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Evaluation of Membrane Fragments Extracted from *Escherichia coli* and *Pseudomonas aeruginosa* on *Campylobacter jejuni* Growth under Normal Atmosphere

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ABSTRACT

Study on extraction of membrane fragments from *Escherichia coli* and *Psuedomonas aeruginosa* were determined for using to support *Campylobacter* cultivation under normal atmosphere. Crude membrane fragments were extracted from 45 strains of *E. coli* and 44 strains of *P. aeruginosa*. Strains that provided highest efficiency of oxygen reduction were selected to purify membrane fragments by French pressure cell and ultracentrifugation. The purified membrane fragments were characterized and investigated for supporting *Campylobacter jejuni* growth in Mueller-Hinton broth. The broth supplemented with and without membrane fragments was incubated under normal atmosphere at 37°C or 42°C. *Campylobacter* growth in the broth containing purified membrane fragments was initially observed within 6 to 12 hours of incubation while the media without supplemented with membrane fragments showed no growth of the bacteria. Therefore, oxygen reducing membrane fragments prepared from the selected strains could support *Campylobacter* cultivation under normal atmosphere.

Key words: oxygen reducing membrane fragments, *Campylobacter jejuni*

INTRODUCTION

Gastroenteritis caused by *Campylobacter* species has been increasing in both developed and developing countries for about 10 years. In USA, an active surveillance indicated that about 15 cases were diagnosed each year for every 100,000 persons in the population. Many cases went undiagnosed or unreported, and campylobacteriosis was estimated to affect over 1 million persons every year. Approximately 100 persons with *Campylobacter* infection may die each year (CDC, 2005). A survey in northern Thailand in 2005 reported that the prevalence of *Campylobacter* in chickens at the farm, slaughterhouse, and market were 64, 38 and 47%, respectively. No *Campylobacter* isolates were obtained from healthy non-farm residents, but isolates were obtained from 5 and 18% of farm workers and children with diarrhea, respectively (Padungtod and Kaneene, 2005).

For conventional technique of *Campylobacter* isolation from foods and other samples, the bacteria need to be cultured and they

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need approximately 5% O_2 and 10% CO_2 for growth. This special condition is called microaerobic condition because an important key for *Campylobacter* cultivation is that the microorganisms need less oxygen for growth. The common method for culturing *Campylobacter* spp. is by using a gas generating pack within a jar to create a microaerobic condition. The other method uses gas replacement with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. These methods need special equipment and complicated processes, and are also expensive. Some bacteria can utilize oxygen for growth via their membrane bound enzymes by catalyzing oxidation reaction causing consumption a large amount of oxygen. These enzymes can be extracted in the form of membrane fragments from oxidative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. Many reports discussed on a success in using commercial membrane fragments to reduce oxygen concentration in culture media and promote growth for *Campylobacter* spp. (Niroomand and Fung, 1994, Tuitemwong *et al.*,1994, Wonglumsom *et al.*, 2001).

The objective of this study is to screen *Escherichia coli* and *Pseudomonas aeruginosa* isolates that produce high activity of oxygen reduction. The characteristics of purified membrane fragments were determined and the efficiency of the fragments for supporting *Campylobacter* growth under normal atmosphere was also evaluated.

MATERIALS AND METHODS

Screening for bacterial isolates with high efficiency of oxygen reduction

Escherichia coli and *Pseudo-monas aeruginosa* isolates were obtained from the stock culture at the Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University. Totally 45 isolates of *E. coli* and 44 isolates of *P. aeruginosa* from clinical specimens were studied. Bacteria were cultured on tryptic soy agar (TSA) plate and incubated at 37°C for 18-24 hours. Gram staining and biochemical tests were performed to confirm bacterial species. The bacteria were subcultured in tryptic soy broth (TSB) and placed on a 37°C shaking incubator at 150 rpm for 18-24 hours. The protocol for crude membrane extraction was modified from Tuitemwong *et al.* (1994). Briefly, bacteria were harvested by centrifugation at 7,000×g, 4°C for 10 minutes and washed twice with sterilized phosphate buffer, pH 7.5. Cell pellets were suspended in steriled 0.01 M of HEPES buffer, pH 7.2. Crude membrane fragments were obtained by breaking the bacterial cells with a sonicator and membrane fragment solutions were stored at –20°C. Protein concentrations were determined by dye binding assay method with spectrophotometer using the wavelengths of 595 nm.

The ability of oxygen reduction of the crude membrane fragments from each isolate was measured with dissolved oxygen (DO) meter and expressed as percent of DO. %DO was recorded every 10 minutes for an hour in 100 ml of TSB. Results were plotted on a graph with y-axis representing %DO value and x-axis representing times in minute. An activity of crude membrane fragments was calculated using the following.

$$activity(U/ml) = \left(\frac{\frac{(\%DOstart - \%DOremain)}{\%DOstart} \times 100\% \times \frac{1}{time(\sec)}}{volume(ml)} \right)$$

Specific activity (U/mg) = activity (U/ml)/protein concentration (mg/ml)

Specific activity for all isolates of *E. coli* and *P. aeruginosa* were determined to find bacterial isolates that provide crude membrane fragments with high effective oxygen reduction. The experiments for the selected isolates were performed again to choose only one isolate from each genus as the provider of membrane fragments for future experiment.

