

Research Article

ISOELECTRIC FOCUSING OF MEMBRANE PROTEINS OF PROBIOTIC *B. COAGULANS* AND ITS BACTERIOPHAGE RESISTANT MUTANTS

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Abstract: Bacteriophages are the most notorious type of infection in the probiotic and dairy fermentations. Two phage resistant mutants viz. *B. co* PIII and *B. co* MIII (*B. coagulans* mutants PIII and MIII) obtained in previous studies (Dubey and Vakil, 2010), were further characterized for their protein profile in comparison with the parental probiotic strain –*B. coagulans*. The cell lysates were subjected to ultra-centrifugation and the purified membrane fractions were resolved using 2D gel electrophoresis. The Isoelectric focussing showed 187, 202 and 154 protein spots for the parental strain, mutant *B. co* PIII and mutant *B. co* MIII, respectively. Ten and 18 protein spots were missing as compared to parent for mutants *B.co* PIII and *B.co* MIII whereas there were 21 and 14 new spots noticed for these two mutants. Eight membrane proteins present only in the phage sensitive parental culture could be tentatively identified by comparison with the complete proteome of *B. coagulans* by use of UniprotKB and then CELLO database. It is quite likely that some of these identified membrane proteins may be also functioning as receptors for phage adsorption followed by entry of nucleic acid into the phage sensitive host cell.

Keywords: 2D gel electrophoresis; Isoelectric focussing; membrane proteins; probiotic; *Bacillus coagulans* mutants.

Note: Coloured Figures available on Journal Website in "Archives" Section

Introduction

Probiotics are live microorganisms which when consumed in adequate amounts confer several health benefits to the hosts as evident from the literature (Harish and Varghese, 2006; Alloui *et al.*, 2013). Probiotics are produced as biomass by the route of fermentation and are used in foods or as medicine. The resultant biomass is harvested, separated from the spent growth medium and then concentrated and preserved as bulk before formulating it as a probiotic (Brüssow, 2001; Shah, 2001; Lacroix and Yildirim, 2007; Ding and Shah, 2009). The fermentation process is prone to contamination by strain specific phages which normally lead to batch failures due to lysis

of actively growing probiotic cultures resulting in huge financial losses (Pandey *et al.*, 2015).

The traditional microbiological strategies to keep phages away from the fermentation process include rotation of cultures, use of phage inhibitory media and maintaining of aseptic processing conditions from laboratory to commercial production area (Brüssow, 2001). A more effective way to prevent the phage infection is to make the cultures genetically resistant. Physical and chemical mutagens have been used effectively to induce changes in the host DNA to make them resistant to phage attacks (Marci *et al.*, 2004; Dubey and Vakil, 2010). Classical mutation and selection programs have been used in the industries for many years and have resulted in screening and isolation of mutants with improved characteristics that have resulted in large productivity increments. Such strain improvement programs have substantially driven

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down the cost of production of a wide range of fermentation products (Pinched *et al.*, 2001).

Proteomic studies help to elucidate the expression, structure and function of all the proteins which are responsible for life's activities (Rappsilber *et al.*, 2002). The main techniques used for separation and identification of expressed proteins are two-dimensional(2-D) gel electrophoresis, 2-D HPLC, MALDI-TOF/MS and bioinformatics (Wang *et al.*, 1999; Garrigues *et al.*, 2005).

Out of the several mechanisms described in literature for bacteriophage resistance, the most common mechanism is alterations in the membrane proteins (Wilson and Murray, 1991; Coffey and Ross, 2004; McGrath *et al.*, 2007; Chopin *et al.*, 2005; Yang *et al.*, 2006). Membrane proteins are strategically localized at the interface between the cell and its external environment, which impart them a role in transport, sensing and communication (Molloy *et al.*, 2000; Santoni *et al.*, 2000). Phages attach to their complementary receptors, usually located on membranes to infect a host (Forde and Fitzgerald, 2003; DeHaard *et al.*, 2005; Tremblay *et al.*, 2006). It is estimated that between 20 to 30% of all genes in bacteria code for membrane proteins (Poetsch and Wolters, 2008). Membrane proteins can be divided into two structural groups -integral proteins that are embedded in the membrane and peripheral proteins that have a lower association with the membrane (Sjoquist *et al.*, 1972).

