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Application of extreme environmental conditions to resuscitation of viable but non culturable *E. coli* DH5α

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ABSTRACT

The resuscitation of Viable But Non-Culturable (VBNC) state in Escherichia coli DH5α as one of the most usable expression host was investigated. The VBNC state in bacteria is defined as while the cells are alive but unable to grow visibly on nonselective growth medium. After collecting several samples, Non-culturable E. coli DH5α (that has undertaken on various recombinant manipulations) were divided into different groups in order to carry-out different experiments. They were treated to heat shock at 42ºC in different periods of time, different concentration of Bile-salts and NaCl and combinational of these methods. The results showed that the almost of resuscitation treatment had positive effect on reactivation of VBNC E. coli DH5α. The combination of these parameters (various NaCl and bile salts concentration and heat shock method at 42 °C in different time) in a binary manner, also inferred to suitable results. Furthermore, by applying the three stresses simultaneously we achieved optical density up to 0.58 and 9×10^8 CFU/ml which had presented the best results. The results show that by applying some alterations in the condition of such recombinant E. coli DH5α, the growth path of these bacteria which remain to a VBNC phase can be changed to the normal status.

Key words: Non-culturable bacteria, *E. coli*, Bile-salts, NaCl, Thermal Shock.

INTRODUCTION

In general, the surroundings of bacteria in nature are very different from those of laboratory and oligotrophic circumstances are more the rule than the exception . To survive such nutritionally harsh conditions, bacteria must adjust their metabolism to a less profligate way of life than that adopted conditions of nutrient surplus [1]. This would assumed that in the case of complete exhaustion of exogenous nutrients they can go into a dormant state, which help the cell to survive for a long time without growth and proliferation and it is now clear that this state

provides an important depository of pathogens in the environment [1,2]. This state is also referred to the "viable but non-culturable" (VBNC) state, where the cells remain viable, but are no longer able to grow on standard laboratory media [1,2]. In spite of their characteristically low levels of metabolic activity, they are again culturable upon resuscitation. Since the pioneering study by Xu et al. (1982) during more than 25 years, a great body of literature has developed from researchers worldwide documenting the presence of a VBNC state in a wide range of bacteria [3]. The medical implications of this truth are numerous [4]. For example, it appears that the 'dormant' or the 'latent' phase of *Mycobacterium tuberculosis* infections depicts the VBNC state in this pathogen [5,6], and that the repetition of tuberculosis years after a person was believed to be tuberculosis free is due to resuscitation of this pathogen from the VBNC state [7]. The inventory of pathogenic bacteria that have adopted this lifestyle as a means of survival is ever rising, and includes *Escherichia coli* (including EHEC strains), *Campylobacter* spp., *Francisella tularensis*, *Legionella pneumophila*, *Helicobacter pylori*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *M. tuberculosis*, several *Shigella* and *Salmonella* spp. and various pathogenic *Vibrio* spp [2,8,9,4]. This state of pathogenic bacteria could be implicated as one of the main hazardous agents for public health [4]. Bacteria are induced to enter the viable but non-culturable state by one or a mixture of environmental stresses, such as temperature shift, starvation, and exposure to a heavy metal [8]. There are several assays used to document the viability of viable but non-culturable cells including observation of chemical reduction of redox dyes (e.g. CTC [5-cyano-2,3-ditolyl tetrazolium chloride] and INT [2-(piodophenyl)- 3-(p-nitrophenyl)-5-phenyl tetrazolium chloride]), direct viable count (DVC), RFLP, PCR-DGGE and RT-PCR [10,7,11,12]. The resuscitation of cells from the VBNC state could be occurs by recruiting a series of physical-chemical changes such as treatment with phosphate buffer, exposure to hypochlorous acid (HOCl), injection to laboratory animal model and culture in medium containing ammonium salt along with certain time and temperature [13-15,7].

To our best knowledge there are a few studies on VBNC state in *E. coli* DH5α. Our objective of this study was to evaluate the resuscitation of recombinant *E. coli* DH5α in VBNC state (that has archived from frequent transformation processes, exposure to low temperature for long time (-80 °C for more than two years and high glycerol concentration) into normal phase using various environmental shocks such as heat and salt shock and enriched media.

