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PCR based screening for *srf*AA ORF1 from local *Bacillus* species

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Abstract

Cyclic lipopeptides including surfactin, iturin and fengycin families are important metabolites produced by *Bacillus* having significant role in disease control, agriculture, medicine, cosmetics, pharmaceuticals, food and petroleum industry. Some of these lipopeptides have been reported to show biosurfactant activities with surfactin being the most studied biosurfactant possessing growth inhibition activity for phytopathogenic bacteria, fungi, virus or mycoplasma. *srf*A operon of surfactin is essential for competence development and the presence of this operon is confined to *Bacillus subtilis, B. amyloliquefaciens* and *B. circulans* only. In the present project, three different *Bacillus* species i.e. *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus sphaericus* were screened for the presence of *srf*AA ORF1 of surfactin synthetase using PCR. Specific primers were designed against this ORF1 region for PCR amplification with the help of online bioinformatics tools and by using already reported GenBank data. Genetic variability in the size of the ORF1 region was observed in the two isolates of *Bacillus subtilis* (~500 bp for MBL-A and ~700 bp for MBL-B) as compared to the reported size. Some nonspecific amplifications of approximately 300 bp size were also seen in *Bacillus thuringiensis* and *Bacillus sphaericus* and may represent some part of surfactin synthetase ORF1. Precise knowledge requires further studies of gene characterization, genetic variations and phylogeny.

Key words: Lipopeptides · Biosurfactant · Surfactin · *Bacillus* species · *srf*A operon

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1. Introduction

Some *Bacillus* species are capable of producing compounds like glycolipids, lipopeptides, enzymes, bacteriocins [9, 12], proteins, lantibiotics [5], metabolites etc. The synthesis of peptides in *Bacillus* is controlled by large multienzyme complexes named peptide synthetases. The multidomain nature of these enzyme subunits has been confirmed by isolating and characterizing genes of several peptide synthetases. Each enzyme subunit is a large protein with molecular weight over 100,000 Daltons containing 1 or more amino acid-activating domains [18]. Lipopeptides are amphiphilic cyclic peptides that are composed of ten or seven amino acids with a long fatty acid side chain. Lipopeptides from *Bacillus* species were recently studied for their great effect in the biocontrol of phytopathogens [7, 20, 14].

Cyclic lipopeptides of the surfactin, iturin and fengycin families are important metabolites produced by *Bacillus* species and they have significant role in disease control. Some lipopeptides possess biosurfactant properties and consist of a hydrophobic fatty acid portion linked to a hydrophilic peptide chain in the molecule. The biosynthetic mechanism is same for all lipopeptides i.e. they are synthesized non-ribosomally, although structures are different [16].

Surfactin, a bacterial cyclic lipopeptide, largely prominent for its exceptional surfactant power, is used as an antibiotic. It was discovered about 35 years ago in an attempt to search for an inhibitor of fibrin clot-formation [10]. This compound was found to lower the surface tension of water from 72 mN $m^{-1}$to 27 mN m^{-1} and proved to have surface activity much better than sodium lauryl sulfate, a synthetic surfactant. The demand for surfactins has increased not only because of their biosurfactant properties but also due to their

bioactivities such as antimicrobial, antitumoral, antiviral, hemolytic and hypercholesterolemic [2, 10, 14, 17].

Surfactin possesses three essential genetic loci, *srfA* operon, *comA* (previously called *srf* B) and *sfp* that together lead to surfactin production in *B. subtilis* [15]. The biosynthesis of the lipopeptide surfactin by *Bacillus subtilis* involves the multienzyme thiotemplate mechanism. *srf*A operon is the part that contains the genes encoding at least part of the multienzyme complex [18]. The role of *comA* and *sfp* is to encode a 4' - phosphopantetheinyl transferase (an activating enzyme of *srf*A multienzyme complex) and a transcriptional activator of the *srfA* gene [15]. For the nonribosomal biosynthesis of surfactin, the presence of *srf*A operon and *sfp* gene is required. *srf*A operon consists of the four genes, *srf*AA (402 kDa), *srf*AB (401 kDa), *srf*AC (144 kDa), and *srf*AD that encode the surfactin synthetase subunits and play a role in the proof reading of peptide synthesis [2, 19]. *srf*A has significant role in the development of genetic competence (cell specialization process in which cell fraction becomes capable to internalize exogenous DNA) in *Bacillus subtilis* [3]. An intermediate step of the competence developmental pathway involves *srf*A operon and is located between the early competence genes, *com*A and *com*P, and the late genes encoding the proteins necessary for DNA uptake [13].

