The Feasibility of GasPak Envelope Production for Anaerobic Bacteria Cultivation

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Abstract

Nowadays, plenty of well known brands of GasPak envelopes are available in the market for microbiological laboratories to generate an anaerobic atmosphere in an anaerobic jar. This work aims to produce low cost GasPak envelopes and monitor their abilities of anaerobic bacteria cultivation under the atmosphere of $O_2 \leq 5\%$ and 10% of CO_2, approximately. The Anaerocult A system (Merck, Darmstadt, Germany) was utilized as a reference chemical system. Consequently, the abilities to decrease oxygen concentration and to liberate carbon dioxide in a jar of both GasPak anaerobic systems, i.e., the Merck Anaerocult A and our new product were measured by Gas Chromatography (GC). We found that the atmospheric systems generated by the Merck Anaerocult A and our new product turned out to have O_2 at 5.73 vol%, and 5.30 vol% respectively, after placing the envelopes for 60 min, while 200 min later CO_2 concentration became stable around 8.92 vol% and 12.29 vol%, respectively. Finally, amounts of the cultivated anaerobic bacteria in both systems were measured by using a spectrophotometer as a tool. It reveals that there is no significant difference in the anaerobic bacteria culture with both kinds of gas generator envelopes. Of great practical importance, our GasPak envelopes are valuable with the benefit of low cost production at only 17 Thai Baht per pack.

Keywords: GasPak envelope, Anaerobic bacteria, Anaerocult A system, Gas Chromatography, Spectrophotometer

1. Introduction

In order to cultivate anaerobic bacteria, a rapid atmospheric generation with very low oxygen level is compulsary. Imhof and Heinzer [1] measured the oxygen concentration in an anaerobic jar (2.5 L) by an oxygen analyzer series 3600 instrument (Orbisphere Laboratories, Neuchatel-Geneva Switzerland). They compared the abilities of several GasPak systems and the evacuation-replacement method for oxygen concentration reduction in an anaerobic jar. They found that the times to reach an oxygen concentration of 0.5% were 60-93 min for the Merck Anaerocult A system. In 1999, Summanen et.al [2] provided an automated evacuation-replacement technique to create an anaerobic or microaerophilic environment in a jar as the Anoxomat system and compared it with the anaerobic chamber and the GasPak Jar system by considering the growth of obligate anaerobes and the recovery of anaerobic organisms from clinical specimens. Their results show that the Anoxomat system appears to be a good alternative to a chamber for clinical laboratories with limited space or unavailability of chambers with high initial cost, with a small continuing cost of its use. In this work, we would like to produce GasPak gas

generator envelopes for providing anaerobic atmosphere, since the imported ones are expensive. It was then preferable to seek a low cost GasPak envelope with comparable quality to use for anaerobic bacteria cultivation in microbiological laboratories. As a result, the Anaerocult A system (Merck, Darmstadt, Germany) was utilized as a reference chemical system and the anaerobic atmosphere produced in a closed jar was measured by using Gas Chromatography (GC). Finally, the comparison of the abilities of anaerobic cultivation in the systems between our new product and the Merck Anaerocult A were performed by measuring cell numbers with a McFarland nephelometer technique and spectrophotometer [3-5].

2. Materials and Methods

Containers: A glass jar of 3 L volume, 0.1 m diameter with a small circle rubber lid at the top was used as an anaerobic jar to test the anaerobic atmosphere produced by each kind of studied GasPak generator systems.

Materials: The commercial costly imported GasPak envelope of the Merck Anaerocult A was used as a reference, while the mixture of the chemical substances of 70 g powdered active oxygen absorber (Wonderkeep: commercial packets generally used in food preservations), 6 g oxalic acid and 8 g sodium bicarbonate packed in two filter paper envelopes with 7x20 cm^2 size was provided as our new GasPak generator system. In general 35 mL of water has to be injected into the Merck Anaerocult A envelope before being placed in the jar, while in the case of the new GasPak envelope, 25 mL of water is needed in the jar of 10 cm diameter before placing two envelopes vertically at a time to control hydro-osmosis in the system. A gas sample was collected in the gas tight syringe for GC analysis as shown in Figure 1.