Purification and characterization of membrane fragments

Cell breakage was performed repeatedly 4 times using French pressure cell at 15,000 psi. The debris was removed by centrifugation at 6000xg in 4°C for 10 minutes. Purified membrane fragments were obtained by ultracentrifugation at 175,000xg in 5°C for 4 hours. The pellet was weighed and added with steriled 0.01 M HEPES buffer, pH 7.5. The solution was sterilized by filtering through a sterilized 0.45 mm-membrane filter. The purified membrane fragment was kept at -20°C until use. The membrane fragments were examined to find oxygen reduction activity under different temperatures at 25°C, 37°C, 42°C and 50°C, and at various pH of 6.0, 7.0, 7.5 and 8.5.

Evaluation of membrane fragments on *Campylobacter* growth

Campylobacter jejuni ATCC 33291 was cultured on blood agar and incubated at 42°C for 24 hours under microaerobic condition using a mixture gas of 5% O_2, 10% CO_2 and 85% N_2. Bacteria were adjusted to achieve a turbidity of OD 0.08-0.1 at 625 nm and diluted with 0.1% peptone buffer to achieve bacterial concentration of approximately 10^7 cell/ml. Ten microliters of the bacterial suspension was inoculated into a screw-cap bottles containing 100 ml of Mueller Hinton broth (MHB) supplemented with 0.05 unit and 0.08 unit of purified membrane fragments. The media added with membrane fragments extracted from *E. coli* and *P. aeruginosa* were incubated at 42 and 37°C, respectively, for up to 24 hours. *Campylobacter* growth was also examined in MHB with no flushing and flushing with the mixed gases. *Campylobacter* counts were performed by 6x6 drop plate method (Chen *et al.,* 2003) on charcoal desoxycholate agar (CDA) plates and incubated at 42°C under microaerobic condition for 24-30 hours.

RESULTS AND DISCUSSION

Screening for bacterial isolates with high efficiency of oxygen reduction

Oxygen reduction activities of crude membrane fragments from 45 strains of *E. coli* and 44 strains of *P. aeruginosa* are shown in Table 1. Most crude membrane fragments from *E. coli* provided the activity around 0.010 U/ml to 0.015 U/ml while four strains had activity above 0.015 U/ml.

Crude membrane fragments from 19 strains of *P. aeruginosa* showed the activity above 0.015 U/ml. *E. coli* no. 3, no. 7 and no. 40 revealed higher specific activity of 0.00174, 0.000789 and 0.000311 U/mg, respectively (Figure 1). *P. aeruginosa* no. 26, no. 30 and no. 33 also had higher specific activity of 0.001121, 0.001698 and 0.001526 U/mg, respectively (Figure 1). With the highest efficiency, *E. coli* no. 3 and *P. aeruginosa* no. 30 were selected for further study.

Characterization of the membrane fragments

The purified membrane fragment from *E. coli* no.3 showed high oxygen reduction activity at temperatures 42°C and 50°C, and at pH 7.5 to pH 8.5. In a different way, the purified membrane fragment from *P. aeruginosa* no. 30 shown high reduction activity at temperatures 37°C and 42°C, and at pH 7.0 to pH 7.5. Membrane fragments from *E. coli* no. 3 and *P. aeruginosa* no. 30 shown different working conditions. Slightly high temperatures of 42°C to 50°C and slightly

Table 1 Oxygen-reduction activity of crude membrane fragments.

Activity level (U/ml)	Numbers of strains	
	E. coli (n=45)	*P. aeruginosa* (n=44)
above 0.015	4	19
0.010-0.015	23	11
0.005-0.010	15	8
below 0.005	3	6

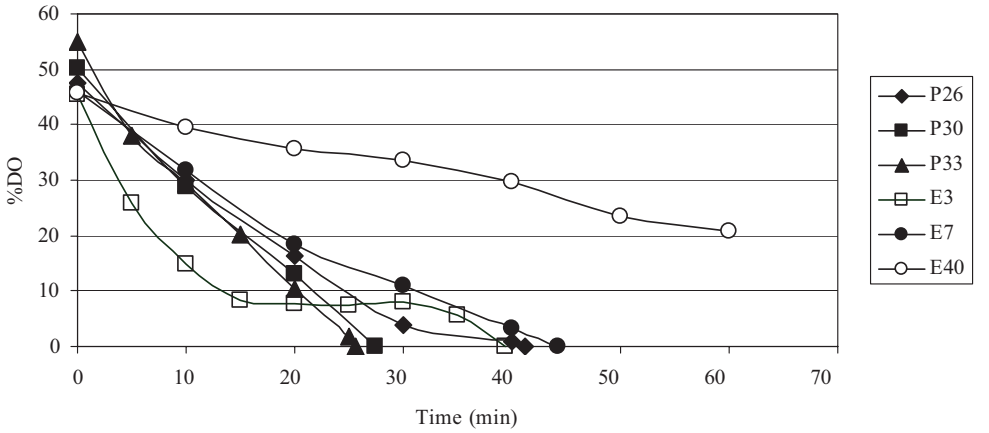

Figure 1 Oxygen reduction curve of crude membrane fragments from *E. coli* no.3, no.7 and no.40 and *P. aeruginosa* no. 26, no.30 and no.3.

