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RESEARCH ARTICLE

TRACING OF BIOSURFACTANT SYNTHESIZING GENES IN *BACILLUS* SP., BY IN VITRO AND IN SILICO TECHNIQUES USING *SRFA* GENE AS MARKER

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ABSTRACT

Biosurfactant production enhances the establishment of bacteria in its environment. It was confirmed among the food borne pathogens of *Bacillus cereus*, *B. licheniformis* and *B. subtilis* isolated from spoiled dairy products. Molecular weights of the purified DNA from these isolates were determined as > 4000 Kb. Restriction digestion of extracted genomic DNA by EcoRI and HindIII and amplification by genus specific 16S rRNA derived primer confirmed the homology among all. Production of biosurfactant by these bacteria was confirmed by drops collapse test, reduction in surface tension of culture media and emulsification properties. Purified biosurfactants from these isolates were characterized as surfactin, lichenysin and plipastatin from *B. subtilis*, *B. licheniformis* and *B. cereus* respectively. BLAST analysis of surfactin synthesizing gene *srfA* from *B. subtilis* showed 80% similarity with surfactant coding gene of lichenysin in *B. licheniformis*, 76% similarity with an unknown non-ribosomal peptidyl protein and 73% with bacitracin synthetase in *B. cereus*. So, the unknown plipastatin coding genes in *B. cereus* predicted as a non-ribosomal in origin and have antimicrobial properties.
Key words: Bacillus, Emulsification, Surfactant, Surfactin synthesizing genes.

INTRODUCTION

Extreme usage of chemical surfactants in industries and discharge of such chemical wastes into the environment leads to the unwanted changes in the ecosystem. Biological alternative to use of such harsh chemical is the biosurfactants that are required for many industrial processes such as foaming, wetting, washing, emulsification, and phase separation. Other advantages of using biosurfactants are their ecological acceptance owing to their lower toxicity, biodegradability and specificity[1]. So, the increasing industrial demand for the biosurfactants requires the large-scale production of biosurfactants using microorganisms. Improved production of biosurfactant in microorganisms can be achieved by gene amplification.

Biosurfactants are synthesized through series of enzymatic cascade and these enzymes are coded by cluster of genes[2]. For cloning and improved production, entire set of genes coding for biosurfactant must be known. In many efficient producers, genes involved in biosurfactants synthesis are not completely known.

One of the major biosurfactant-producing organisms belongs to the genus Bacillus[3]. The genus Bacillus is common food spoilage causing bacteria; representative species are *B. licheniformis*, *B. subtilis* and *B. cereus*. Biosurfactants production among these species enables them in coordinated way of establishment in the contaminated food through biofilm formation and emulsification of food. Researchers recognized that in biofilm, biosurfactants maintains channels between multicellular structures and in dispersal of cells through quorum sensing[4].

B. subtilis produces a known lipopeptide biosurfactant called as surfactin, which is coded by four open reading frames (ORFs) named as *srfA*, *srfB*, *srfC* and *srfD*[5]. Similarly, lichenysin is a lipopeptide biosurfactant produced by *B. licheniformis* coded by lichenysin operon (*lchA*) consists of three peptide synthetase genes *licAA*, *licAB*, *licAC* and *licAD*[6]. *B. cereus* produces a lipopeptide biosurfactant called plipastatin[7]. But biosurfactant plipastatin coding genes in *B. cereus* is still under study.

The present work has been carried out to establish the role of biosurfactant produced by food spoilage causing genus Bacillus and deducing the genes coding for biosurfactant especially plipastatin by comparing the similarity between biosurfactants genes of *B. licheniformis* and *B. subtilis* using in silico technique.

MATERIALS AND METHODS

Isolation and identification of Bacillus

Spoiled, canned dairy product was collected, homogenized and about 10 g of homogenized sample was inoculated in 100 ml of minimal media with 2% paraffin as carbon source. After 48 h of incubation, inoculum was spread over minimal agar media. The plates were incubated at room temperature for 24 h. Morphologically distinct colonies were isolated and sub-cultured. Isolates were subjected to microscopic, specific biochemical and metabolic characterization tests following the procedures of Bergy's classification system[8].

Measurement of biosurfactant activity

Isolates were sub-cultured on minimal medium and biosurfactant activities of the isolates were determined qualitatively by drops collapse test[9]. Quantitative surfactant activities were determined by burette method and emulsification properties by emulsification activity and emulsification index[10].

Structural characterization of the biosurfactants

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Biosurfactants produced by each isolate was purified from the culture supernatants prepared by centrifuging the culture broth at 12000 rpm for 30 min at 4ºC. Then it was mixed with methanol (2:1) and incubated overnight at 4ºC. After incubation, precipitated biosurfactants was collected by centrifugation at 6000 rpm for 15 min at 4ºC. Biosurfactants were further purified by extraction with 250 µl of chilled acetone. Ionic nature of the purified biosurfactants was determined[11].