Gas Analysis: A Shimadzu Gas Chromatography (GC-17A) equipped with a packed column (Unibeads c) and a thermal conductivity detector was utilized for analyzing the anaerobic atmosphere in the jar [6]. A 0.5 mL gas tight syringe as mentioned above was used for gas injection with He carrier gas flow rate of 35 mL/min and applied 80 mA electrical current at the detector. The GC oven temperature was set from 31 to 150 °C. Scotty Analyzed Gases (Sulpelgo, Bellefont, USA) were used as standard gas for GC calibration, and is composed of H_2 4.05 vol%, O_2 5.0 vol%, N_2 5.0 vol%, CO 5.06 vol%, CO_2 5.06 vol%, CH_4 4.05 vol% and He 71.64 vol%. The quantitative gas analyses of O_2 and CO_2 were run from 0^{th} to 60^{th} min, 1360^{th} to 1660^{th} min and 2710^{th} to 2910^{th} min with 50 minute intervals for all periods of each GasPak anaerobic systems. Triplicate measurements of each experiment were done, and the normal atmosphere in a jar before placing each GasPak envelope was measured, so the precision of the analysis can be monitored by relative standard deviation.

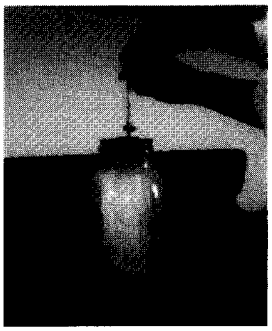

Figure 1 Our new GasPak envelopes were placed into the jar for providing the anaerobic atmosphere. Then, a gas sample was collected for GC analysis.

Growth of Anaerobes: A Jenway 6400 spectrophotometer was provided to measure the 620 nm wavelength light absorption constant of the solution, where the light of 620 nm is suitable for this kind of solution. Amounts of anaerobic bacteria measurements are obtainable conveniently by McFarland nephelometer technique [3-4]. Hence four species of anaerobe, i.e. *Clostridium difficile, Clostridium perfringens, Clostridium bifermentans* and *Bacteroides fragilis* were inoculated in three sets of three test tubes filled with brain heart infusion broth for each bacterial species, and all were incubated at 37 °C for 48 hr. Each set of four species was incubated in jars with different GasPak generator systems, i.e. the Merck Anaerocult A, the new GasPak envelopes and the normal atmosphere without any GasPak envelope as a control system. The calibration curve between absorption constant and corresponding bacterial colony forming unit per mL was performed by preparing standard solutions following McFarland nephelometer technique. The absorption constants of each

solution were obtained using a spectrophotometer, the colony forming, grown under both GasPak generator systems were compared to each other. This experiment was repeated 3 times.

3. Results and Discussion

Figures 2a-2c show chromatograms of three systems measured by GC. Meanwhile the quantitative gas analyses of normal atmosphere in a jar using GC are O_2 20.99 vol%, N_2 77.87 vol% with > 1.5 % relative standard deviation of all measurements. This reveals that the gas analyses for such atmospheric system using GC are reliable. Nevertheless the quantitative gas analyses of O_2 and CO_2 in GasPak generator systems at every certain time interval with 3 replications as reported in Table 1, indicate very high relative standard deviation because of the extremely low concentrations and small numbers of experimental repetitions, especially on the analytical results of the Merck Anaerocult A system. This large statistical error was attributed to the sensitivity of gas generation in the jar, to chemical processes between water and GasPak substances of Merck Anaerocult A system, where the lower relative standard deviations were found in our new GasPak system. Figure 3 shows the time evolution of atmosphere in the anaerobic jar for both systems. Moreover, histograms of the equivalent growth in both chemical systems are shown in Figure 4b whereas the data concerning amounts of anaerobic bacteria are available from the calibration curve between absorption constant and corresponding bacterial colony forming units (CFU) per mL as shown in Figure 4a. In addition the cultivation results of each system are shown in Table 2. Finally, for our new GasPak envelope, the total production cost was estimated at only 17 Thai Baht per pack.

