Gram-Positive Agar Formulation

An Honors Thesis (HONR 499)

by

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April 2023

Expected Date of Graduation

May 2023

Abstract

Selective media which allows for the growth of specific bacteria while inhibiting the growth of others is an important tool used daily by microbiologists. There exists excellent media for the selective growth of Gram-negative bacteria, yet there are minimal options for the selective growth of Gram-positive bacteria. While there are Gram-positive selective media, they may contain antibiotics, be species specific, or allow the growth of some Gram-negative organisms. This poses an issue to students in microbiology classes who may struggle to isolate Gram-positive bacteria from a mixed or contaminated sample potentially due to improper streaking techniques. An effective Gram-positive selective media would also be beneficial in the research or clinical setting where it could improve the efficiency of isolation. The aim of this study was to create a new formulation for Gram-positive selective agar (GPA) which does not contain antibiotics and allows for the growth of most Gram-positive bacteria while inhibiting the growth of most Gram-negatives. To do this, a formulation previously created by McKillip and Drake was used as an initial guideline. This GPA utilized 2-phenylethanol as the main selective ingredient due to its documented inhibition of Gram-negative growth. The formulation was mixed, and plates were inoculated with different species of Gram-positive or Gram-negative bacteria. The plates were statically incubated at 37℃ for 96 hours and were checked every 24 hours for growth. Based on the presence or absence of growth, the components and their concentrations were adjusted to address undesired results. This process was repeated for different GPA formulations. The study was ceased before optimal results were achieved due to time constraints. The final formulation contained 33.6 g/L sodium chloride, 15 g/L sodium glycerophosphate, 12 g/L lithium chloride, 0.31% 2-phenylethanol, 15 g/L glucose, 10 g/L tryptone, 10 g/L yeast extract, 19 g/L agar, and 5% sheep’s blood. While a perfect formula has

not yet been achieved, considerable progress has been made. Further work on GPA is required to create a media which addresses the needs of scientists in a suitable manner.

Acknowledgements

I would like to thank Dr. John McKillip for entrusting me with this project. His guidance, expertise, and support proved invaluable to the success of this experiment.

Process Analysis Statement

Bacteria can be broadly categorized as Gram-positive or Gram-negative based on how their cell wall is stained during the Gram staining procedure. A bacterial Gram stain classification is an important property and is commonly used as a descriptor. Because different bacteria have different mechanisms for survival and have different tolerances to certain environments, some media are classified as selective because they only allow the growth of a certain species or genus. There are currently excellent options for selectively growing Gram-negative bacteria such as MacConkey agar, but this is not the case for Gram-positives. This project was based on a previous publication by McKillip and Drake (1999) in which they created a method of creating an agar which was selective for Gram-positive bacteria using 2-phenylethanol. However, the media required further work as it only allowed for the growth of certain Gram-positives while not inhibiting all Gram-negatives (McKillip and Drake, 1999). In my project, I worked on formulating an efficient Gram-positive selective agar (GPA) which overcame these limitations. I hypothesized that I would be able to formulate a GPA which would inhibit the growth of most Gram-negative bacteria while allowing for the growth of most Gram-positives using 2-phenylethanol as the primary selective agent.

This study was conducted based on trial and error. I would make one batch of media and see which bacteria grew on it. I would then adjust the formulation based on the results. For example, when Gram-negatives grew, I increased the amount of the components that inhibit their growth. When Gram-positives did not grow, the amounts of these selective components were decreased. I did this because the components such as 2-phenylethanol, lithium chloride, and sodium chloride are selective for Gram-positive bacteria because these species have a higher

tolerance for them. However, if they are present in a high enough concentration, they also inhibit the growth of Gram-positive bacteria.

Conducting this study taught me about bacterial culture and growth requirements. Over the past year, I have become an expert in how different types of bacteria react to different compounds. I can predict how a change in the formulation will affect the growth of Gram-positive and Gram-negative bacteria. I hold a greater understanding of the differences between these groups of bacteria and how these inconsistencies can be taken advantage of to promote selective growth.