Presence of lipids in the purified biosurfactants were confirmed by TLC using the solvent system hexane: petroleum ether: acetic acid (60:40:1) and iodine vaporization. Amino acids in the peptides of biosurfactants were hydrolysed by boiling with 6N HCl for 6 h and the content was run in TLC using a solvent system of butan-1-ol: acetic acid: water (80:20:20) and ninhydrin reagent was used as a detecting agent. Protein content in each sample was estimated by Lowry et al. method[12].

Invitro analysis of biosurfactants synthesizing genes

Genomic DNA was isolated from the *Bacillus* sp. by enzymatic hydrolysis of cell wall, extraction by chloroform – isoamylalcohol (24:1) mixture and purification by 70% of ethanol. The purified DNA (2 µl of each) was subjected to restriction by restriction enzymes of 1 µl Eco RI and 1 µl Hind III in 2.5 µl of 10X TE buffer. The mixtures were incubated at varying temperatures of 37°C for 2 h, 65°C for 2 h and 70°C for 20 min and stored at 4°C. Then the products were amplified by PCR using the primer derived from 16S rRNA sequence of *B. circulans* (Gen Bank accession No: X60613) for selective amplification of DNA of *Bacillus* sp. from mixed samples. It can amplify 546 bp of sequence[13].

Primer type	Primer Sequence
BCF1	CGGGAGGCAGCAGTAGGGAAT
BCR2	CTCCCCAGGCGGAGTGCTTAAT

The PCR product was visualized in 2% agarose gel electrophoresis applying an electric current of 50V. Sizes of the PCR products were determined by comparing with a narrow range DNA ladder in agarose gel electrophoresis.

Table 1: Biosurfactant activities of bacterial isolates

Organisms	EA (OD at 610 nm)	EI (%)	Surface tension (Nm^{-1})
Bacillus cereus	0.035	70	**0.39**
B. licheniformis	0.064	59	**0.31**
B. subtilis	0.045	67	**0.30**
control	0.000	00	**0.72**

In silico analysis of biosurfactants synthesizing genes

The surfactin synthetase gene, *srfA* (X65835) is 865 bp in length. It is involved in the synthesis of biosurfactant surfactin in *B. subtilis*[5] and retrieved from GenBank. A basic local alignment search (BLAST 2.2.24) was performed for *srfA* gene with genomic DNA of *B. cereus* AH820 (NC_011773) and *B. licheniformis* ATCC14580 (NC_006270).

RESULTS AND DISCUSSIONS

Assay of biosurfactant activity

Food spoilage causing bacteria establishes in the food and resists the attack by chemical preservatives through the biofilm formation. Bacterial cells in biofilm communicate in more complex patterns of co-operative behavior that are termed as 'quorum sensing', bacterial cell-to-cell communication which is attributed by biosurfactants[14]. Biosurfactants also enables the bacterial population to trigger a unified response that is advantageous to its survival. This response improves access to complex nutrients or environmental niches and develops collective defense mechanisms against alternative competitive microorganisms or agents [15].

Biosurfactants production by *B. cereus*, *B. licheniformis* and *B. subtilis* isolated from biofilm of spoiled food were confirmed by collapsing of drop within a minute in drops collapse test. Culture supernatant from isolates, collapsed/emulsified the water droplet on a mineral oil platform within 10sec. Surfactant present in the drop spreads or collapses the drop by force or reduction in interfacial tension between the liquid drop and the hydrophobic surface. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension [16]. As the time required for collapsing and surfactant concentrations are indirectly correlated, collapsing of drop within 10sec confirmed presence of higher concentration of biosurfactant by all the isolates.

Surfactant and emulsification activities of the isolates were tabulated in table 1. Results showed that surface tensions of culture media were reduced from 0.072 N/m to less than 0.040 N/m and formed stable emulsion of above 60%. The efficient surfactant and emulsification properties of the purified biosurfactants of these isolates confirmed it's foaming, wetting, washing and emulsification properties needed for industrial processes.

Structural characterization of the biosurfactants

White, powdery biosurfactant of 10 mg/ml of media was purified from *B. subtilis* and *B. cereus* and 20 mg/ml of media from *B. licheniformis*. Based on the formation of line of precipitation, ionic natures were determined as zwitter ionic in *B. subtilis*, anionic in *B. cereus* and cationic in *B. licheniformis*. Development of yellow colour positive bands confirmed the presence of lipids in lipid chromatography, but R_f values of bands varied with each sample due to the linkage of peptides with varying length of fatty acids. Presence of amino acids valine, isoleucine, leucine and asparagine were confirmed using nin-hydrin in TLC plates and further by development of colour in Lowry et al. assay. So, the purified biosurfactants from the

isolates were confirmed as lipopeptides. The results of above experiments were tabulated in table 2.