4. Conclusion

From Figure 3, it can be seen that our new GasPak envelope can generate an anaerobic atmosphere as well as the Merck Anaerocult A one. Besides it is able to be reproduced easily by technicians in a small laboratory. Additionally, we can assure the reliability of using Gas Chromatography for quantitative gas analyses, and also the effect of water absorption in the chemical process within the Merck Anaerocult A envelope on gas generation in the system, which influenced the high level of relative standard deviation of triplicate measurements in quantitative gas analyses. From the chromatograms in Figure 2b-2c, the position of the O_2 peak and N_2 peak are very close together, while the CO_2 peak is situated on high base line. These consequences cause rather high relative standard deviation of those low concentration analyses as well.

5. Acknowledgement

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6. References

[1] Imhof, A. and Heinzer, I., Continuous Monitoring of Oxygen Concentrations in Several Systems for Cultivation of Anaerobic Bacteria, *J. Clinical Microbiology*, Vol.34, No. 7, pp.1646-1648, 1996.

[2] Summanen, P.H. et al., Comparison of Recovery of Anaerobic Bacteria Using the Anoxomat, Anaerobic Chamber, and GasPak Jar Systems, *J. Anaerobe*, 5, pp. 5-9, 1999.

[3] Skoog, A.D., Principles of Instrument Analysis. 4th Edition, Saunders College Publishing, pp. 90-100, 1992.

[4] Lennette, E.H. et al., Manual of Clinical Microbiology, 4th Edition, Washington DC: American Society for Microbiology, p.1095, 1985.

[5] Ingraham, J.L et al., Introduction to Microbiology. Belmont: Wadsworth Publishing, pp. 205-207, 1995.

[6] Kenkel, J., Analytical Chemistry for Technicians, New York: Lewis Publishers ACR C Press Company, 2003.

Table 1 Comparison of O_2 and CO_2 concentrations in two kinds of GasPak gas generator systems measured by GC technique.

Time (Min)	Merck Anaerocult A (vol%)		Our New GasPak Envelope (vol%)	
	O_2 [RSD%]	CO_2 [RSD%]	O_2 [RSD%]	CO_2 [RSD%]
0^{th}	20.58 [2.69]	-	21.00 [0.88]	-
10^{th}	17.52 [14.16]	5.20 [35.90]	15.30 [9.91]	0.88 [149.55]
60^{th}	5.73 [52.43]	8.13 [25.36]	5.30 [16.48]	6.75 [75.12]
110^{th}	2.51 [95.88]	8.75 [28.05]	2.68 [19.41]	12.29 [12.46]
160^{th}	1.40 [99.97]	8.89 [30.75]	1.65 [36.13]	12.18 [14.20]
210^{th}	1.00 [100.01]	8.99 [33.84]	1.24 [17.68]	11.81 [16.48]
260^{th}	0.89 [100.02]	8.92 [34.39]	1.22 [12.03]	10.70 [28.52]
1360^{th}	1.24 [23.76]	11.36 [31.31]	1.04 [8.41]	9.21 [7.55]
1410^{th}	1.23 [20.38]	11.40 [31.14]	1.01 [11.43]	9.21 [7.86]
1460^{th}	0.77 [99.94]	11.29 [29.31]	1.00 [4.72]	9.11 [11.75]
1510^{th}	1.21 [28.66]	11.04 [30.76]	1.09 [8.66]	8.76 [9.06]
1560^{th}	1.24 [24.82]	11.04 [30.57]	1.06 [10.77]	8.88 [11.01]
1610^{th}	1.29 [31.01]	10.77 [31.19]	0.99 [4.93]	8.86 [12.58]
1660^{th}	0.96 [99.99]	10.60 [32.73]	0.95 [3.67]	8.88 [13.26]
2710^{th}	1.42 [19.86]	5.84 [10.59]	1.13 [11.81]	8.32 [7.70]
2760^{th}	1.52 [31.42]	5.96 [5.31]	1.00 [5.08]	8.66 [6.79]
2810^{th}	1.45 [31.48]	5.85 [5.95]	1.01 [0.69]	8.59 [9.73]
2860^{th}	1.21 [99.97]	5.42 [18.72]	1.15 [24.85]	8.49 [14.75]
2910^{th}	1.23 [18.27]	5.34 [21.53]	1.00 [3.00]	8.60 [12.72]

RSD = Relative Standard Deviation

Table 2 The abilities of anaerobic cultivation in brain heart infusion broth under three kinds of atmospheric systems at 37 °C for 48 hrs. Each experiment was repeated 3 times.