MATERIALS AND METHODS

Bacterial strains

The recombinant *E. coli* DH5α strains (transformed bacteria by *ipad*-pET 28a(+) plasmid) were selected randomly under the following conditions: long time preservation at -80°C, high glycerol concentration (80%) and those which were cultured on oligotrophic media.

To determine total bacterial count estimation, cells were rinsed with phosphate buffer saline (PBS) and the optical density of cells was measured at OD 600 using UV spectrophotometer device (Pico200 Picodrop™, UK).

VBNC assessment assay

In order to ensure the VBNC state on the above mention *E. coli DH5α* strains, the following tests were carried out. The non-culturability of cells was confirmed by culturing 150 µl of VBNC bacteria in enriched LB agar (LB agar supplemented with 0.2% glucose and 1% Caso-amino acids) and broth (as same for enriched LB agar minus agar) and incubated at 37 °C for 48 h. Bacteria were considered to be VBNC states when there were lower than 0.1 $CFU.ml^{-1}$.

The morphological study was conducted for assessment of any changes in cellular morphology as described by M T Fera et. al [16].

The viability of cells was assessed using a 5-cyano-2,3-ditolyl tetrazolium chloride reduction method as described previously [17].

Resuscitation procedures

Various experiments were carried out in order to resuscitate the VBNC bacteria. At first, eight VBNC suspensions were prepared in enriched LB broth (0.2% glucose and 1% Caso-amino acids), then various concentration of NaCl and bile salts (15, 30 and 45 mM of each) were added to these suspensions separately and incubated overnight at 37 °C with shaking speed at 150 rpm (Table 1). In another experiments, the heat shock method at 42 °C at different

time (1, 1.5 and 2) were carried out to VBNC suspensions after incubation overnight at 37 °C with shaking speed at 150 rpm (Table 1). Culturability of these cells was determined by plating on enriched LB agar and then incubated at 37 °C for 24 h. Finally, the combination of first experiment series were designed from the best results obtained of the above mention experiments (Table 1).

RESULTS

Initially, the suspensions of recombinant *E. coli* DH5α (transformed bacteria by *ipad*-pET 28a(+) plasmid) were tested for VBNC state by culturing on enriched LB agar. The results showed that by direct plating of bacteria in enriched LB agar, no colony was observed after incubation at 37 °C for 48 h while the whole direct counts maintained constant at ca. 10^7 cells/ml. In order to confirm the viability of VBNC bacteria, the 5-cyano-2,3-ditolyl tetrazolium chloride reduction method was conducted and results revealed that these bacteria were able to reduce this substance. Also, the morphological studies showed that the most of VBNC bacteria had reduced cellular size and were in coccoid form than in bacilli form. In order to resuscitate the VBNC bacteria after the confirmation tests, we conducted various reactivation experiments including chemical (different concentrations of NaCl and bile salts) and physical (heat shock) treatments (Table 1). As can be seen on Table 1, the 15 and 45 mM concentration of bile salt and NaCl and also heat shock method at 42 °C for 2 minutes had the most significant effects on resuscitation of VBNC bacteria. Therefore, for improvement of the resuscitation rate, the combinations of the best results from the initial experiment were opted for combination model. This combination model was consisted of two by two of each treatment and also the combination of all three treatments (Table 1). As depicted in Table 1, the two salt-salt and salt-heat shock stresses had largely effect on reactivation rate of VBNC bacteria than in comparison of each alone. Among them the best result was obtained from combination of all three treatment in which the resuscitation rate was 9×10^8 CFU/ml (Table 1).

DISCUSSION

VBNC state is recognized for years, but up to the present moment, not much is known about the mechanisms relying induction and resuscitation of bacteria from this state. Most of the research performed so far was regarding induction of VBNC state on identification of VBNC bacteria on various substrates or species environmental microorganisms and also without particular relevance for those bacteria (e.g. *E. coli* DH5α) that had undertaken on various manipulation procedures. There are several empirical strategies that have used for resuscitation of VBNC state in bacteria. For example, such researchers used supernatants of exponentially growing bacteria such as *Mycobacterium tuberculosis* and *Micrococcus luteus* [18,7]. It was hypothesized that a nonproteinaceous molecule named enterobacterial autoinducer [19,9] a protein nominated resuscitation-promoting factor may take part in resuscitation processes [20], though our initial experiment did not show the effectiveness of this procedure (data not shown) [2]. In this study, the bacterial cells that had undergone on variety of manipulations (very low temperature and high glycerol concentration and also transformation processes) were chosen for their ability to form active state. Our preliminary experiment showed these conditions could induce VBNC state in bacteria. These findings are inconsistence with those of Oliver D. James *et al* who reported that recombinant manipulation of bacteria (plasmid insertion) along with temperature stress at 4 °C and room temperature had no effect on entering VBNC state [2]. The mechanism of this phenomenon is still remain unknown whereas the effect of low temperature is duo to the retaining of culturability for a longer period of time. It is hypothesized that low temperature can decrease the metabolism of the bacteria enabling them to sustain culturability for longer [21].