Despite their functional relevance the study of membrane proteins is notoriously difficult because of their hydrophobicity and embedment in the lipid bilayers. SDS-PAGE alone cannot resolve membrane proteins which are of such diverse nature and in minute quantities (Wilkins *et al.*, 1998). Advanced technique of 2-dimensional electrophoresis has been used to separate and purify membrane proteins after their separation from rest of the cell components, cell debris and growth media components by ultracentrifugation (Pasquali *et al.*, 1997; Rabilloud *et al.*, 1997; Molloy *et al.*, 2000; Garrigues *et al.*, 2005). During isoelectric focussing proteins carrying different charges, depending on the amino acid composition, travel towards the oppositely charged electrode under the influence of

electricity. In this course, the migrating proteins reach a pH value where their net charge is zero, i.e. zwitterionic form. This pH value is the isoelectric point (pI) of the protein (Simpson, 2006). Thus, IEF involves separation of proteins based on their characteristic pI values. IEF is generally followed by second dimension SDS-PAGE using 15% resolving gel where the proteins are further separated according to their molecular weights (M_r , relative molecular mass) (Simpson, 2006). Each spot on the resulting two-dimensional gel potentially corresponds to a single protein species in the sample (GE manual, 2013). Using bioinformatics tools identification of the probable proteins of interest can be effectively carried out.

The UniProt KnowledgeBase (UniProtKB) is the central hub for collection of functional information on proteins with accurate, consistent and rich annotation (<http://www.uniprot.org/help/uniprotkb>). CELLO is a subCELLularLOCALization predictive system (<http://cello.life.nctu.edu.tw/cgi/readme.txt>) which can be used for identification of proteins according to their subcellular locations.

To overcome the issue of recurring phage attacks, industrial probiotic culture *B. coagulans* was mutated earlier and several phage resistant mutants were obtained (Dubey and Vakil, 2010). However, the non-specific random mutations may have introduced some unintended and undesirable mutations (Friefelder, 1987). Current work is an effort to focus and separate the membrane proteins from the parent *B. coagulans* and its 2 phage resistant mutants- *B. co* MIII and *B. co* PIII. Further aim was to identify the differences in their spot profiles after 2-D gel electrophoresis so as to correlate it with the phage resistance.

Materials and Methods

Microbiological media and biochemical indicators were obtained from Himedia® India Ltd., Molecular biology kits and enzymes from Invitrogen Inc., USA. Chemical reagents and stains required were procured from Merck® Specialty Chemicals Limited and SRL labs. IPG strips, buffer and other consumables were procured from GE India, Pvt. Ltd.

Bacterial strains

Parental strain was a phage sensitive *B. coagulans* culture received from Microbax Pvt. Ltd., a pharmaceutical company from Hyderabad, India and the phage resistant mutants B. co. MIII and B. co. PIII were obtained by mutation treatment with UV irradiation and MMS (Methylmethanesulfonate), respectively (Dubey and Vakil, 2010). The three cultures were maintained on glucose yeast extract medium and preserved as suspension in 15% glycerol at -20 °C.

Proteomic characterization

Proteomic characterization of probiotic strains was carried out as depicted in Figure 1.

Sample preparation

Cell growth

To 500 ml flasks containing 100 ml of GYEB medium, 250 µl of actively growing ($A_{540nm}=0.5$) *Bacillus coagulans* cells (parental and mutant), were inoculated and the flasks were incubated at 37°C, at 150 rpm on an orbital shaker. The cells were allowed to grow till an OD of $A_{540nm}=2.0-3.0$ was achieved (20-24 h) and the biomass was harvested by centrifugation at 13000 g for 10 min at 4 °C (Pandey and Vakil, 2016). Supernatant was discarded while the pellet was stored on ice for

10 min followed by resuspending it in ice cold PBS to obtain a homogenous suspension. The cells were washed twice by resuspension of pellet in the same buffer followed centrifugation.