However, I think that this project has given me the greatest insight into what it means to be a scientist in a laboratory. I have really enjoyed improving my laboratory techniques. I am able to now confidently streak for isolation, pour agar plates, and stain colonies. I also liked having such an active role in my research. While I did not create the initial formulation, I did make every adjustment based on the previous results. This allowed me to use my critical thinking skills to look at the results of a trial, consider my knowledge of the components of GPA, and determine the appropriate changes in the formulation. I could then see the direct result of the changes that I made and was able to further improve the product based on what I had learned. While it could be frustrating to have so many failures, I eventually learned to see every undesired outcome as an opportunity rather than a disappointment. With each trial, I became more knowledgeable and discovered something new about the tolerance of bacteria for the components of GPA. Another thing that this project taught me was how to ask for help. Coming into this experiment, I felt lost. I had experience in the lab but was working on a completely different project, so I felt as though my skills did not carry over. I had to learn that it is okay if I am not sure how to do something and that there is nothing wrong with asking for help. This project also

has improved my writing skills. While I still prefer the actual experimentation to the writing about it, I feel a lot more comfortable doing scientific writing. This is a skill which will prove to be useful as I enter medical school and hope to conduct and publish research during my time there.

The project did not come without its share of difficulties. What I struggled with the most during this study was the waiting. After streaking the plates, I had to wait 96 hours before I had definitive results and could continue with the next trial. This meant that I could only do one trial per week. If I was unable to mix and pour my GPA at the start of the week due to illness, work, or other commitments, I missed out on the entire weeks' worth of results. My project was also placed on pause due to difficulties obtaining resources, such as the sheep's blood. It was hard to sit and wait for weeks until it finally arrived. Also, there were multiple trials which I had to do multiple times because they became contaminated. This was particularly frustrating because I felt as though I had wasted time and resources. However, patience is a virtue. I learned to let go of the things which are out of my control and focus my attention on what I can do.

Gram-Positive Agar Formulation

INTRODUCTION

One of the most prevalent issues which microbiologists face are impure samples. Whether it be that the original specimen has an unknown bacterial composition or that the pure culture of known bacteria has been contaminated, microbiologists must determine ways of isolating and purifying the desired bacteria. Because it is such a large and diverse group, most bacteria are categorized as either Gram-positive or Gram-negative based on the result of a Gram stain. This stain provides insight into the cellular wall of the bacteria. Those which are said to be Gram-positive appear purple after staining due to the lack of outer lipid membrane and their thick outer layer of peptidoglycan. The Gram-negative bacteria are pink after being stained because of the thin peptidoglycan layer which is covered by the outer lipid membrane. Due to these major differences, Gram-positive and Gram-negative bacteria have different sets of inhibitors, some of which are utilized in selective media. Selective media allow for the growth of one type of organism while inhibiting the rest. One of the most common examples of selective media is MacConkey agar which allows for the growth of Gram-negative bacteria. MacConkey agar is useful when a microbiologist has a mixed sample and wants to isolate the Gram-negatives. However, there are currently no effective selective media for most species of Gram-positive bacteria. While there are different media available, these contain antibiotics, are species-specific, or allow for the growth of various Gram-negatives (McKillip and Drake, 1999). The aim of this pilot study was to create a Gram-positive agar (GPA) which will allow for the growth of most Gram-positive bacteria while inhibiting most Gram-negatives using 2-phenylethanol as the primary selective agent.

MATERIALS AND METHODS

The first batch of GPA was prepared according to McKillip and Drake (1999) using 0.34% 2-phenylethanol (**Table 1**). This formulation contained D-mannitol and bromocresol purple which were included to allow the media to differentiate between bacteria which can and cannot ferment mannitol. Sodium chloride was included in the formulation because Gram-positives have a higher tolerance for salt than Gram-negative, making it selective (Tesone et al, 2011). Bacteria require phosphorus acquired in the form of phosphate to maintain their membrane, build genetic code, and gain energy (Yao et al, 2016). To meet this demand, sodium glycerophosphate was used in the GPA formulation. Lithium chloride was added into the formulation as another selective agent as Gram-positive bacteria can withstand a higher concentration due to the teichoic acid in their cell walls (Cubillos et al, 2018). The primary selective component in this agar was 2-phenylethanol which inhibits DNA synthesis in Gram-negative bacteria, thus not allowing growth (Berrah and Konetzka, 1962). To promote growth of bacteria, bacto-tryptone was added as an additional source of carbon, nitrogen, and amino acids (Puhm et al, 2022). Similarly, Bacto yeast extract was added as a growth stimulator (Li et al, 2011). Lastly, agar powder was added to solidify the mixture so that it could be used for streaking. The plates were aseptically inoculated with pure cultures of *Staphylococcus aureus, Streptococcus pyogenes, Bacillus cereus, Bacillus megaterium, Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Citrobacter freundii,* or *Pseudomonas aeruginosa* which were streaked for isolation. The plates were statically incubated at 37℃ for 96 hours and were checked every 24 hours for growth. The remainder of the trials were completed following the same protocol with changes being made to the formulation (**Table 2**).