*srf*AA (the first open-reading frame of *srf*A) encodes the enzyme that has ability to activate the first amino acid, glutamate and also contains amino acid-activating domains for Leu and d-Leu of peptide portion of surfactin. The presence of spacer motif in *srf*AA region proposed that *srf*AA alone may not initiate surfactin synthesis and possibly the enzyme activating the β-hydroxy fatty acid moiety is required for the synthesis of surfactin. The other two open-reading frames of *srf*A contain amino acid-activating domains for different amino acids as *srfAB* have domains for Val, Asp and d-Leu; *srf*AC for l-Leu whereas *srf*AD encodes a product having sequence similarity to the *grs*T gene product [18, 3].

The project was designed to check the presence of *srf*AA ORF1 in the local isolates of three different species of *Bacillus* i.e. *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus sphaericus* to screen out species capable of competence development due to the existence of this ORF1.

2. Material and Methods

2.1. Bacterial strains and growth conditions for bacterial culture

Three locally isolated strains of *Bacillus* species i.e. *Bacillus subtilis, Bacillus thuringiensis* and *Bacillus sphaericus* were used for the isolation of *srf*AA ORF1. Two isolates of *Bacillus subtilis* were collected from Molecular Biochemistry Lab (MBL) that were previously isolated by our research group and named as MBL-A and MBL-B while the isolates of other two species were taken from Department of Microbiology. The strain identification was also confirmed by the Department of Microbiology, University of Agriculture, Faisalabad. The above mentioned species of *Bacillus* were grown in three separate 250 mL Erlenmeyer flasks on prepared Luria-Bertani media consisting of 10 g tryptone, 5 g yeast extract and 10 g NaCl per 1000 mL. The pH of the medium was maintained to 7.2 with 0.1 N HCl/NaOH and then autoclaved at 121°C at 15 psi pressure for 20 minutes. A loopful culture of each *Bacillus* species from sporulation medium was shifted aseptically into the flask and incubated in orbital shaker (140 rpm) for 24 hours at 37 °C.

2.2. DNA isolation

All the three *Bacillus* species were subjected to genomic DNA isolation following DNA extraction protocol of Wilson (1997) [11]. RNase was used to remove traces of RNA in the genomic DNA. For the confirmation of DNA isolation, 1% agarose gel (w/v in 1X TAE) was prepared and the gel electrophoresis was done at 80 volts for 45 minutes. Later on, the gel was stained with ethidium bromide for the visualization of results. Syngene GeneGenius Gel Light Imaging System was used for the purpose of gel documentation. DNA was quantified via spectrophotometric analysis at 260 nm and the purity of the DNA was also calculated by 260/280 ratio. This DNA was used as template DNA for PCR amplification or stored at -20 °C.

2.3. Polymerase chain reaction (PCR) conditions

ORF1 of surfactin synthetase (*srf*AA) was amplified by PCR using the specifically designed primers from the genomic DNA of locally isolated

Bacillus species. PCR mixture ingredients were used according to user defined protocol of PCR (Fermentas). The reaction mixture included template DNA, 10X PCR buffer, 2 mM dNTPs, forward and reverse primers, $MgCl_2$, *Taq* DNA Polymerase (1.25 units) and nuclease free water adjusting the final volume up to 50 µL. Considering the Tm of primers and length of gene to be amplified, the PCR thermal cycling conditions were set as 95 °C for initial denaturation for 3-4 minutes followed by 35 cycles of repeated denaturation at 94 °C for 1 min, polymerization at 72 °C for 1 min and final extension of 5 min at 72 °C to amplify the desired amplicon. Annealing temperature was adjusted some degrees lower than the melting temperature of primers used.