Cell disruption by sonication

The pellet was re-suspended in 25 ml ice cold extraction buffer [50 mM Tris (pH 7.3), 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol] and sonicated at 40 % amplitude, 10 Sec ON and 5 sec OFF cycle, for 20 min under cold conditions to obtain a homogeneous cell lysate which was centrifuged at 13000 g for 10 min at 4 °C and pellet was discarded (Clarke and Hill, 1990). Supernatant was transferred into pre-chilled falcon tube. Protein content in the sample was estimated by Bradford's colorimetric method (Kruger, 1994). Samples were stored at - 20 °C till further processing.

Ultracentrifugation

The lysate obtained (as described above) was centrifuged in an ultracentrifuge (Model hMac, Beckman Coulter) at 100000 g for 90 min as per the instructions of the centrifuge manufacturer (Lustig *et al.*, 2000). The supernatant (cytosolic fraction) was discarded and the pellet consisting of membrane proteins was washed with extraction buffer and centrifuged at 100000 g at 4 °C for 90 min. Solubilization buffer was added (7

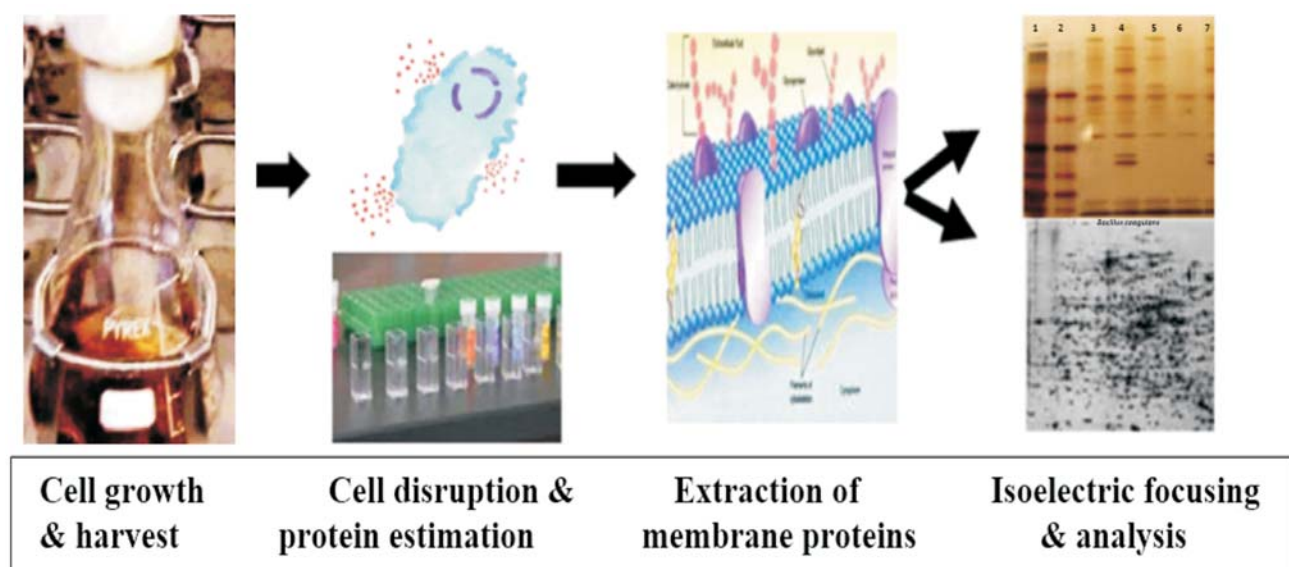


Figure 1: Schematic outline of proteomic study.

M Urea, 2 M Thiourea, 2% Triton X-100) and incubated for an hour with occasional vortexing (GE, 2DE Manual, 2013). The content was recentrifuged at 100000 g at 4°C for 90 min and the supernatant was retained as the membrane fraction for further proteomic analysis. Fractions were stored at -80 °C till used for further work.

SDS-PAGE

Proteins from whole cell lysate were analyzed on electrophoretic apparatus (Make: Technosource) with discontinuous gel system (15% resolving and 5% stacking gels) (Hames, 1998). Samples were subjected to heat shock at 95-100°C for 10 min before loading 5 µg of sample (Simpson, 2006). Two µl of protein ladder (GE manual, 2013) was loaded in the last well. The gel was electrophoresed at 120 V till tracking dye reached the bottom of the gel. The gel was silver stained (Heukeshoven and Dernick, 1985).