Presence of valine, isoleucine, leucine and asparagine in purified biosurfactants confirmed as surfactin in *B. subtilis*, which is a cyclic lipoheptapeptides consists of (L-Glutamine --> (L - Leucine)$_2$ --> Valine --> L – Aspartic acid --> D – Leucine --> L – Leucine --> L – Valine --> L- Isoleucine) and β - hydroxy fatty acid [17]. Lichenysin is a similar biosurfactant produced by *B. licheniformis* differs by the presence of isoleucine in the C–terminal end instead of leucine and asparagines residue instead of aspartic acid in the surfactin peptide[18]. Plipastatin is a lipopeptides biosurfactant produced by *B. cereus* [7].

Table 2: Characters of biosurfactants purified from bacterial isolates

Bacterial source of biosurfactant	**R_f values for lipid**	**Con. of protein (µg/ml)**	**Ionic detection**
Bacterial source of biosurfactant	**R_f values for lipid (cm)**	**Con. of protein (µg/ml)**	**Ionic detection**
B. cereus	0.48	0.29	Anionic
B. licheniformis	0.58	0.30	Cationic
B. subtilis	0.12	0.21	Zwitterion

DNA analysis

DNA isolated from *B. cereus*, *B. licheniformis* and *B. subtilis* showed molecular weights above 4000 Kb. Extracted genomic DNA were subsequently subjected to restriction digestion and the products were amplified in PCR using genus specific primers. So that isolated bacterial genus Bacillus was characterized using the combination of culture and non-culture techniques for confirmation of homology among isolated genus Bacillus[19]. Restricted products of genomic DNA from each isolate resulted in similar banding pattern (100 bp in length) (Fig. 1), which confirmed that surfactant-synthesizing genes are not interfering in genus specific genes and are not interfered by the restriction digestion by EcoRI and HindIII.

In silico analysis of biosurfactants synthesizing genes

The BLAST result of *srfA* with *B. licheniformis* ATCC14580 (NC_006270) showed 83% similarity with *lchAA* gene and 72% of similarity with *lchAB*. Similarly, 76% of similarity with non-ribosomal peptidyl synthetase protein coding gene, 76% of similarity with long chain fatty acid CoA ligase gene and 73% of similarity with bacitracin synthetase 1 gene of *B. cereus* AH820 (NC_011773). So, the surfactin and lichenysin coding gene clusters are quite similar. Whereas, in *B. cereus*, showed identity with bacitracin (an antibiotic) synthetase gene. So the biosurfactant plipastatin may have antimicrobial activity and can control bacteria and viruses through disruption of cell membrane through physiochemical interaction[20] and is non-ribosomal in origin.

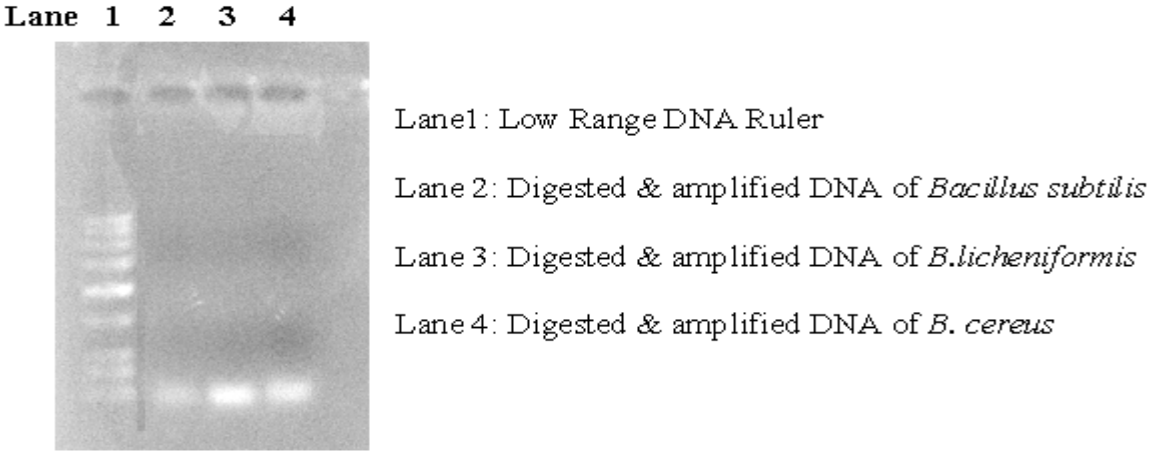

Figure 1: PCR amplified restricted DNA

CONCLUSION

The food-spoilage causing genus *Bacillus* produces lipopeptide biosurfactants. That enables in establishment of bacterial cells in denser media and competitively inhibits the growth of other population. Purified lipopeptide biosurfactants were recognized as surfactin, lichenysin and plipastatin from *B. subtilis*, *B. licheniformis* and *B. cereus* respectively. DNA isolated from these isolates showed homology and from the known *srfA* of *B. subtilis*, the unknown plipastatin coding genes in *B. cereus* was identified as non-ribosomal in origin and has antimicrobial properties. Thereby concludes that lipopeptide surfactants produced by the bacterial genus Bacillus presents a great potential for pharmaceutical and industrial application. The knowledge of gene clusters coding for the biosurfactants paves the new way to develop recombinants for the overproduction of biosurfactants to meet the industrial needs.

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