The PCR products were confirmed on 1% agarose gel in 1X TAE buffer. Favorprep gel purification mini kit of FAVORGEN Biotech Core (Cat. No. FAGPK 001-1; 200 preps.) containing FAGP Buffer, Wash Buffer (conc.), Elution Buffer, FAGP columns and 2 mL Collection tubes were used to get the PCR products in pure form free of PCR ingredients and salts following the user defined protocol (FAVORGEN). The results of purification were confirmed through gel electrophoresis, documented and analyzed.

3. Results and Discussion

Many strains of *Bacillus* species produce surface-active substances just like surfactins, and *Bacillus subtilis* specifically have been viewed as one of the most efficient biosurfactant producer [19]. Surfactin is a highly effective biosurfactant with a high surface activity and a variety of fascinating biological activities [21].

The potential of *srf*AA to develop competence had drawn our attention towards designing such a project in which we attempted to amplify the open reading frame, *srf*AA of surfactin synthetase from three different local isolates of *Bacillus* species including *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus sphaericus*. *B. subtilis* species (MBL-A and MBL-B). First of all, we isolated the genomic DNA of *B. subtilis* (MBL-A), *B. subtilis* (MBL-B), *B. thuringiensis* and *B. sphaericus* following bacterial DNA extraction protocol [11] with some modifications. In the initial step, cells were treated with lysozyme in addition to TE buffer and incubated at 37 °C for 1 hour. Lysozyme helps to break the bacterial cell walls by hydrolyzing 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan. Incubation time of 1 hour helped to completely break the membrane structures and regulate the osmolarity and acidity. Another modification was the use of more concentration of proteinase K to completely cleave the peptide bonds present in the membrane proteins. The pellet was dissolved in distilled water and stored at -20 °C until analyzed further. The results were confirmed on agarose gel (Fig.1 & Fig.2) and the concentration of isolated genomic DNA was quantified by UV-VIS spectrophotometer at 260 nm using the formula: Conc. (µg/mL) = A_{260} x 50 x Dilution Factor. The quantified concentrations of genomic DNAs of MBL-A, MBL-B, *B. thuringiensis* and *B. sphaericus* were found to be 14.0, 5.0, 6.0 and 5.0 µg/mL respectively. 260/280 ratio was also calculated for estimation of protein or RNA contamination and the results indicated that DNA samples were almost pure without any RNA or protein contamination.

3.1. PCR Primers and gene amplification

To isolate the *srf*AA ORF1 from *Bacillus* species, degenerate oligodeoxyribonucleotides were designed by using online bioinformatics software and tools based on the CDS sequence of *srf*AA of surfactin synthetase reported by Fabret *et al*., (1995) in *Bacillus subtilis* with accession number X72672 [4]. Firstly the sequence was cleaned from unwanted spaces/gaps by using CLEANER hosted tool (www.justbio.com). Then primer parameters were calculated by OLIGOCALC and to get reverse primer, COMPLEMENTOR tool was utilized that gave complementary reverse sequence of primer. To confirm the hybridization of primers with DNA template sequence, PRIMER ANEAL tool was used. CUTTER tool provided the information about restriction enzymes that cut the sequence on appropriate locations and thus help during cloning. In this way, with the help of all these tools, specific primers both forward primer and reverse primer were designed manually for *srf*AA ORF1. Primer3 tool also helped in designing primers (http://primer3.ut.ee/) and provided the information about primer parameters such as Tm, GC content, length etc. The designed primer pair

used for amplification of ORF1 from different *Bacillus* species is given below:

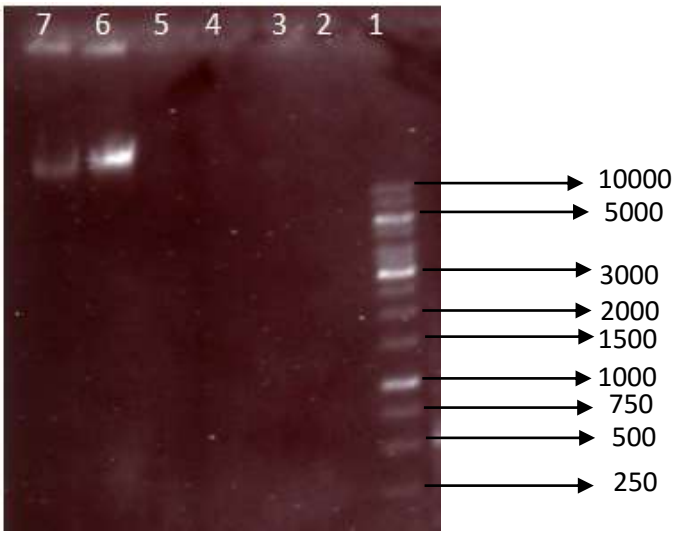

Figure.1. Isolation of genomic DNA from *Bacillus subtilis* MBL-A and MBL-B using protocol defined by Wilson (1997). Lane 1 shows the marker (M) i.e.1 kb DNA Ladder (Fermentas). Lane 6 and 7 shows the genomic DNA sample of *Bacillus subtilis* MBL-A and MBL-B isolates respectively.

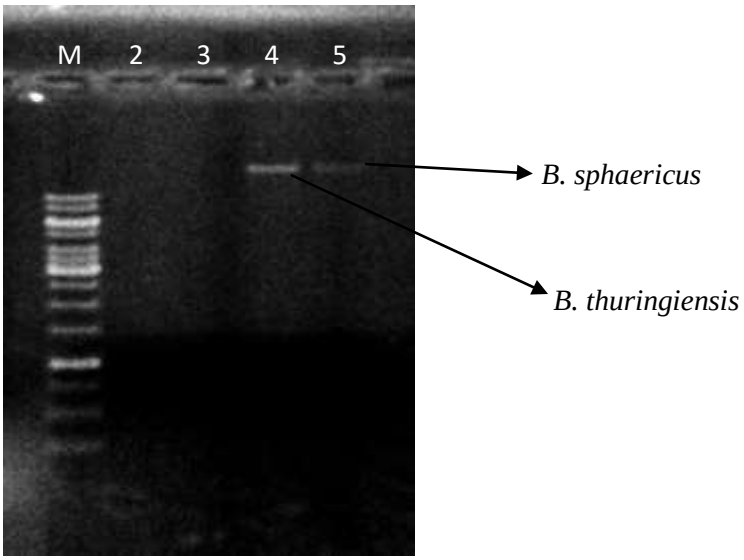

Figure.2. Isolation of genomic DNA from *Bacillus thuringiensis* and *B. sphaericus*. M on left side represents the marker, 1 kb DNA Ladder (Fermentas) and lanes 4 and 5 contain the DNA samples isolated from *Bacillus thuringiensis* and *B. sphaericus* respectively.