Two-Dimensional gel electrophoresis

1st dimension: Isoelectric focussing (IEF)

Sample was prepared by adding 62.5µl of rehydration buffer containing 0.7mg of DTT to equal volume of membrane fraction of cells. Destreak buffer (1.3µl) was added to the mixture (GE manual, 2013). The content was centrifuged

in a tap spin for 30sec and the total volume was withdrawn and used as sample for isoelectric focussing as per the scheme shown in Figure 2.

The proteins were focused using NL (non-linear) IPG strip of 7.0 cm length between the pH of 3-11 (Hames, 1998). For equilibration the IPG strip was placed in a 25 ml falcon tube. Ten ml of SDS equilibration buffer (Composition = urea: 6 M, TrisHCl (pH 8.8): 75 mM, glycerol: 29.3%, SDS: 2%, 1% Bromophenol blue: 0.002%, total volume: 50 ml), containing 100 mg of DTT was used for first step of equilibration for 15 min. The tube content was poured off without disturbing the strip. Second equilibration step was carried out for another 15 min with SDS equilibration buffer containing 250 mg of iodoacetamide. The gels obtained were silver stained (Heukeshoven and Dernick, 1985).

The focussing program was divided into 7 steps as represented in Table 1. The voltage gradient of the focussing program was increased from 500 to 2500 V and the focussing occurred at 1500 Vh. The process of focussing of proteins took 18-20 h.

Second dimension: SDS-PAGE

After 1st dimension separation, the samples were subjected to 2nd dimensional separation, where

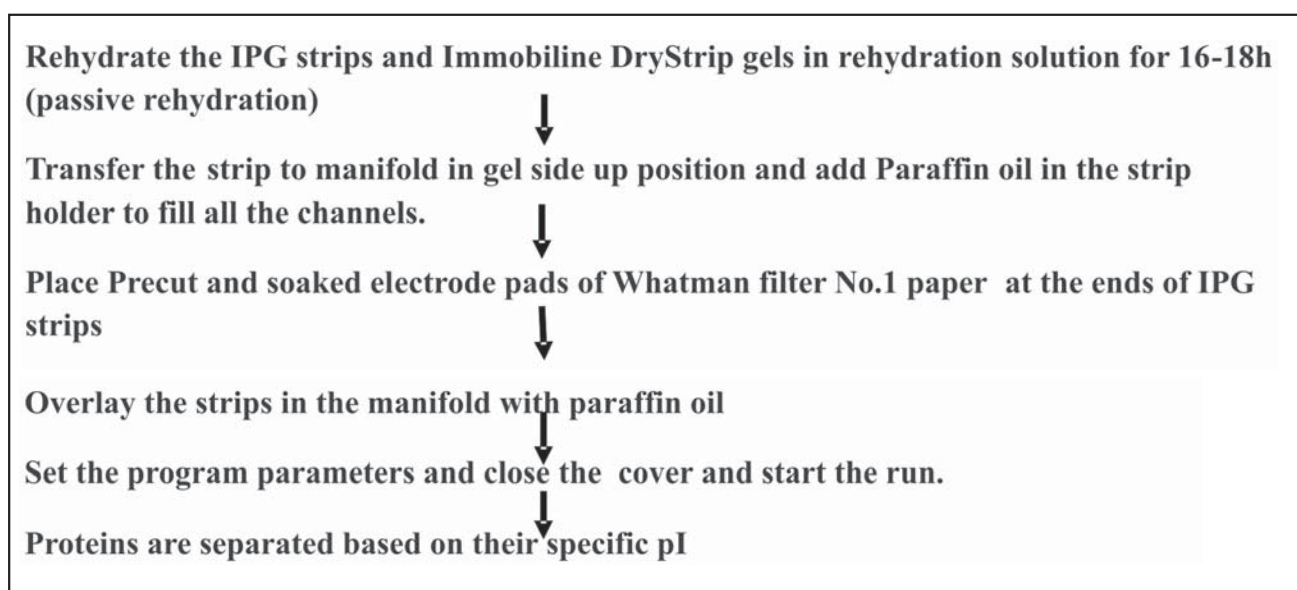


Figure 2: Schematic representation of isoelectric focussing procedure (GE manual, 2013)

Table 1
Program for isoelectric focussing of protein fraction

Steps	Volts (Step/Gradient)	Time(h)
1	50V (step)	1
2	200V (step)	0.5
3	500V(step)	0.5
4	2500V (Gradient)	0.5
5	5700V (Gradient)	1500Vh
6	7000V (step)	1
7	50V(step)	20

proteins were further separated based on molecular mass. SDS-PAGE (2nd dimension) was carried out with 12 % resolving gel (Hames, 1998). The isoelectrically focussed IPG strip was placed horizontally in contact with the gel and electrophoresis was carried out. The gel was run using 125 V till tracking dye had travelled almost 90 % of the gel length and it took 45-50 min to complete the run (GE manual, 2013).

