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Induction to Viable but Non Culturable State and Resuscitation of *Bacillus cereus* and *E. coli*

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Abstract

E.coli and *B. Cereus* were induced to Viable but Non Culturable State by starvation and temperature upshift to 50°C. The change in osmolarity by addition of more than 10% NaCl also resulted into induction of VBNC state of both *B.cereus* and *E.coli.* In the VBNC state the cultures lost their capacity to reduce nitrate *B.cereus* also lost the capacity to ferment maltose, fructose, hydrolysis of starch and citrate utilization. On the other hand for *E.coli* the capacity to ferment arabinose, sorbitol and sucrose was lost. While serum and egg yolk could not help the resuscitation of the cultures but favourable temperature between 35° C - 40°C and enrichment of media could help in resuscitation of these cultures. There were distinct differences in the VBNC state with respect to enzyme activity.

Keywords: *E. Coli* | *B. cereus* | Culture

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Introduction

It is not necessary for all living systems to be in an environment which favours their survival. Various types of stress conditions may be experienced by microbes in different living conditions. The adaptability of the microbes to such stress conditions depends on various factors. A bacterial cell may enter into a starvation mode or enter into a physiological stress , wherein it is viable but not dead but multiplies by such a low rate that a visible colony is not formed . Such a state wherein the bacteria are viable but are not in a condition to grow well and hence non culturable can be defined as Viable but Non Culturable State or simply VBNC state. Conditions such as temperature, osmolarity, nutrient availability, pH and hydration are common factors leading to induction in the VBNC state.

Once a bacterial cell enters into VBNC state the question remains to be answered is that whether the state is a permanent state or the state can be reverted back to a culturable state.The change of non culturable bacteria back to culturable state is known as 'Resuscitation.' This has been observed in

many pathogens like *Mycobacterium tuberculosis* (Laam li *et al.,* 2014) *Legionella sps* (Steinert *et al.,* 1997; Al Bana *et al.,* 2013). *Vibrio sps*. (Baffone *et al.,* 2003; Sun *et al.,* 2008) When VBNC cells in the latent phase of a disease gets resuscitated the culturable cells then retain their virulence potential. A number of virulence and toxin genes are expressed even in the VBNC state.(Vora *et al.,* 2005).

Sinc many of the common pathogens including food pathogens are known to enter into the non culturable VBNC state and later resuscitate in the host body. It is important to have a detailed investigation of the various stress responses of the commonly known pathogens.

Materials and Methods

Induction into VBNC state using Starvation and Nutrient Deprivation

The isolates were subjected to nutrient deprivation and starvation by either prolonged incubation or by altering the media composition. This was found to be the most effective inducing factor for getting the VBNC state .the media composition was altered by gradually changing the contents from 100% to 10% For *e.g.*: The nutrient content of nutrient broth was reduced 13 gm / ltr to upto 0.5 gm per ltr (Lemke and Leff, 2006; Rehman *et al.,* 2001).

Induction into VBNC state using osmotic pressure

Salt and sugar concentration in the medium were increased from 2% to 10% for generating severe osmotic shock. The isolates were grown in high osmotic conditions by incubating at 37°C till the VBNC state is achieved (Inglish and Savripanth, 2006).

Induction of VBNC state using elevated temp of incubation

The isolated were grown at high temp of incubation upto 50°C fir getting VBNC states (Reissvrodt *et al.,* 2002)

Resucitation of the VBNC states of the Isolates

Resuscitation of VBNC state was carried out by different methods . The media composition was altered and enriched with either egg yolk or serum. The VBNC organisms were grown in the enriched media and incubated at 37°C until resuscitation occurs. In some of the cases the resuscitation was done by temperature modulation or temperature downshift as done by many workers . (Mukamolova *et al.,* 1998 and Downing *et al*., 2005; Reissbrodt *et al*., 2002)

Comparative Morphological, Biochemical Characterization ,Carbohydrate fermentation and Enzyme Studies were done to compare the Normal state, VBNC and Resuscitated state.