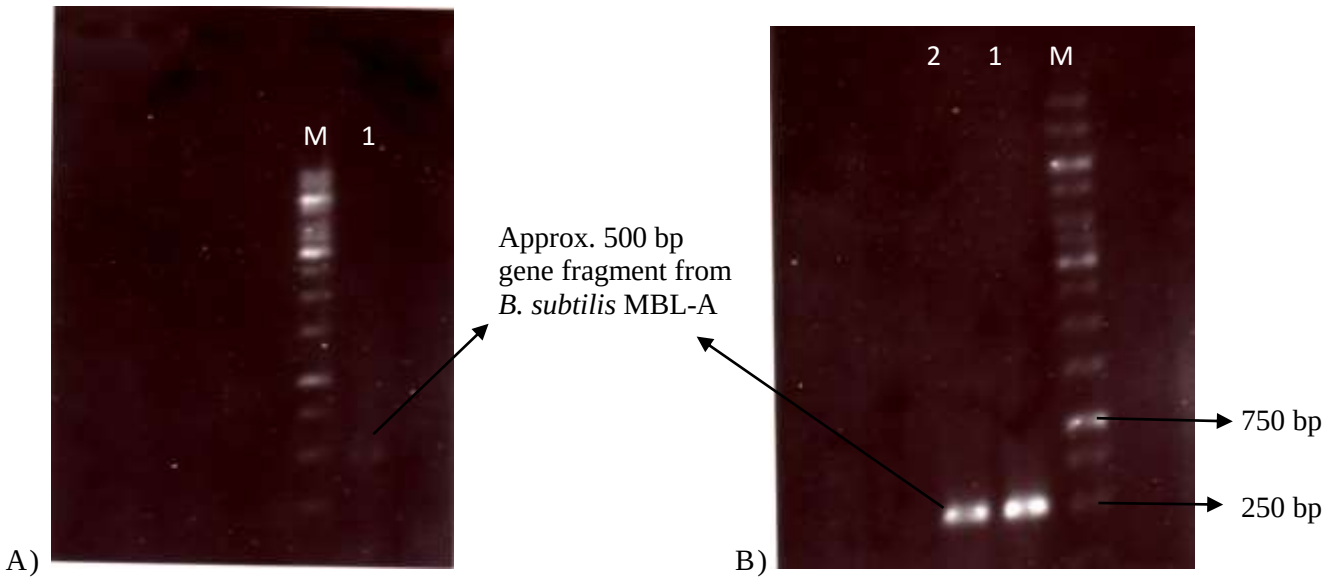

Figure.3. Amplification of ORF1 *srf*AA from *B. subtilis* MBL-A at different annealing temperatures: A) Lane 1 shows low intensity amplification of *srf*AA ORF1 using primers (srf F/srf R) from MBL-A strain of *Bacillus* at 47 ^{o}C annealing temperature. **B)** Lanes 1, 2 shows intense bands of ORF1 *srf*AA gene amplicon from MBL-A at 49 ^{o}C. M is 1 kb marker (Fermentas).

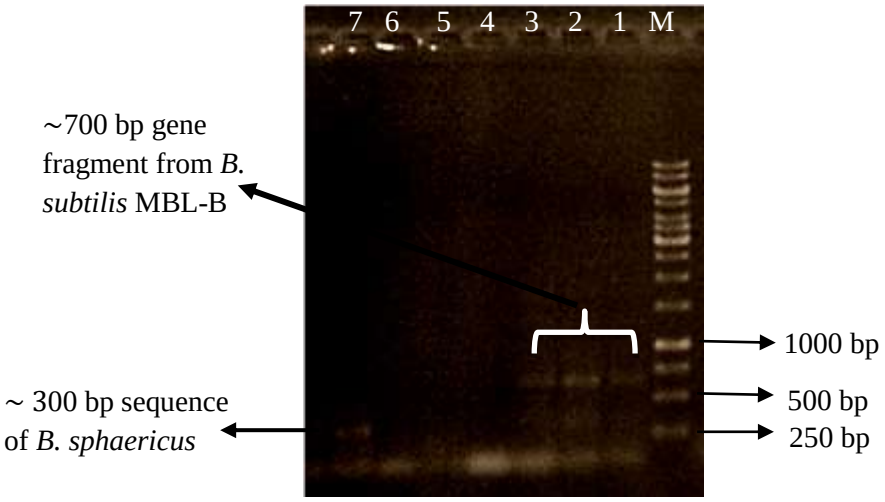

Figure.4. PCR results of *srf*AA amplification from *B. subtilis* MBL-B and *Bacillus sphaericus*: Lanes 1, 2 and 3 are representing PCR replicates of *B. subtilis* MBL-B. Lane 7 shows gene fragment amplification from *Bacillus sphaericus*. M represents 1 kb DNA ladder (Fermentas)