Relative mobility of proteins

It is the migration distance M_r of proteins with respect to the tracking dye. It was estimated using the formula given below.

$$M_r = \frac{\text{Migration distance of interested protein spot (cm)}}{\text{Migration distance of dye front (cm)}}$$

On the basis of a graph constructed, M_r of protein spot(s) of interest was located on the X-axis and used to obtain its corresponding molecular mass (Simpson, 2006).

pI of the proteins

The first dimension lengths were determined by measuring length of the immobilized dry strip gel and positioning the spot on the second-dimension gel. Spot positions were plotted (as a % of gel length) versus pH and the pI read off from the graph of the pH gradient (GE manual, 2013).

Probable identification of the proteins

The full proteome of *B. coagulans* 36D1 (size 3275) was retrieved from UniProt database. The CELLO subcellular localization prediction tool was used to identify the proteins that were classified as membrane proteins as literature

shows that proteins involved in phage adsorption are cytoplasmic membrane proteins. The criteria for selection of proteins from the CELLO database was to take the proteins whose values confirmed in final prediction with more than score of 7 for cytoplasmic membrane and more than scores of 9 for cytoplasmic. These are the threshold values for selection keeping the statistical significance in mind. Thus from the full proteome data of about 3,275 proteins 415 proteins were selected. Further the in-silico data retrieved from CELLO database was compared with the Mr Values of the protein which in turn were based on the pI values obtained from the wet lab data. Thus 8 proteins were predicted which were called as "Probable predicted proteins" of interest. Further analytical laboratory work as well as software based image analysis and mass spectrometry based molecular wt. determination work coupled with bioinformatics approach will be needed to characterize the short-listed 8 proteins.

Results and Discussion

SDS-PAGE analysis of protein fractions

Proteins extracted after cell lysis include proteins from cytoplasm as well as organelles and membrane. Ultracentrifugation technique was resorted to for obtaining the membrane proteins from the cell lysate. Before proceeding to isoelectric focussing, the proteins were checked for separation by SDS-PAGE and visualized by silver staining (Heukeshoven and Dernick, 1985).

As may be noted from Figure 3 compared to parent *B. coagulans*, mutants *B. co* PIII and *B. co* MIII displayed fewer numbers of bands (20-30) and were stained rather lightly. Some streaking effect was also noticed. Streaking could have been a result of poor solubilization of some of the hydrophobic membrane proteins. As expected, number of bands in membrane fractions was fewer compared to the cytosolic proteins. Larger number of proteins in the cytosolic fractions could have caused poor resolution in case of cytosolic fractions. The trial SDS-PAGE runs assisted in determining the right percentage of resolving gel suitable for separation of membrane fractions for the 2 D gel electrophoresis. It also helped in assessing the quality of separation of membrane proteins.

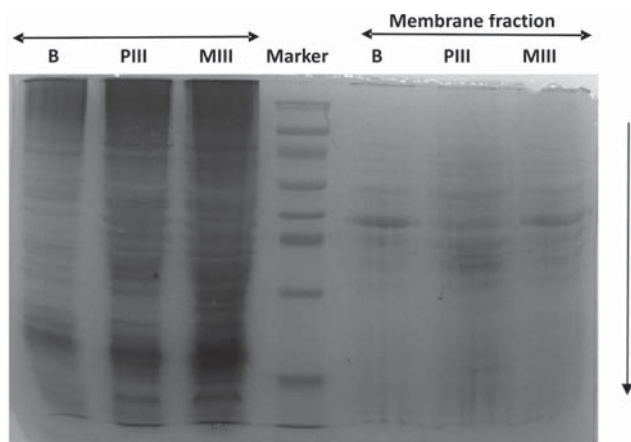


Figure 3: Cytoplasmic and membrane proteins separation using SDS-PAGE on 12% resolving gel (Lanes 1-3: cytosolic fraction of parent and mutants *B. co* PIII and *B. co* MIII respectively, Lane 4 -protein marker Lanes 5-7- membrane fractions of parent and mutants *B. co* PIII and *B. co* MIII, respectively)