Results and Discussion

In case of Nutrient deprivation or starvation almost all the organisms entered into VBNC state when the nutrient supplied was reduced to around 60%. Similarly the temperature modulation showed a very distinct variation whereas the temperature upshift to about 50°C resulted in induction into VBNC state in *E. coli* VBNC state was detected at 4-6°C of incubation. It was noted that the difference in the total and viable counts was starkly different in case of nutrient deprivation in comparision to temperature upshift . Therefore it seems that starvation is better for induction into VBNC

state. When osmotic pressure was varied by the addition of salt and sugar it is observed that addition of sugar did not induce any of the cells into VBNC state. Both *Bacillus cereus,*and *E. coli,* tolerated the additional supplementation of sucrose. However, addition of sodium chloride at 10% resulted in induction into VBNC state. Lower than 10% of sodium chloride addition did not result in induction into VBNC state.

The resuscitated cultures showed morphology very similar to their corresponding normal state. Both *Bacillus cereus* and *E .coli* lost their capacity to reduce nitrate in their VBNC state and all these cultures upon resuscitation regain this property upon resuscitation. In case of *Bacillus cereus* loss of citrate utilization was observed. It can be seen from table 15 that *Bacillus cereus* loses the capacity to ferment maltose, fructose and does not show even hydrolysis of starch. In case of *E. coli* arabinose, sorbitol and sucrose are not fermented in the VBNC state as shown in table 16.

As can be seen from table 8 that none of the organisms could be resuscitated in 72 hrs by the addition of serum in the medium . The cfu/ml did not rise above 10/ml in any of the cultures . This indicates that serum does not act as a stimuli for resuscitation. Most of the colonies that grew on the cfu plates were of diminished size and did not show the typical colony characteristics of both the cultures under study. The serum contents are not able to provide the conducive environment or nutrient for resuscitation.

Egg yolk is generally considered to be rich in proteins and other growth factors. However supplementation of egg yolk at around 10 ml level could partially resuscitate as cfu rise above 10 /ml marginally as can be seen from table 9. In comparision to serum egg yolk was able to resuscitate partially but did not sufficiently resuscitate the VBNC cultures . The colony characteristics on the cfu plates were much larger in size in comparision to the colony size with serum addition.

Temperature upshift between 35 to 40°C resuscitated as the cultures with increase in cfu /ml as can be seen from table 10. Both the cultures showed typical colony characteristics upon resuscitation.

Enrichment of the media by adding additional amount of dehydrated medium reduced the starvation effect and increased the cfu/ml thereby resuscitating both the cultures. The colony characteristics of both the cultures were found to be same as the characteristics shown by normal viable and culturable cells.

In Biochemical Tests both *Bacillus cereus* and *E.coli* lost their capacity to reduce nitrate in their VBNC state and both the cultures upon resuscitation regain this property upon resuscitation. In case of *Bacillus cereus* loss of citrate utilization was observed. While comparing the carbohydrate fermentation of both the cultures in their VBNC state and their resuscitated state it can be observed that a number of sugars are not fermented in their VBNC state. It can be seen from table 15 that *Bacillus cereus* loses the capacity to ferment maltose, fructose and does not show even hydrolysis of starch. In case of *E.coli*

arabinose, sorbitol and sucrose are not fermented in the VBNC state as shown in table 16. Both *B.cereus* and *E.coli* loses the catalase property the lack of catalase in the VBNC state clearly states the necessary stress that is responsible for entry into the VBNC state.Apart from this *Bacillus cereus* also loses the arginine dehydrolase and esculin hydrolysis activity in VBNC state.Similarly *E.coli* also shows distinct biochemical variations in a large number of enzyme activity. For *e.g.* in the VBNC state it loses arginine dehydrolase , acetate utilization , ornithine decarboxylase and lysine activities.

In the present study nutrient deprivation induced both *Bacillus cereus* and *E.coli* to enter into VBNC state. As can be seen from table 1 when nutrients are very low the ratio between the viable count and the total count is very high and cfu/ml is far below 10. Similar difference between the viable and total count has been reported by other workers. (Chaveerach *et al.,* 2003). Prolonged incubation resulted in successful establishment of VBNC state. The elevation of temperature of incubation overall 50°C results in induction into VBNC state but low temperature did not favour induction into VBNC state except for E.coli. Osmotic pressure due to addition of salt resulted in induction into VBNC state but additional sugar addition did not act as a factor for induction into VBNC state.