Anaerobic Bacteria Species	Average of Bacterial Colony Forming Units per mL ($x10^8$) [RSD*%]								
	I			II			III		
	1	2	3	1	2	3	1	2	3
Clostridium difficile	4.6 [1.31]	3.9 [10.78]	5.0 [20.38]	6.2 [8.09]	5.7 [5.83]	5.5 [5.50]	0.7 [3.52]	0.7 [3.52]	0.7 [3.52]
Clostridium perfringens	7.1 [7.08]	7.0 [0.29]	6.8 [4.43]	7.0 [4.26]	7.1 [3.52]	6.9 [1.45]	0.5 [11.09]	0.5 [11.09]	0.5 [11.09]
Clostridium bifermentans	6.3 [3.04]	3.9 [1.28]	6.1 [8.14]	6.9 [4.35]	6.7 [6.01]	6.7 [1.49]	0.8 [10.61]	0.8 [10.61]	0.8 [10.61]
Bacteroids fragilis	10.5 [0.57]	10.2 [7.85]	10.4 [3.07]	10.7 [3.74]	10.7 [2.43]	10.5 [2.85]	0.9 [4.57]	0.9 [4.57]	0.9 [4.57]

I: The Merck Anaerocult A system
II: Our New GasPak System
III: Control System (normal atmosphere)
* triplicate cultivation for each species of anaerobic bacteria.