D-Mannitol and bromocresol purple were no longer used after the first trial. D-glucose anhydrous was added as an easily digestible carbon source (Bren et al, 2016). Sheep's blood was also added to facilitate growth by providing nutrients. The sheep's blood was added to the agar after it was autoclaved, before being poured except for trial eight. The amount of sodium glycerophosphate was tripled from 5.0 g/L in trial one to 15.0 g/L in trial two (**Table 1**, **Table 2**). The amount of agar was increased from 15.0 g/L to 19.0 g/L for trial three (**Table 2**). The concentration of 2-phenylethanol was decreased from 0.34% to 0.315% for trial four (**Table 2**). The amount of lithium chloride was doubled from 10.0 g/L to 20.0 g/L for trial five (**Table 2**). The lithium chloride was then decreased by 25% from 20.0 g/L to 15.0 g/L for trial six (**Table 2**). The concentration of 2-phenylethanol was decreased from 0.315% to 0.310% and the amount of lithium chloride was decreased from 15.0 g/L to 12.5 g/L for trial seven (**Table 2**). Lithium chloride was again decreased from 12.5 g/L to 12.0 g/L for trial eight (**Table 2**). Trial eight agar was poured onto previously made 5% sheep's blood agar and the blood was not directly added to the formulation. The amount of sodium chloride was increased by 10% from 32.0 g/L to 35.2 g/L for trial nine (**Table 2**). Sodium chloride was then decreased to 33.6 g/L for trial ten (**Table 2**).

Trial eight was also conducted in broth. The formulation was prepared without the addition of 19.0 g/L agar powder. The broth was pipetted into test tubes in the amount of 9.5 mL before it was autoclaved. 0.5 mL of sheep's blood was then pipetted into each test tube. 10 mL tubes of tryptic soy broth (TSB) were prepared. The broths were then aseptically inoculated with pure cultures of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Bacillus megaterium*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Citrobacter freundii*, or *Pseudomonas aeruginosa*. The broths were statically incubated at 37℃ for 96 hours and were checked every 24 hours for growth.

Table 1 Ingredients and their amounts per liter included in the first Gram-positive agar (GPA) formulation

Component	Amount per L
Sodium Chloride	32.0g
Sodium Glycerophosphate	5.0g
Lithium Chloride	10.0g
2-Phenylethanol	3.40mL
Mannitol	15.0g
Tryptone	10.0g
Yeast Extract	10.0g
Bromocresol Purple	0.02g
Agar	15.0g

Table 2 Ingredients and their amounts per liter included in Gram-positive agar (GPA) formulations two through ten

	Trial (per L)								
Component	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sodium Chloride	32.0g	32.0g	32.0g	32.0g	32.0g	32.0g	32.0g	35.2g	33.6g
Sodium Glycerophosphate	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g
Lithium Chloride	10.0g	10.0g	10.0g	20.0g	15.0g	12.5g	12.0g	12.0g	12.0g
2-Phenylethanol	3.40 mL	3.40 mL	3.15 mL	3.15 mL	3.15 mL	3.10 mL	3.10 mL	3.10 mL	3.10 mL
Glucose	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g	15.0g
Tryptone	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g
Yeast Extract	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g	10.0g
Sheep's Blood	50.0 mL	50.0 mL	50.0 mL	50.0 mL	50.0 mL	50.0 mL	X	50.0 mL	50.0 mL
Agar	15.0g	19.0g	19.0g	19.0g	19.0g	19.0g	19.0g	19.0g	19.0g