In our study resuscitation could not be attained by the addition of serum or egg yolk. However temperature modulation resulted in rsuscitation within 72 hrs .Similarly the increase in the nutrient content resulted in resuscitation. The resuscitation should be improved further by increasing the incubation period.Such work has been reported by many workers. (Pawlowski *et al.*. 2011; Amel *et al.,* 2008; Panutdaporn 2006; Bovill and Mackey 1997)

In the present study distinct changes were observed in the biochemical characteristics of the organisms . While catalase activity was absent in VBNC cells the lack of catalase must have resulted in stress of reacting oxygen species .Distinctly nitrate reduction and argine dehydrolase activity have been markedly accepted in the VBNC state.

It is very essential to examine a large number of food items that are available in the market to ascertain the existence of VBNC state of different microbes . The improvement of human health necessitates a thorough and deep study in this field.

S. No	**Organism**	**Number of Colonies (cfu/ml)/Total Count (a*10^3)**								**Induction into VBNC state**
		12gms	10gms	8gms	6gms	4gms	2gms	1gms	0.5gms	
1	B.cereus	30/46	30/48	10/45	7/49	7/51	6/51	5/44	5/52	+
2	E.coli	32/38	25/39	8/37	7/36	6/37	6/37	5/38	2/39	+

+ = Positive Reaction: - + Negative Reaction ; ND=Not Detected

Table 1: Induction into VBNC state of different organism by Nutrient Deprivation

S. No	Organism	Morphology	Gm Reaction
1.	B.cereus	Very short coccoid	Gm +ve
2.	E.coli	Coccoid	Gm-ve

Table 2: Morphological variation in VBNC State

S.No	Organism	Incubation in days	Number of colonies/ Total Count(a*10^3)	Morphology
1	B.cereus	45	04/56	Very short cocccoid
2	E.coli	40	03/58	Coccoid

Table 3: Induction into VBNC state by prolonged incubation

S. No	Organism	No of colonies(Cfu/ml)/ Total Count (a*10^3)				Induction into VBNC State
		39°C	42°C	46°C	50°C	
1	B. cereus	40/48	40/49	20/46	8/49	+
2	E. coli	50/52	40/50	20/52	7/51	+

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 4: Induction into VBNC state by High Temperature Incubation

S. No	Organism	No of colonies (Cfu/ml)/Total Count (a*10^3)				Induction into VBNC State
		20°C	10°C	6°C	4°C	
1	B..Cereus	ND	ND	ND	ND	ND
2	E.coli	180/210	10/160	2/49	1/47	+

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 5: Induction into VBNC state by Low Temperature Incubation

S. No	Organism	Number of Colonies (Cfu/ml)/ Total Count (a*10^3)					Induction into VBNC State
		2%	4%	6%	8%	10%	
1	B.cereus	40/48	37/49	37/46	15/48	07/49	+
2	E.coli	45/49	42/49	37/48	16/46	06/48	+

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 6: Induction into VBNC state by Osmotic Pressure using Salt

S. No	Organism	Number of Colonies (Cfu/ml)/Total Count(a*10^3)					Induction into VBNC State
		2%	4%	6%	8%	10%	
1	B.cereus	40/44	42/46	42/45	40/46	38/46	ND
2	E.coli	32/41	35/45	37/42	32/40	32/41	ND

+ = Positive Reaction: - + Negative Reaction ;ND=Not Detected

Table 7: Induction into VBNC state by osmotic pressure using Sugar

S. No	Organism	Concentration of Serum in ml								
		0.1	0.5	1	2	4	6	8	10	Incubation Period
		No. of Colonies(Cfu/ml)/Total Count(a*10^3)								
1	B.cereus	1/46	2/44	2/48	2/46	3/44	6/48	8/47	8/46	72 hrs
2	E.coli	1/45	3/48	3/46	3/48	4/48	6/47	6/43	6/44	72 hrs

Table 8: Resuscitation by addition of Serum

S.No	Organism	Concentration of Egg yolk								
		0.1	0.5	1	2	4	6	8	10	Incubation Period
		No of Colonies(Cfu/ml)/Total Count ($a*10^3$)								
1	B.cereus	0/46	0/48	2/47	2/44	5/47	7/48	12/46	12/46	72 hrs
2	E.coli	0/44	0/46	0/44	2/48	4/49	10/46	10/44	12/48	72hrs