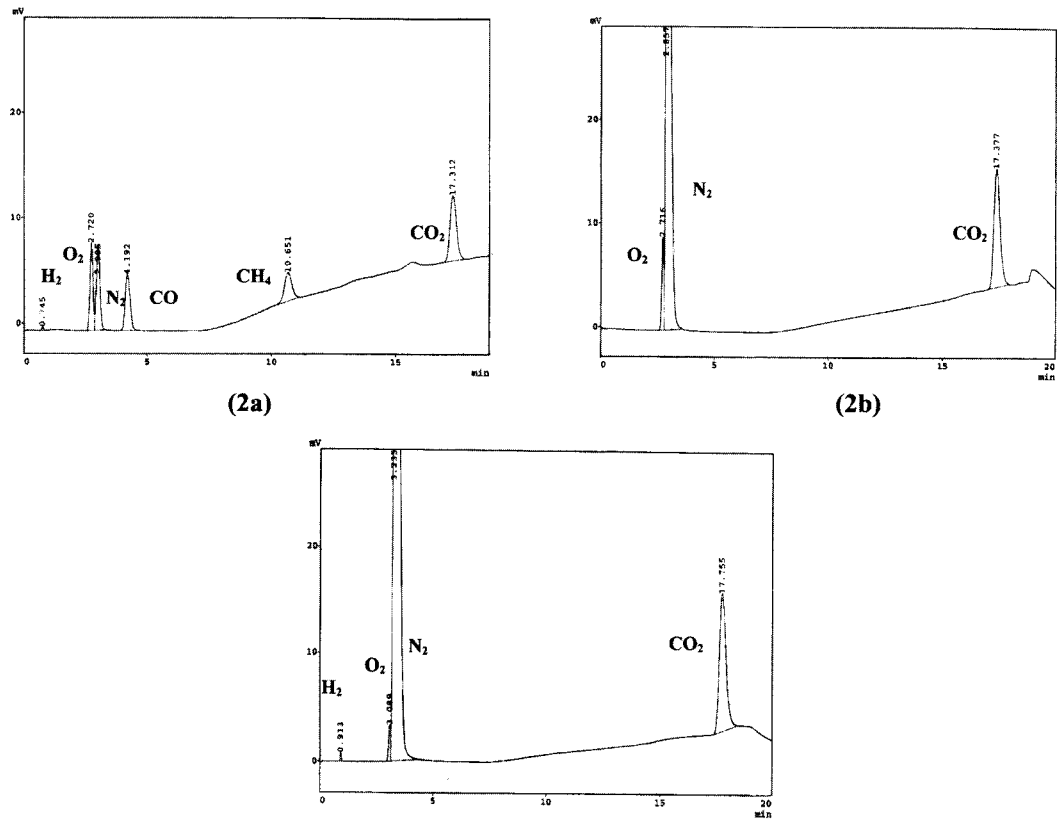


Figure 2 Chromatograms (as shown by detector response (mV) for Y-axis versus retention time (min) for X-axis) of three systems measured by GC **(2a)** the standard gas, **(2b)** the Merck Anaerocult A jar system at 60th min, and **(2c)** our new GasPak jar system at 60th min. All measurements were done under the conditions of 80 mA electrical current for the detector and 31 °C for starting temperature of a packed column.