RESULTS

There was no bacterial growth in the first trial (**Table 3**). There continued to be no growth in the second trial (**Table 3**). However, there was growth of the Gram-positive *B. megaterium* and the Gram-negative *P. mirabilis* and *P. aeruginosa* after 96 hours in trial three (**Table 3**). In trial four, all bacteria exhibited growth after 48 hours except for the Gram-positive *S. pyogenes* which had growth only after 96 hours (**Table 3**). Trials five and six had no bacterial growth (**Table 3**). Trial seven had only growth of Gram-positive *B. cereus* after 96 hours of incubation (**Table 3**). The Gram-positives *S. aureus* and *B. cereus* along with the Gram-negative *P. mirabilis* had growth after 24 hours in trial eight (**Table 3**). In trial nine, the Gram-positive *S. aureus* had growth after 48 hours while there was no growth in trial ten (**Table 3**).

Table 3 The time it took for first bacterial growth to occur in each trial while statically incubating at 37°C

	Trial									
Bacteria	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
S. aureus	-	-	-	++	-	-	-	+	++	-
S. pyogenes	-	-	-	++++	-	-	-	-	-	-
B. cereus	-	-	-	++	-	-	+++	+	-	-
B. megaterium	-	-	++++	++	-	-	-	-	-	-
E. coli	-	-	-	++	-	-	-	-	-	-
P. mirabilis	-	-	++++	++	-	-	-	+	-	-
K. pneumoniae	-	-	-	++	-	-	-	-	-	-
C. freundii	-	-	-	++	-	-	-	-	-	-
P. aeruginosa	-	-	++++	++	-	-	-	-	-	-

- No growth after 96 hours of incubation
+ Growth after 24 hours of incubation
++ Growth after 48 hours of incubation
+++ Growth after 72 hours of incubation
++++ Growth after 96 hours of incubation

DISCUSSION

There is a lack of adequate Gram-positive selective agar which can be used in educational environments. I attempted to create an efficient GPA which does not use antibiotics and allows for the growth of most Gram-positive bacteria while inhibiting the growth of most Gram-negatives. This was done by creating the GPA, inoculating it with bacteria, then adjusting the formula based on the results.

The first trial had no observable bacterial growth. Initially, the GPA was to be selective for Gram-positive bacteria as well as differential for mannitol fermentation. However, the lack of growth on the first trial indicated that the mannitol was an insufficient carbon source for the

bacteria. To give the bacteria more nutrients, D-mannitol was replaced by D-glucose anhydrous, and sheep's blood was incorporated. Even after these changes were implemented, there was still no growth in trial two. This was unsurprising, however, as the GPA was very soft, and the inoculating loop was gouging the media. To remedy this, I increased the amount of agar powder used for trial three. This yielded improved results with some growth after 96 hours. However, only one species of Gram-positive bacteria grew while two species of Gram-negative did. The lack of Gram-positive growth led me to speculate that the amount of 2-phenylethanol was so high as to inhibit the growth of all bacteria, not only Gram-negatives. I decreased the amount used in the fourth trial which led to the growth of all bacteria. I then decided to increase selectivity by increasing the amount of lithium chloride in trial five rather than readjusting the 2-phenylethanol. This increase proved to be too high, however, as no bacteria grew. I decreased the lithium chloride but there was still no growth in trial six. For trial seven, I decreased both the lithium chloride and 2-phenylethanol to limit the amount of strain being put on the Gram-positives. This resulted in the growth of only one Gram-positive species, *B. cereus*. For the eighth trial, the lithium chloride was decreased further. Additionally, the GPA was made without the addition of sheep's blood but was rather poured over previously made 5% sheep's blood agar plates. This formulation allowed the growth of two Gram-positive species and only one Gram-negative, so it was created as a broth for further experimentation. However, all bacteria had growth in the broth after 24 hours. The amount of sodium chloride was increased for trial nine due to the growth of Gram-negatives in the broth cultures. This led to the growth of only one species of Gram-positive bacteria, *S. aureus*. For the tenth and final trial, the sodium chloride was decreased because the previous amount had inhibited the growth of most Gram-positives. This, however, led to the unexpected result of no bacterial growth.

While significant strides have been made, the work on creating an efficient Gram-positive selective agar is not complete. If I did not have time constraints, I would continue the process of making the GPA then adjusting the formulation based on the results. For the next trial, I recommend further decreasing the amount of sodium chloride until Gram-positive growth is no longer inhibited.

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