Table 9: Resuscitation by addition of Egg Yolk

S. No	Organism	Temperature of Incubation				
		4+-2°C	10+-2°C	37+-2°C	40+-2°C	Incubation Period
		No of Colonies(Cfu/ml)/Total Count ($a*10^3$)				
1	B..cereus	6/46	10/48	40/47	42/45	72hrs
2	E.coli	0/44	5/50	50/54	55/58	72 hrs

Table 10: Resuscitation by Temperature modulation

S. No	Organism	Concentration of Nutrient media (gms/ltr)			Incubation Period
		13	35	40	
		No. of Colonies(Cfu/ml)/ Total Count($a*10^3$)			
1	B.cereus	2/44	19/46	20/44	72 hrs
2	E.coli	4/46	20/44	25/45	72 hrs

Table 11: Resuscitation by increase in Nutrient Content

S. No	Organism	Gram Reaction	Motility	Spore Staining	Morphology
1	B. cereus	Gm +ve	Sluggishly motile	Sporulating	Very short coccoids
2	E. coli	Gm -ve	Motile with zigzag motion	Non sporulating	Coccoid

Table 12: Morphological Characterization of Resuscitated Cultures

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Indole	-	-	-
2	Methyl Red	-	-	-
3	VogesPrauskauer	+	+	+
4	Citrate	+	-	+
5	Nitrate reduction	+	-	+
6	H_2S	-	-	-

+ = Positive Reaction: - + Negative Reaction ;
ND=Not Detected

Table 13: Comparative Biochemical characteristics of *B.cereus*

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Indole	+	+	+
2	Methyl Red	+	+	+
3	VogesPrauskauer	-	-	-
4	Citrate	-	-	-
5	Nitrate reduction	+	-	+
6	H_2S	-	-	-

+ = Positive Reaction: - + Negative Reaction;
ND=Not Detected

Table 14: Comparative Biochemical characteristics of *E.coli*

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Glucose	+	+	+
2	Galactose	-	-	-
3	Maltose	+	-	+
4	Mannitol	-	-	-
5	Lactose	-	-	-
6	Inulin	-	-	-
7	Fructose	+	-	+
8	Arabinose	-	-	-
9	Sorbitol	-	-	-
10	Starch	+	-	+
11	Sucrose	+	-	+
12	Xylose	-	-	-

+ = Positive Reaction; - + Negative Reaction;
ND=Not Detected

Table 15: Comparative Carbohydrate fermentation of *B.cereus*

S. No	Characteristics	Organism		
		Normal State	VBNC state	Resuscitated state
1	Glucose	+	+	+
2	Galactose	ND	ND	ND
3	Maltose	-	-	-
4	Mannitol	+	+	+
5	Lactose	+	+	+
6	Inulin	-	-	-
7	Fructose	-	-	-
8	Arabinose	+	-	+
9	Sorbitol	+	-	+
10	Starch	ND	ND	ND
11	Sucrose	+	-	+
12	Xylose	ND	ND	ND

+ = Positive Reaction; - + Negative Reaction;
ND=Not Detected

Table 16: Comparative Carbohydrate fermentation of *E.coli*

S. No	Characteristics	Organism		
		Normal state	VBNC state	Resuscitated state
1	Oxidase	-	-	-
2	Catalase	+	-	+
3	Urease	-	-	-
4	Arginine dehydrolase	+	-	+
5	Esculin hydrolysis	+	-	+
6	Acetate utilization	-	-	-
7	Phenylalanine deaminase	-	-	-
8	Ornithine decarboxylase	-	-	-
9	Lysine	-	-	-

+ = Positive Reaction; - + Negative Reaction;
ND=Not Detected

Table 17: Comparative enzyme study of *B.cereus*

S. No	Characteristics	Organism		
		Normal state	VBNC state	Resuscitated state
1	Oxidase	-	-	-
2	Catalase	+	-	+
3	Urease	-	-	-
4	Arginine dehydrolase	+	-	+
5	Esculin hydrolysis	ND	ND	ND
6	Acetate utilization	+	-	+
7	Phenylalanine deaminase	-	-	-
8	Ornithine decarboxylase	+	-	+
9	Lysine	+	-	+

+ = Positive Reaction: - + Negative Reaction;
ND=Not Detected

Table 18: Comparative enzyme study of *E.coli*

Conclusion

Both *B.cereus* and *E.coli* could be induced to VBNC state and were resuscitated by suitable stimulating factors.

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