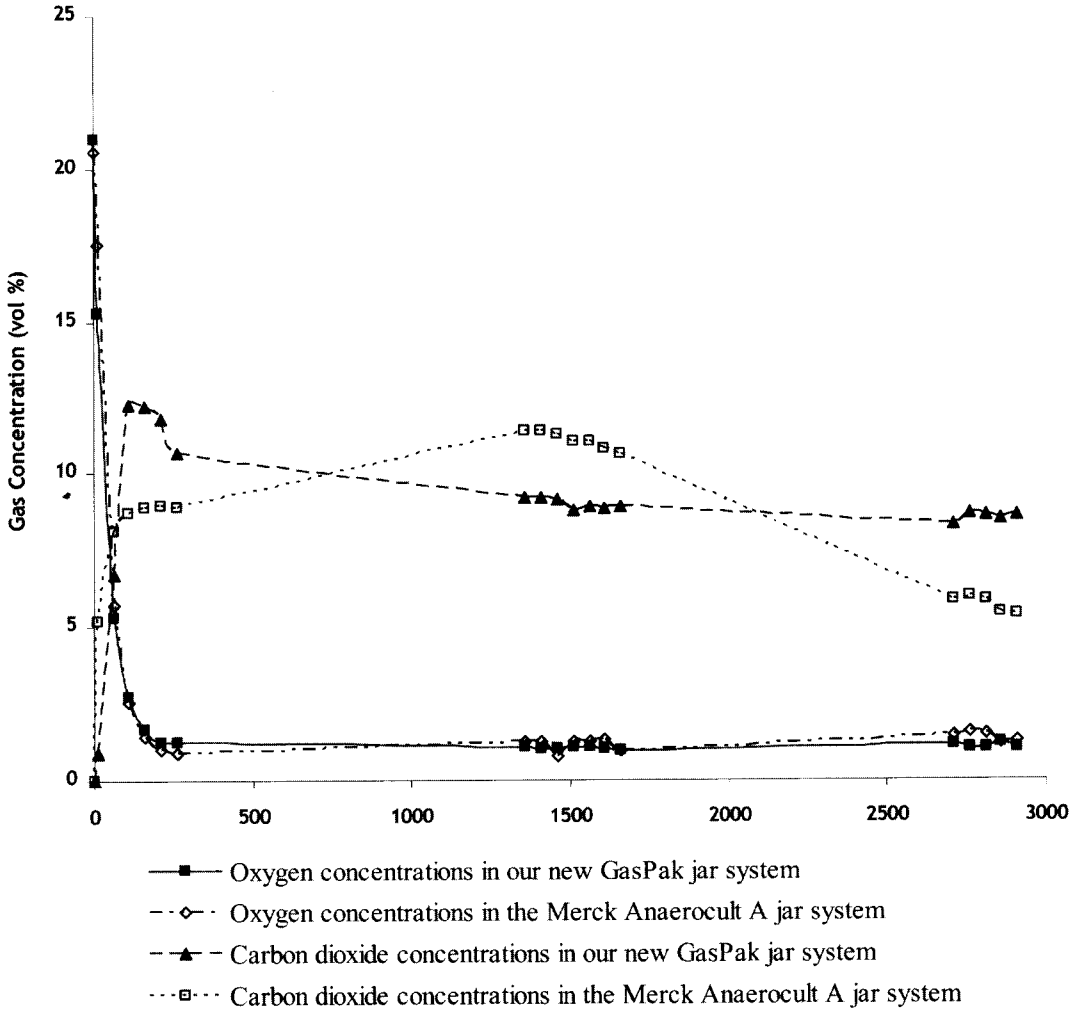
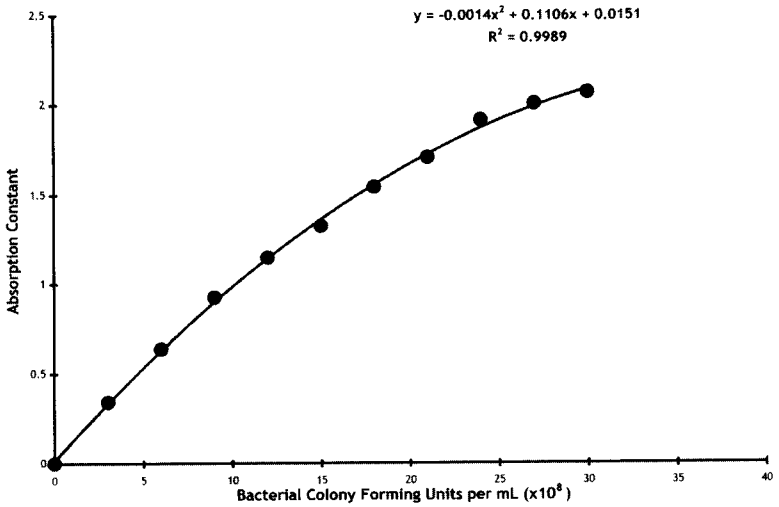
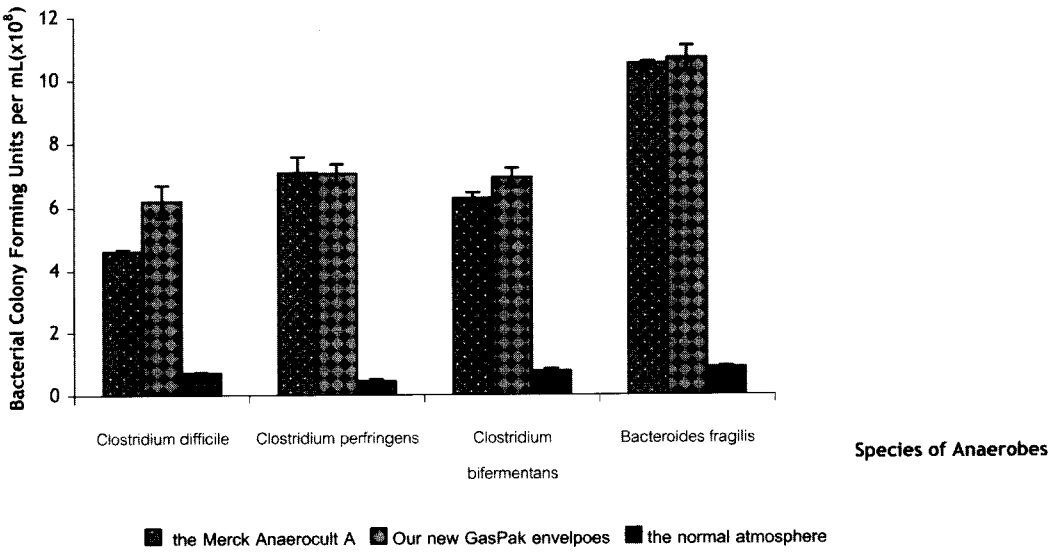


Figure 3 The rate of change of O_2 and CO_2 concentrations in the Merck Anaerocult A jar system and our new GasPak jar system are plotted correspondingly with the data shown in Table 1.



(4a)



(4b)

Figure (4a) The calibration curve between absorption constant and corresponding bacterial CFU per mL **(4b)** the growth of Anaerobes in the anaerobic atmosphere generated with the Merck Anaerocult A system, our new GasPak envelope and normal atmosphere. The case of normal atmosphere is referred to as the control system to express the starting amount of Anaerobes for this experiment.