Various stress shocks including NaCl, bile salt and heat shock were analyzed for reactivation of VBNC bacteria. Our results illustrated that only 30 and 45 mM of NaCl and 15 and 30 mM of bile salt had positive effect on VBNC resuscitation. This event reflects the medium composition that was used for VBNC suspension in which contained the enriched LB broth (containing Caso-amino acids). As it was reported by Pinto *et al*, the treatment of VBNC suspension with both NaCl and several individual amino acids (but not each amino acid alone) led to reactivation of VBNC bacteria. This similar pattern has also been found for spore germination in *Bacillus subtilis* and resuscitation processes by *Vibrio* spp. and *E. coli* O157:H7 [8]. Bile salt as another tested chemical factor has also positive effect on recovery of VBNC bacteria and thus suggested this substance could binds to IpaD in *Shigella* spp. which induces the expression of two main gene clusters leading to VBNC resuscitation [22].

The influence of heat shock was also evaluated which we capable to recuperate viable bacteria from the nonculturable phase. It was theorized that heat shock may leads to expression of some chaperonin classes which

may contribute in the new growth of bacteria [14]. Our outcomes also showed that the combination of all these three stresses had significant synergistic impact on recovery of VBNC bacteria.

Table 1: Reactivation of *E. coli* strain DH5α from a nonculturable state by various chemical and physical treatments

Treatment Procedure	Viable cell count (cfu.ml^{-1})			
	Direct Plate Culture	Direct Broth Culture	Initial Cell no.	Final Cell Number After Treatment
1ml of bacteria+ 15 mM bile salt	ND*	ND	1×10^{8}	1×10^{4}
1ml of bacteria+ 30 mM bile salt	ND	ND	1×10^{8}	0.01×10^{4}
1ml of bacteria+ 45 mM bile salt	ND	ND	1×10^{8}	ND
1ml of bacteria+ 15 mM NaCl	ND	ND	1×10^{8}	ND
1ml of bacteria+ 30 mM NaCl	ND	ND	1×10^{8}	0.1×10^{4}
1ml of bacteria+ 45 mM NaCl	ND	ND	1×10^{8}	0.6×10^{4}
Heat shock at 42 °C for 1 min in 1 ml bacteria	ND	ND	1×10^{8}	1.2×10^{4}
Heat shock at 42 °C for 1.5 min in 1 ml bacteria	ND	ND	1×10^{8}	2.3×10^{4}
Heat shock at 42 °C for 2 min in 1 ml bacteria	ND	ND	1×10^{8}	3.1×10^{4}
1ml of bacteria+ 15 mM bile salt+ 45 mM NaCl	ND	ND	1×10^{8}	1×10^{7}
1ml of bacteria+ 15 mM bile salt+ Heat shock at 42 °C for 2 min	ND	ND	1×10^{8}	3×10^{8}
1ml of bacteria+ 45 mM NaCl+ Heat shock at 42 °C for 2 min	ND	ND	1×10^{8}	1.2×10^{8}
1ml of bacteria+ 15 mM bile salt+ 45 mM NaCl+ Heat shock at 42 °C for 2 min	ND	ND	1×10^{8}	9×10^{8}

Each treatment was done in enriched LB broth (0.2% glucose and 1% Caso-amino acids) medium for 12 h.
**ND: Not Defined*

CONCLUSION

In conclusion, this work illustrates the provocation of VBNC state on recombinant *E. coli* DH5α by stress conditions that are part of some problems in molecular biology. We believe that our findings are the first to recruit extensively of such stresses to overcome the VBNC states which may occur in laboratories. Therefore, this revitalization of culturability under special environmental circumstances constitutes a challenge for such researchers attempting to utilize such strategies to conquer of this problem.

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