alkalinity of pH 7.5 to pH 8.5 were the appropriate conditions for membrane fragments extracted from *E. coli* no. 3. In contrast, lower temperatures and lower alkalinity were appropriate conditions for membrane fragments extracted from *P. aeruginosa* no. 30. A difference in characteristics of the membrane fragments were possibly resulted from different properties of membrane bound enzymes that contains in each bacteria such as lactate dehydrogenase from *E. coli* had optimum pH at 8.0-9.0 (Futai, 1973) while D-gluconate dehydrogenase from *P. aeruginosa* had optimum pH at 5.0-6.0 (Matsushita *et al.,* 1979).

Evaluation of membrane fragments on *Campylobacter* growth

The purified membrane fragment extracted from *E. coli* no.3 was used for culturing *Campylobacter* at 42°C while using the purified membrane fragment from *P. aeruginosa* for cultivation at 37 °C. Growth of *C. jejuni* at 42°C in Mueller Hinton broth (MHB) supplemented with purified membrane fragment from *E. coli* no. 3 (MHB+E3) at concentrations of 0.05 unit and 0.08 unit was compared with the growth in MHB only and in MHB with mixed gases flushing (Table 2). At 6, 12 and 24 hours of incubation, bacterial count could be observed in MHB with membrane fragment and MHB with gas flushing, while MHB only showed no growth. Growth of *C. jejuni* at 37 °C in MHB supplemented with purified membrane fragment from *P. aeruginosa* no. 30 (MHB+P30) at concentrations of 0.05 unit and 0.08 unit was compared with MHB only and MHB with mixed gases flushing (Table 3). MHB only showed no bacterial growth at 6, 12 and 24 hours of incubation. At incubation periods of 6 and 12 hours, bacterial counts using MHB added with both 0.05 unit and 0.08 unit of membrane fragments from *P. aeruginosa* and those using MHB with mixed gases flushing were not significantly different showing the differences less than 0.5 log cfu/ml. Wonglumsom *et al.* (2001) reported no significant differences between using 0.15 U/ml of commercial membrane fragments and using Hunt Broth with gas flushing for recovery of *C. jejuni* from artificially inoculated ground beef.

By generating microaerophilic condition from oxygen scavenging process, oxygen reducing membrane fragments from *E. coli* and *P. aeruginosa* could work well in supporting the growth of *C. jejuni* ATCC 33291. Their results were similar to that using MHB with mixed gases flushing while having only MHB could not support the growth of *Campylobacter*

Table 2 A comparison of *C. jejuni* growth at 42 °C in different media conditions.

	Campylobacter count (log cfu/ml)					
	1st Experiment			2nd Experiment		
Time (hour)	MHB+E3 0.05 U	MHB only	MHB+gas flushing	MHB+E3 0.08 U	MHB only	MHB+gas flushing
0	2.65	2.74	2.70	1.40	1.70	1.40
6	2.95	no growth	3.44	1.88	no growth	2.48
12	3.11	no growth	3.61	2.40	no growth	3.47
24	4.64	no growth	7.45	4.90	no growth	6.35

Table 3 A comparison of *C. jejuni* growth at 37 °C in different media condition**.**

	Campylobacter count (log cfu/ml)					
	1st Experiment			2nd Experiment		
Time (hour)	MHB+P30 0.05 U	MHB only	MHB+gas flushing	MHB+P3 0 0.08 U	MHB only	MHB+gas flushing
0	1.88	1.88	1.70	2.84	2.87	2.70
6	2.10	no growth	2.30	3.03	no growth	3.00
12	2.54	no growth	2.90	3.10	no growth	3.50
24	3.65	no growth	4.65	6.51	no growth	7.06

becausehigh oxygen level still remained in the media. In addition, the ingredient of Mueller-Hinton broth used in this study no chemical reducing agents. In some studies had shown that membrane fragments worked very well in culture media added with reducing agents such as ferrous sulfate, sodium metabisulfite and sodium pyruvate (Niroomand and Fung, 1994, Raben and Slavik, 1994). Using 0.08 unit of membrane fragments in culture media showed slightly higher bacterial counts than that supplementing with 0.05 unit of the membrane fragments. These results agreed with the study of Niroomand and Fung (1994) which stated that *Campylobacter* growth increased as the amounts of membrane fragments in the broth increased.

CONCLUSION

In-house preparation of oxygen-reducing membrane fragments from *Escherichia coli* no.3 and *Psuedomonas aeruginosa* no.30 could support the growth of *Campylobacter jejuni* ATCC 33291 under normal atmosphere in culture media without chemical reducing agents. The optimal pH of media supplemented with the membrane fragments from both strains was at pH 7.0 to pH 7.5. However, the appropriate incubating temperature was at 42°C for the membrane fragment extracted from *E. coli* and at 37°C for the membrane fragment extracted from *P. aeruginosa*.

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