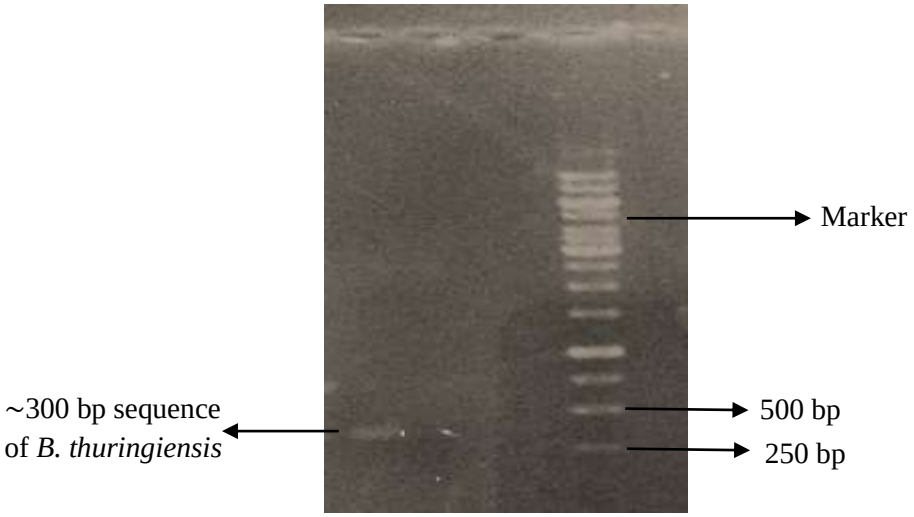

Figure.5. Gene fragment amplification from *B. thuringiensis*: Amplification of PCR product from *B. thuringiensis* shown in lane 3 on 1% agarose gel.

srf F: 5' - TCACTTGCTTCTTGCCATTCAC -3'

(length: 22 bp, Tm: 57 °C, GC content: 45%)

srf R: 5' - GATTTTTTGCTCATATGCCACCTC - 3'

(length: 24 bp, Tm: 58 °C, GC content: 42%)

The size of ORF1 of surfactin synthetase from *Bacillus subtilis* reported by Fabret and his co-workers was 1,024 bp and we also designed the primer pair to amplify 1,024 bp region however, 1,040 bp amplification of *srf*AA gene fragment has also been reported by Bais and his team through PCR from *B. subtilis* 6051 chromosomal DNA [8]. By taking into consideration the work of Fabret and Bais, we attempted to amplify *srf*AA ORF1 in this project utilizing three different species of *Bacillus*.

Before starting the 1st PCR reaction for amplification, 10 µM solution of primers was prepared by diluting stock solution for further use. In the first experiment, *Bacillus subtilis* MBL-A was subjected to the amplification of the ORF1 region using 50 ng of template DNA with 0.2 µM of each primer, 2.0 mM $MgCl_2$ at annealing temperature of 47 ^oC. PCR product was confirmed through gel electrophoresis. A PCR product of nearly 500 bp was obtained but the amplification was not very intense to be seen on gel (Fig.3.A). In order to get better results, optimization of annealing temperature was done to check the best optimized temperature for amplification and after some trials, it was set to 49 ^oC. The concentrations of $MgCl_2$, primer and template DNA were also optimized for the attainment of more appropriate results and these optimized conditions were used later on for all the next amplifications. $MgCl_2$ concentration was raised to 3.0 mM from 2.0 mM, primer concentration to 0.5 µM from 0.2 µM and template DNA to 100 ng from 50 ng.

In the second trial, the isolation of *srf*AA region from *Bacillus subtilis* MBL-A was proceeded by using 100 ng of template DNA, 0.5 µM of each primer, 3.0 mM of $MgCl_2$ at annealing temperature of 49 ^oC. This annealing temperature was optimized after some initial trials and the results showed that this set of parameters and optimized temperature worked well. Amplification of nearly 500 bp fragment was intense in MBL-A when the above mentioned conditions were applied (Fig.3.B). In 2010, Tapi and his colleagues also reported the amplification of nearly 500 bp PCR product from *Bacillus subtilis* using primer pair As1-F/Ts2-R but at a different temperature (45 ^oC) however, their amplified fragment showed high homology with surfactin synthetase family genes [1].

The same thermocycling conditions and concentrations of reagents were used to amplify the *srf*AA ORF1 from genomic DNA of *Bacillus subtilis* MBL-B and *B. sphaericus* in the next experiment. For *Bacillus subtilis* MBL-B, result was an approximately 700 bp fragment which was different from that of MBL-A (Fig.4). An amplification of approximately similar size (675 bp) has been reported by Tapi *et al*. in 2010

reporting the *sfp* gene (a part of *srf*A operon) from *B. subtilis* at annealing temperature of 46 ^{o}C using PCR [1]. In another report, 629 bp *sfp* gene was isolated via PCR from *B. subtilis* NH-160 and *B. subtilis* NH-217 strains by Hsieh and his team in 2004 [6]. The two reports of isolation of *sfp* gene from *B. subtilis* present a possible resemblance of our PCR product with the *sfp* gene due to some similarity in size however it will be confirmed in future through proper sequencing of the gene fragment. *B. sphaericus* exhibited a nonspecific amplification of ~300 bp fragment. There were no amplifications of 500 bp or 700 bp as observed in case of *Bacillus subtilis* (Fig.4).