The 2 D gel electrophoresis

Figure 4 displays the 2 D spot profiles of *B. coagulans* and mutants *B.co* PIII and *B.co* MIII. The parental culture run appeared slightly over-stained hence looked darker. Streaking was also observed. A total of 187 spots were obtained membrane fraction of *B. coagulans*. There were far more bands in the upper half of the gel indicating presence of higher molecular weight proteins. Major proteins were localized in the pH range of 4.0-8.0. Streaking effect was observed indicating likely ionic contamination from the buffers used in the purification process. Some areas of low molecular weight unresolved proteins were also observed. There are several reasons which impart high resistance to protein mobility and hence streaking and blotching in the gel (O'Farrell, 1975). Some of the factors include protein aggregation, high salt content in the sample, hydrophobicity (especially in case of membrane proteins), inappropriate protein solubilisation *etc.*

Use of de-streaking solution for samples of mutants *B. co* PIII and *B.co* MIII significantly reduced the problem of streaking. Also protein spots were better visualized because of high contrast due to clear background. Very few spots were present at the extreme pH values (3-5 and 8-11). Numbers of spots in *B.co* PIII gel were 202. Several spots (minimum 10 No.) were missing when compared to the parental profile. Also some

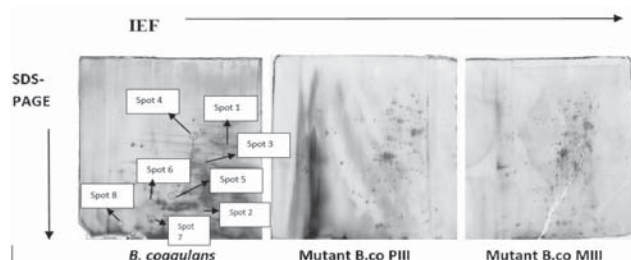


Figure 4: The 2D protein spot profiles of the parental culture *B. coagulans* and mutants *B. co* PIII and *B. co* MIII.

new spots (minimum 21) were noticed. In case of another mutant -*B.co* MIII 14 new spots were noticeable while 18 spots were missing making a total of 154 spots observed. Table 2 represents the number of total protein spots present in the 3 gels including the number of missing and newly identified spots.

Based on the comparison of M_r for the parental protein spots obtained experimentally with the M_r of proteins in the CELLO database 8 membrane proteins were noted to be present in case of phage sensitive parental culture of *B. coagulans* but absent in the profiles of both the mutants. These proteins were tentatively identified by comparing their PI values and M_r with the CELLO database. These proteins are marked as spots in Figure 4 and these are also listed in Table 3-showing their reported functions. The list includes enzymes, transporter and membrane proteins having varied roles to play. The phage resistant mutants might carry different types of mutations like single nucleotide polymorphisms (SNPs), small insertions or deletions, leading to nonsense codons, frame-shifts or other mutations. These alterations may have caused inactivation of one or more membrane proteins from parental cells some of which may have a role to play in phage infection.

Table 2
Distribution of protein spots in the 3 probiotic membrane fractions

Cultures	Total number of spots	Missing spots	New spots
Parent <i>B. coagulans</i>	187	-	-
Mutant <i>B.co</i> PIII	202	10	21
Mutant <i>B.co</i> MIII	154	18	14

Thus, it is conceivable that inactivation/modification of such gene product(s) may prevent receptor specific adsorption of phage particles and subsequent transportation of phage nucleic acid across the bacterial membrane.

