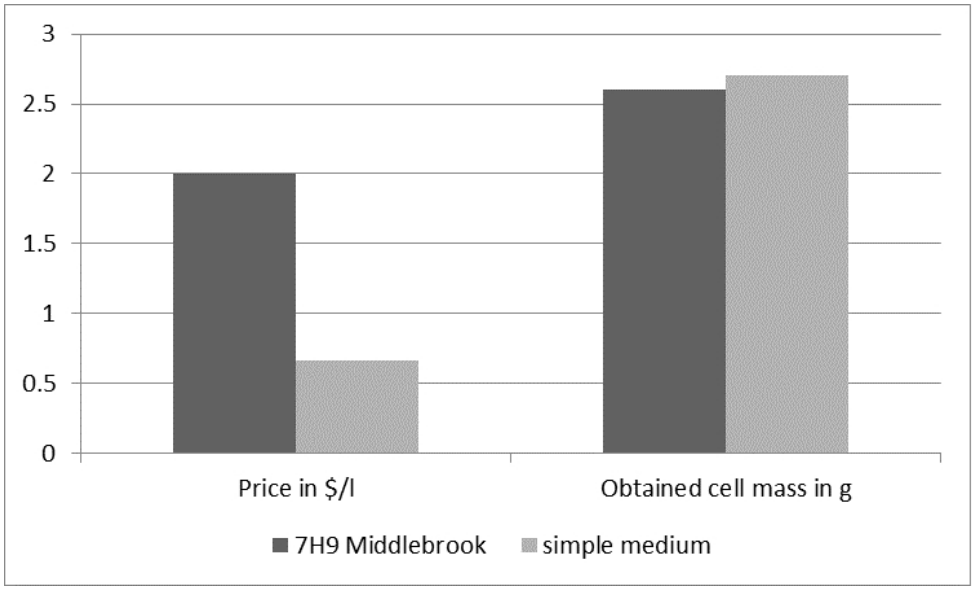

Fig. 8. Comparison of the 7H9 Middlebrook medium and the simple medium developed here

4. CONCLUSION

Experimental design was used to optimize a medium by varying nitrogen and glucose simultaneously to arrive at a minimized cost of the medium while maintaining high biomass- and ultimate target protein yield. Cell mass yield from fermentation with the optimized medium was comparable to results with 7H9 (Figs. 7 & 8.) but the medium cost was lowered by about 60%. Glucose fed was reduced by 40%. An additional cost benefit was garnered from replacing hygromycin with a copper compound [18].

For future work, the scale up to an aerated and agitated bioreactor with pH control would be desirable, since the impacts of those environmental conditions on the growth of *M. smegmatis* have not been investigated. Substituting glucose with an inexpensive carbohydrate source should also be considered. Even after the optimization, glucose accounts for over 50% of the costs per liter medium, which offers further potential for cost reduction.

ACKNOWLEDGEMENTS

This material is based upon work supported by the National Science Foundation under Award No. EPS-0903806 and matching support from the State of Kansas through Kansas Technology Enterprise Corporation. The authors thank Dr. Michael Niederweis, Department of Microbiology, University of Alabama at Birmingham for generously providing the mc2 155 strain of *Mycobacterium smegmatis*.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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