Following the above mentioned reaction concentrations of reagents and optimized conditions of thermal cycling (3-4 minutes initial denaturation at 95 °C followed by 35 cycles of repeated denaturation at 94 °C for 1 min, 1 min annealing at 49 °C, 1 min polymerization at 72 °C and 5 min final extension at 72 °C), *Bacillus thuringiensis* was also screened for the presence of *srf*AA ORF1 and this species also gave results similar to *B. sphaericus* but different from both samples of the *Bacillus subtilis*. Nonspecific amplification of ~300 bp fragment was seen on the gel in *Bacillus thuringiensis* (Fig.5) and there is possibility that these 300 bp fragments of *B. sphaericus* and *B. thuringiensis* might be representing some part of *srf*AA ORF1 but confirmation requires sequencing that will be done in future.

Table.1. The screened *Bacillus* species with their respective sizes of *srf*AA ORF1 region amplified through PCR using primers (srf F/srf R).

***Bacillus* species**	**Approximate amplified size (bp)**
Bacillus subtilis (MBL-A)	500
Bacillus subtilis (MBL-B)	700
Bacillus thuringiensis	300
Bacillus sphaericus	300

Our results of PCR products exhibited variation in size of the ORF1 region (~500 and ~700 bp) as compared to already reported size in case of *Bacillus subtilis* and ~300 bp in *B. thuringiensis* and *B. sphaericus* that was neither identified before nor reported earlier in literature. The reason for this difference in size can be attributed to the genetic variation as the genomic DNAs were isolated from different sources. The different sizes of amplifications from the two different isolates of the same species (*B. subtilis* MBL-A and MBL-B), amplified by following the same protocol had also shown this genetic variance. One of them showed amplification of ~500 bp (MBL-A) and the other isolate (MBL-B) gave amplification of ~700 bp of same OFR1 with the primers that were designed specifically for this region. Many attempts were made for the amplification of desired gene fragment size but the results remained unchanged every time. The PCR products of *srf*AA ORF1 from different *Bacillus* species were subjected to purification using Favorprep gel purification mini kit (FAVORGEN Biotech Core). After purification, sequencing will be done to get better understanding and confirmation of our results.

4.0. Conclusion

Three different species of *Bacillus* were subjected to screening for the presence of *srf*AA ORF1 using PCR. Previously the surfactin gene had been found in various *Bacillus* species such as *Bacillus subtilis, B. amyloliquefaciens* and *B. circulans* but the presence of surfactin gene or *srf*AA ORF1 was not reported in *B. thuringiensis* and *B. sphaericus*. During our experimental work, we found amplification of nearly 500 bp from one isolate of *Bacillus subtilis* and approximately 700 bp amplification from the second isolate of the same species that shows the possibility of different variants in different isolates of the same species. Another possibility was the presence of some part of *srf*AA ORF1 in *B. thuringiensis* and *B. sphaericus* as some nonspecific amplifications were also observed in these two species using same primers that were used for amplification of *srf*AA ORF1 from *Bacillus subtilis*. The detail of amplified *srf*AA region from the four *Bacillus* species is given in table.1. The difference in genetic makeup of isolates could be a reason for the variability in size of ORF1 region in different isolates. The presence of *srf*AA ORF1 in these species may prove helpful in competence development in them and hence their usage in cloning. This region may also initiate the synthesis of the whole surfactin gene and hence may prove beneficial for the production of this bioactive compound with versatile applications in various fields. Sequencing will be done in future and the present project will be used for gene

expression studies and purification of recombinant products along with its detailed characterization. Hence, the research work will prove helpful to get best out of surfactin's commercial potential to be used in health care and environmental issues.

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