Scanty reports are available on proteomic characterization of bacteriophage resistant strains of *B. coagulans*. Two of the 8 membrane proteins, YhgE/Pip N-terminal domain protein (pI 8.40 and $M_r=94680$) and Methyl-accepting chemotaxis sensory transducer protein (pI 6.65 and $M_r=66075$) are both transmembrane proteins and reported to have been associated with development of phage resistance in *Clostridium difficile* and *Escherichia coli* respectively (Labrie *et al.*, 2010; Sekulovic *et al.*, 2015). Whereas endolysin R (pI 7.50, M_r 103706) an enzyme coded by bacteriophage genes has been postulated to play a role in lysis of host cells (Loessner *et al.*, 1997). It is quite conceivable that there will be several other transmembrane proteins besides the 3 members mentioned in Table 3, which may play a role in phage infection or resistance.

A polypeptide bacteriophage receptor was studied in *B. sphaericus* strain P-1. *B. sphaericus* cells were mutated and their cell walls were examined. Mutant strains did not adsorb the phage. The amino acid composition of the T

layers, which is the outer layer of bacterial cell wall, was altered owing to mutagenesis. It has significant role in display of receptor proteins where phages attach to infect a host cell (Howard and Tipper, 1972). One of the most probable causes for development of phage resistance is alterations in receptor proteins at membrane surfaces. Sao-Jose and coworkers (2004) were the first to demonstrate membrane-bound protein acting as a phage receptor in *B. subtilis*. They further suggested an additional involvement of the *yukE* operon in a step subsequent to irreversible adsorption. Davidson *et al.*, (2005) reported identification of a protein (GamR), a surface-anchored protein which is involved in the bacterial receptor for the ϕ phage. They deleted a single gene from *B. anthracis* and this rendered it resistant to phage infection. In another study (Bishop and co-workers, 2012) reported development of phage resistance in *B. anthracis* by substitution of a highly conserved histidine residue in CsaB with an alanine residue (H270A) protein, indicating the role of CsaB in phage infection, most likely in phage adsorption. Thus, we can conclude that the membrane proteins which can act as receptors for phage attachment have vital role in phage infection and alterations in these proteins due to mutations can cause development of phage resistance.

Table 3
Eight membrane proteins present only in parental phage sensitive *B. coagulans*

Sr. No	pI	M_r (Da)	Probable protein	Reported function / role
Spot 1	8.40	94680	YhgE/Pip N-terminal domain protein	Integral component of the membrane structure.
Spot 2	8.40	56866	Sugar transporter	Membrane protein involved in transport of oligosaccharides and amino acids
Spot 3	7.50	103706	Endolysin R	Enzyme with probable role in phage infection
Spot 4	6.85	120350	Methyl malonyl-CoA mutase, large subunit	Catalyzes the conversion of acids to succinyl Co-A
Spot 5	6.65	66075	Methyl-accepting chemotaxis sensory transducer	Transmembrane signalling receptor activity.
Spot 6	6.14	53652	GamR protein	Gamma receptor protein involved in transmembrane transport
Spot 7	6.05	35913	UDP-glucose polyglycerol teichoic acid glucosyltransferase	Involved in transmembrane transport
Spot 8	4.80	53729	Polysaccharide biosynthesis protein	Involved in biosynthesis of polysaccharide layer of the cells

Conclusions

Two-dimensional gel electrophoresis for the membrane proteins from *B. coagulans* and its mutants has been performed for the first time. Using ultra-centrifugation, membrane proteins of the 3 strains: *B. coagulans*, mutant B.co PIII and mutant B.co MIII, were isolated and purified. These proteins were resolved using 2D gel electrophoresis. With the help of databases UniProtKB and CELLO 8 of the proteins present only in the parental phage sensitive culture were identified. Presence of new spots as well as absence of many spots in the 2 mutants- B. co PIII and B.co MIII suggests that random mutagenesis had quite an effect on the genetic profile of parental strain which led to development of phage resistance. It may be concluded that random mutagenesis can be a very easy and economical way to obtain phage resistant mutants for use on commercial scale. As the genetic change is of permanent nature it can offer substantial cost saving on commercial scale as against the use of physical and chemical methods

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Abbreviations

B. coagulans, *Bacillus coagulans*; B. Co PIII, Bacteriophage resistant mutant PIII; B. Co. MIII, Bacteriophage resistant mutant MIII; MMS, Methylmethanesulfonate; IEF, Isoelectric focussing; pI, isoelectric point; 2-D, 2 dimensional; M_r, relative molecular mass (Da). DTT: Dithiothritol.

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